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BIOLOGICAL ACTIVITIES OF *ADIANTUM CAPILLUS-VENERIS* COLLECTED FROM DUHOK PROVINCE (IRAQ)

FALAH SALEH MOHAMMED, MUSTAFA SEVİNDİK, CELAL BAL, HASAN AKGÜL and ZELİHA SELAMOĞLU

ABSTRACT. This study determined the DPPH free-radical scavenging activity, total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI), DNA-protective activity, antiproliferative activity, antimicrobial activity and phenolic contents of methanol (MeOH) and dichloromethane (DCM) extracts of *A. capillus-veneris* leaves collected from the province of Duhok (Iraq). As a result of the studies, it was determined that the MeOH extract of *A. capillus-veneris* had a 49.74% free-radical scavenging activity at 2 mg/mL concentration. It was found that the extracts were effective against the test microorganisms at a concentration level of 200-400 µg/mL. TAS, TOS and OSI values were 3.086±0.066, 21.532±0.525 and 0.698±0.002, respectively. The DNA-protective activity of the extracts was found to be weak compared to the positive control. It was found that, depending on the increase in concentration, the extracts showed antiproliferative activity on A549 cells. Furthermore, the HPLC analyses found Catechin, Cinnamic acid, Chlorogenic acid, Caffeic acid, p-Coumaric acid, Rosmarinic acid and 4-Hydroxybenzoic acid with various ppm values. Consequently, it was determined that *A. capillus-veneris* could be a potential natural source pharmacologically.

1. INTRODUCTION

Plants have always been used by people to improve medical conditions or to lessen their impact [1]. Plants have drawn the attention of many research teams due to their role in fighting various medical conditions such as atherosclerosis, cerebral cardiovascular events, diabetes, hypertension and Alzheimer's disease [1,2].

Previous studies by different research groups have found that different plants have different antibacterial, antiviral, antifungal, antiproliferative, anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic, antidepressant, and antitumor properties [3-8].

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A. capillus-veneris commonly grows in warm, tropical climates with a high moisture content. It has creeping rhizome roots and an aromatic fragrance. The leaves of the plant are generally double-rowed, tender, glabrous, and can grow up to 50 cm (Figure 1). As a cosmopolitan species, the plant is widely distributed in suitable climates [9].

FIGURE 1**.** *Adiantum capillus-veneris* L.

This study used MeOH and DCM extracts from the leaves of *A. capillus-veneris.* Phenolic content, antioxidant potential, oxidant potential, antimicrobial potential, DNA-protective activity and antiproliferative action of the extracts were determined. Within this scope, it was found that the plant could potentially be used as a natural source for pharmacological applications.

2. Material And Method

2.1. Collection of plants and laboratory studies

The study material *A. capillus-veneris* was collected from the Duhok province of Iraq. Herbarium samples are preserved in the herbarium of Zakho University, Science Faulty, Biology Department. The plant parts were cleaned with distilled water and dried under favorable conditions. Later, components of the plant were pulverized using a mechanical grinder. After the grinding process, 15 g of the plant components were extracted with methanol (MeOH) and dichloromethane (DCM) in

a Soxhlet extractor at 50 ^{0}C for approximately six hours. Then, crude extracts were obtained by evaporating the solvents of the extracts using a Rotary Evaporator.

2.2. Antioxidant Activity Tests

Stock solutions were prepared at concentrations of 0.25, 0.5, 1 and 2 mg/mL using DMSO (Dimethyl sulfoxide). 50 µL of the prepared solutions was added to 160 µL 0.039% DPPH. The prepared solutions were incubated for 30 minutes. Following the incubation process, a reading for absorbance at 517 nm was obtained. These processes were repeated for all specified concentrations [10]. Rosmarinic acid (RA) and ascorbic acid (AA) were used as reference antioxidants. DPPH free radical scavenging percentages were determined using the following formula: inhibition% = [(Abs control-Abs sample)\Abs control]x100.

TAS, TOS and OSI values of the plant extracts were determined using Rel Assay branded commercial kits (Rel Assay Diagnostics Kits, Turkey). Trolox and hydrogen peroxide were used as the TAS calibrator and TOS calibrator, respectively. TAS results were expressed in mmol Trolox equiv./L. TOS results were expressed in µmol H_2O_2 equiv./L [11,12]. The OSI (AU: Arbitrary unit) value was calculated based on the following formula (1):

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

2.3. Determination of Phenolic Contents

The phenolic contents of the plant were scanned using an HPLC device. A DAD detector was used as detector. The injection volume was set to 20 µL. A: 3% acetic acid and B: methanol was used in the mobile phase. The flow rate was set to 0.8 mL per minute. Chromatographic separation was carried out with an Agilent Eclipse XDB-C18 column (250x4.6 mm; id 5 µm) at 30 ^{o}C [13].

2.4. Antimicrobial Activity Tests

Tests for the antimicrobial activity of MeOH and DCM extracts of the plant components were conducted using the agar dilution method recommended by the

Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The minimum inhibitory concentration (MIC) for MeOH and DCM extracts was determined against standard bacterium and fungus strains. *Staphylococcus aureus* ATCC 29213, *S. aureus* MRSA ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as bacterium strains. *Candida albicans* ATCC 10231, *C. krusei* ATCC 34135 ATCC 13803, *C. glabrata* ATCC 90030 were used as fungus strains. All extracts were tested at 800-12.5 µg/mL concentrations and all dilutions were performed using distilled water. Bacterium and fungus strains were obtained from the American culture collections. Colony formation was interpreted as the presence of growth and the absence of colony was interpreted as inhibition. Additionally, control plates were used for each study series. The lowest dilution that prevented bacteria and fungi reproduction was the minimum inhibitory concentration (MIC) [14-18].

2.5. DNA-Protective Activity Test

Standard solutions were prepared from MeOH and DCM extracts of the plant parts at concentrations of 25, 50, 100 and 200 µg/mL, and their DNA-protective activity was determined using pBR 322 supercoiled DNA. 0.5 µg plasmid pBR 322 supercoiled DNA was put into Eppendorf tubes and 10 µL of the standard solutions of the extracts was added. 10 µL Fenton agent (30 mM H_2O_2, 50 µM ascorbic acid and 80 µM $FeCI_3$) was added to them and incubation was performed at room temperature for 10 minutes. The mixture was prepared with a final volume of 20 mL and set aside to rest at 37 °C for 30 minutes. Subsequently, it was analyzed by electrophoresis on 1% agarose gel containing DNA ethidium bromide [19].

2.6. Antiproliferative Activity Test

An MTT test (3- [4, 5-dimethylthiazol-2-il] -2, 5-diphenyl-tetrazolium bromide) was performed in order to find the cell habitability on A549 cells of MeOH and DCM extracts of the plant components. After 70-80% unification was achieved, cells were separated using a 3.0 mL Trypsin-EDTA solution (Sigma-Aldrich, MO, ABD). Following the separation process, they were planted on the plates. After the planting process, they were incubated for 24 hours. Following the incubation process, the extracts were subjected to a dilution process at different concentrations (25, 50, 100 and 200 µg/mL) and the cells were incubated for 24 hours. Controls were applied

with a growth medium that was not supplemented with FCS. After an incubation period of 48 hours, the supernatants were dissolved in the growth medium and replaced with 1 mg/mL MTT (Sigma). Then incubation was performed at 37 °C until a purple precipitation was formed. The supernatants were collected and dissolved by adding dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, ABD) to MTT which was absorbed by the cells. Then the plates were measured at 570 nm using the Epoch spectrophotometer (BioTek Instruments, Winooska, VT) [20].

3. Results And Discussion

3.1. Antioxidant Activity

Compounds with antioxidant properties play an essential role in the body's defense system against reactive oxygen species (ROS). ROSs may cause early ageing, cancer and cardiovascular diseases. The antioxidant defense system plays a role in suppressing or dampening the effects of ROSs. When elements of the antioxidant defense system produced by the body cannot adequately reduce the effects of ROSs, supplementary natural antioxidants may be taken. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of many diseases. The increased intake of dietary antioxidants may help support the process of limiting antioxidant concentrates and also may support the normal functioning of various physiological systems [21,22]. Therefore, the natural antioxidant potential of *A. capillus-veneris* leaves was assessed in this study. In the present study, DCM extracts of *A. capillus-veneris* leaves did not demonstrate any antioxidant properties while MeOH extracts were found to be have antioxidant properties in parallel with increases in the concentration levels. The results are shown in Table 1.

TABLE 1. DPPH Free Radical Scavenging Activity

Concentration (mg/mL)	Ascorbic acid (%)	Rosmarinic acid (%)	MeOH	DCM
0.25	27.717	25.501	5.750	0.095
0.5	69.398	43.744	12.283	0.439
1	95.721	79.694	23.018	1.356
2	95.740	93.849	49.742	7.660

Previous studies reported that Ether and MeOH extracts of *A. capillus-veneris* demonstrated normal DPPH free radical scavenging activities [23,24]. Another study on Ethanol, Butanol, Ether and MeOH extracts of *A. capillus-veneris* found that the extracts demonstrated DPPH free radical scavenging activities at normal levels [25]. This study found that the DCM extract of *A. capillus-veneris* demonstrated no DPPH free radical scavenging activities while the MeOH extract demonstrated DPPH free radical scavenging activities by 49.74% at 2 mg/mL concentration. According to the obtained data, it was determined that the leaves of *A. capillus-veneris* had antioxidant potential and could be used as a natural antioxidant source.

Oxidative stress is a condition where the ROS concentration is temporarily or continuously increased and, consequently, cellular metabolism and regulation as well as cellular components are damaged. [26]. Oxidative stress index (OSI) shows the extent to which endogenous oxidant compounds are suppressed by endogenous antioxidant compounds. In the current study, the TAS and TOS values of *A. capillus-veneris* were determined. Also, OSI values were determined on the basis of the TAS and TOS values. According to the results of the study, TAS, TOS and OSI values of *A. capillus-veneris* were 3.086±0.066 mmol/L, 21.532±0.525 µmol/L and 0.698±0.002, respectively. This is the first study that has examined the TAS, TOS and OSI values of *A. capillus-veneris.* In previous studies conducted on plants, the TAS, TOS and OSI values of *Mentha longifolia* subsp. *longifolia* were reported to be 3.628 ± 0.234 mmol/L, 4.046 ± 0.615 µmol/L and 0.112 ± 0.025, respectively [27]. Additionally, the TAS, TOS and OSI values of *Rhus coriaria* var. *zebaria* were reported to be 7.342±0.189 mmol/L, 5.170±0.525 µmol/L and 0.071±0.009, respectively [28]. In another study, the TAS values of ethanolic extracts of *Calendula officinalis* were reported to be 5.55±0.41 mmol/L [29]. It can be seen that *A. capillus-veneris* used in our study has lower TAS values than *M. longifolia* subsp. *longifolia*, *R. coriaria* var. *zebaria* and *C. officinalis*. Also, it was found that *A. capillus-veneris* had higher TOS and OSI values than *M. longifolia* subsp. *longifolia* and *R. coriaria* var. *zebaria*. Consequently, it was determined that *A. capillus-veneris* produced more oxidant compounds than these plants; and that the antioxidants responsible for suppressing these were in inadequate amounts, and therefore the plant had higher OSI values.

3.2. Phenolic Contents

In the current study, the phenolic contents of *A. capillus-veneris* were scanned using an HPLC device. The results obtained in the study are shown in Table 2.

TABLE 2**.** Phenolic content of *A. capillus-veneris*

	A. capillus-veneris (ppm)
Catechin	56.21
Cinnamic acid	19.87
Chlorogenic acid	56.76
Caffeic acid	1.59
p-Coumaric acid	4.47
Rosmarinic acid	130.8
4-Hydroxybenzoic acid	16.32

The plants produce a wide range of biologically active chemicals, secondary metabolites, playing a role in the fight against pests and diseases [30]. Catechin is reported to have antioxidant, antimutagenic, antimicrobial and anticarcinogenic properties [31-34]. Chrologenic acid is reported to have antioxidant, antibacterial, anticarcinogenic, anti-inflammatory and DNA-protective properties [35-39]. It has been reported that caffeic acid has antioxidant, antibacterial, anti-inflammatory, anticarcinogenic and hepatoprotective properties [40-44]. Rosmarinic acid is reported to have antiviral, antibacterial, anti-inflammatory, anticarcinogenic and antioxidant properties [45]. Cinnamic acid is a natural organic acid with several biological activities. Cinnamic acid and its derivatives are significant compounds due to their antibacterial, antiviral and antifungal properties [46]. Coumaric acid has been reported to have antioxidant, anticancer, antimicrobial, antiviral, anti-inflammatory, anti-thrombocyte aggregation, anxiolytic, antipyretic, analgesic properties as well as many other biological activities including mitigating effects against diabetes, obesity, hyperlipemia and gout [47]. It has been reported that hydroxybenzoic acid has antioxidant properties and is beneficial for preventing and reducing the risk of diabetes, coronary heart disease, cancer, Alzheimer's disease and cataracts [48]. The HPLC scans in our study found varying degrees of Catechin, Cinnamic acid, Chlorogenic acid, Caffeic acid, p-Coumaric acid, Rosmarinic acid and 4-Hydroxybenzoic acid in *A. capillus-veneris.* The therapeutic potential of medicinal plants depends on the presence of bioactive components that create specific physiological and pharmacological activities [49]. Within this scope, it was found that *A. capillus-veneris* could be a natural source in terms of the compounds determined in this study.

3.3. Antimicrobial activity

Since pathogens have recently developed resistance against currently available antibiotics, the search for new alternative sources for the treatment of communicable diseases has become inevitable. Plants are rich sources of secondary metabolites with antimicrobial effects [50]. Within this scope, the antibacterial and antifungal potential of *A. capillus-veneris* were evaluated in this study. The results are shown in Table 3.

TABLE 3. Antimicrobial Activity of *A. capillus-veneris*

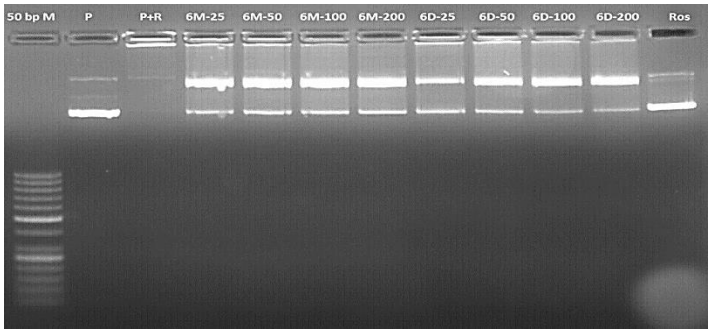
	A	B	C	D	E	F	G	H	J
MeOH	800	800	800	800	400	400	800	400	400
DCM	400	400	800	800	200	200	800	200	200
Ampicillin	1.56	3.12	1.56	3.12	3.12	-	-	-	-
Amikacin	-	-	-	1.56	3.12	3.12	-	-	-
Ciprofloksasin	1.56	3.12	1.56	1.56	3.12	3.12	-	-	-
Flukanazol	-	-	-	-	-	-	3.12	3.12	-
Amfoterisin B	-	-	-	-	-	-	3.12	3.12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

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

Previous studies have reported that the methanol extracts of *A. capillus-veneris* had antimicrobial effects against *Bacillus, Escherichia coli, Staphylococcus, Proteus, Pseudomonas* and *Candida* at concentrations of 0.5-2 mg/mL [51]. Also, another study found that methanol extracts of *A. capillus-veneris* was effective against *Micrococcus luteus, Bacillus subtilis, B. cereus, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Candida albicans, Cryptococcus albidus, Trichophyton rubrum, Aspergillus niger, A. flavus, A. spinulosus, A. terreus* and *A. nidulans* at different concentrations [52]. In the current study, MeOH and DCM extracts of *A. capillus-veneris* were used and it was determined that the extracts were effective on the tested microorganisms at concentrations of 200-800 µg/mL. In addition, as opposed to other studies in the literature, it was found that the MeOH and DCM extracts of *A. capillus-veneris* were also effective against *E. faecalis, A. baumannii, C. glabrata* and *C. krusei* at different concentrations.

3.4. DNA-Protective Activity

In the current study, the DNA-protective activity of MeOH and DCM extracts obtained from *A. capillus-veneris* leaves at concentrations of 25, 50, 100 and 200 µg/mL were tested using pBR322 supercoiled DNA. The results are shown in Figure 2.



* P: DNA, 6: *A. capillus-veneris*, M: Methanol, D: Dichloromethane

FIGURE 2**.** DNA Protective Activity of *A. capillus-veneris*

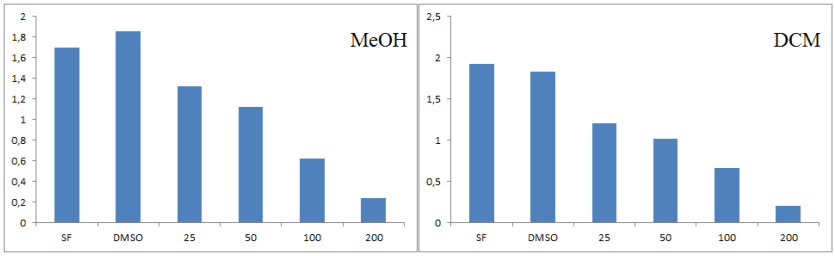
It was determined that the MeOH and DCM extracts from *A. capillus-veneris* leaves had lower DNA-protective properties compared to the positive control at all concentrations applied. In previous studies, various plant types have been reported as having DNA-protective properties [28, 53,54]. The protective effect of *A. capillus-veneris* against DNA damage was researched for the first time in this study and found to have a low impact.

3.5. Antiproliferative activity

Various plant components with anticarcinogenic properties have been identified in many regions of the world. Today, approximately 75% of anticancer drugs accepted worldwide are derived from plants or other natural products [55].

Cell viability was tested with the lung cancer cell line A549 by preparing standard solutions of both MeOH and DCM extracts from plant samples at concentrations of 25, 50, 100 and 150 µg/mL. The results are shown in Figure 2. Despite recent developments in the treatment of cancer, there is a need for new alternative drugs. Therefore, plants still constitute a significant source for the discovery of new drugs.

Moreover, less than 10% of flowering plants have been investigated in an analytical and pharmacological manner for their potential medical value [56]. In previous studies, ethanol extracts of *A. capillus–veneris* were reported to have antiproliferative effects on human gastric carcinoma SGC-7901 cells [25]. In the current study, MeOH and DCM extracts from the leaves of *A. capillus-veneris* were examined for their antiproliferative properties against the lung cancer cell line A549. The results are shown in Figure 3.



*25, 50, 100 and 200 extract concentration

FIGURE 3. Cytotoxic Effects of *A. capillus-veneris*

In the current study, the MeOH and DCM extracts of *A. capillus-veneris* leaves were examined and it was found that their antiproliferative effects on the lung cancer cell line A549 increased depending on the increase in concentration. In this context, it was determined that *A. capillus-veneris* could be a natural cancer-fighting source.

4. Conclusion

In this study, the pharmacological potential of the leaves of *A. capillus-veneris* have been investigated. As a result of the studies conducted, it was determined that the plant components showed antioxidant activity and DNA-protective and anticancer potential. It was also found that they displayed antibacterial and antifungal potential against test microorganisms. Additionally, it was determined that it could be a natural source in terms of the compounds found in its content. Consequently, it was found that *A. capillus-veneris* can be considered a natural source in the manufacture of natural pharmacological drugs due to its biological activities.

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BIOLOGICAL ACTIVITIES OF *ADIANTUM CAPILLUS-VENERIS* COLLECTED FROM DUHOK PROVINCE (IRAQ)

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THE FIRST RECORD OF *CREPIDOTUS CROCOPHYLLUS* FROM TURKEY

Deniz ALTUNTAŞ, Hakan ALLI, Ilgaz AKATA

ABSTRACT. In the current study, *Crepidotus crocophyllus* (Berk.) Sacc. is reported for the first time for Turkish mycobiota. Short description of the new record together with its drawings related to macro and micromorphologies were given and discussed briefly.

1. Introduction

Crepidotus is a genus of the family *Inocybaceae* within the order *Agaricales* (*Basidiomycota*) and it possesses over 150 widely distributed species [1]. Pleuroid fruiting body with lateral stipe, circle, semicircle, fan, kidney or spatula-shaped pileus with fibrillose, tomentose, scaly or glabrous surface, yellow-brown, clay coloured, brown or cinnamon spore print, hyaline, light brown or brown basidiospores with or without ornamentation and the presence of cheilocystida are the characteristics of the genus members [2-4].

Nine *Crepidotus* species (*C. calolepis* (Fr.) P. Karst., *C. caspari* Velen., *C. cesatii* (Rabenh.) Sacc., *C. cinnabarinus* Peck, *C. epibryus* (Fr.) Quél., *C. luteolus* Sacc., *C. mollis* (Schaeff.) Staude, *C. variabilis* (Pers.) P. Kumm. and *C. vulgaris* Hesler & A.H. Sm.) have hitherto been registered from Turkey but there was not any record of *C.crocophyllus* (Berk.) Sacc. [5-11]. The purpose of the present study is to contribute to Turkish *Crepidotus*.

2. Material And Method

Fresh fungi samples were collected from Sinop province on 28th of September 2014. Necessary morphological and ecological characteristics of the samples were noted in their natural habitats. In the laboratory, initially the spore prints of the samples

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were taken, and then, the microstructural data were gathered by light microscopy. Some reagents such distilled water, 5 % KOH, H_2O, H_2SO_4, congo red, cotton blue, etc. were utilized. We benefited from the currently existing literature for the identification of the species [2-4]. The identified specimens were deposited to the herbarium of Ankara University (ANK).

3. Results

The systematics of the species follow Kirk et al. (2008). Short description, ecologies, and distributions, localities, collection dates, drawings related to its macro and microstructures were provided.

Basidiomycota Whittaker ex R.T. Moore

Agaricales Underw.

Inocybaceae Jülich

Crepidotus crocophyllus (Berk.) Sacc., (Figure 1).

Syn.: *Agaricus crocophyllus* Berk.

Macroscopic and microscopic features

Pileus 20-30 mm broad, sessile, laterally attached to the substratum, spathulate to flabelliform, hemispherical, convex when young, later plano-convex, with inflexed margin, later becoming straight and smooth, surface cream yellow to yellowish brown, darker with age covered with brownish, orange-brown, rust or cinnamon fibrils or scales. **Flesh** thin, brownish to orange-brown. **Taste** mild. **Odor** not distinctive. **Lamellae** close to crowded, adnexed to narrowly adnate, grayish-yellow, yellowish-brown, grayish, grayish brown or brownish-orange. **Spores** 5.5-7 × 5-7 µm, globose to subglobose, punctate, warty, yellowish-brown to pale brown, **Basidia** 25-35 × 6-8 µm, cylindrical to narrowly clavate, four-spored and clamped. **Pleurocystidia** absent. **Cheilocystidia** 40-60 × 6-10 µm, clavate, cylindrical, more rarely lageniform. **Pileipellis** a cutis consisting of cylindrical, thick-walled, hyaline hyphae in distilled water, yellow to brown-pigmented in KOH, intracellular and incrusting in hyphae of squamules on pileus, all hyphae with clamps.

Ecology

Solitary to gregarious, on stump, fallen branches, decaying log or dead bark of hardwood, June to October, rare [2,3].

Material examined

TURKEY-Sinop: Bozburun, Abalı village, on common hornbeam (*Carpinus betulus* L.), 20 m, 28.09.2014, Allı 5675.

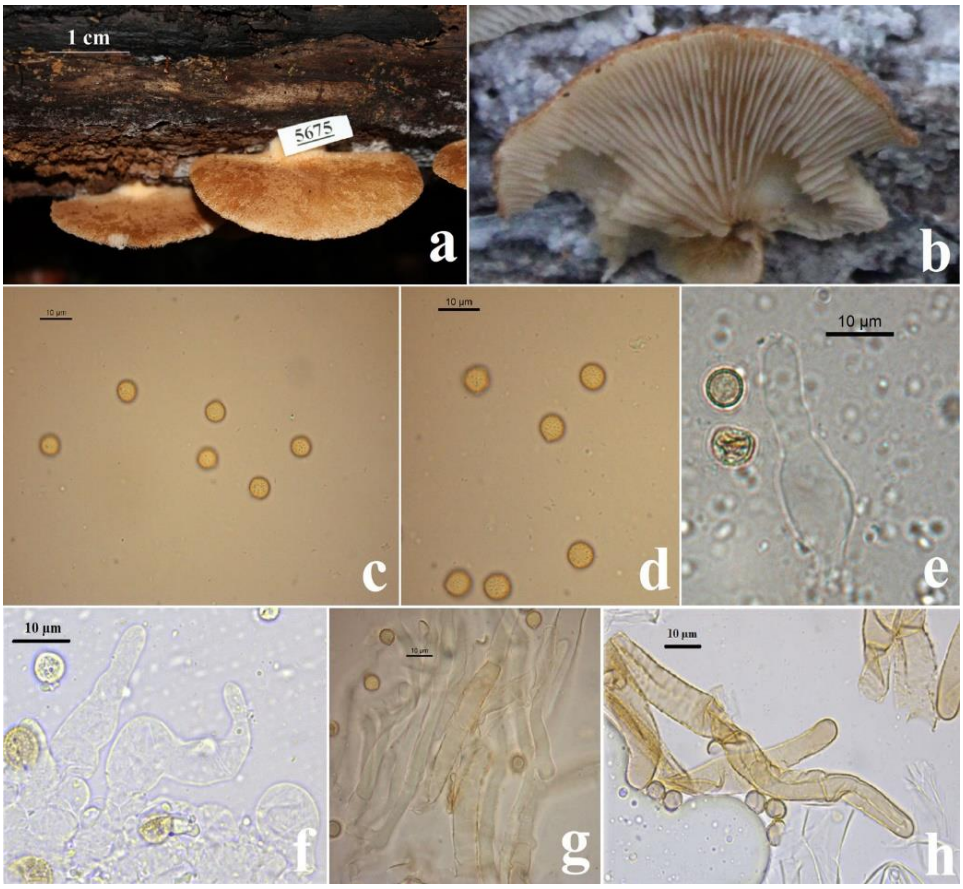

FIGURE 1**.** *Crepidotus crocophyllus*: **a,b.** fruit body. **c,d.** spores. **e,f.** cheilocystidia. **g,h.** pileipellis.

4. Discussion

C. crocophyllus could be recognized by a pileus covered with brown-pigmented fibrils or scales, punctate, warty, globose to subglobose spores, clavate, cylindrical or lageniform cheilocystidia, lack of pleurocystidia, pileipellis as a cutis, pigmented, thick-walled and generally incrusted hyphae [1]. *C. mollis* macroscopically resembles *C. crocophyllus*. Both species produce flabelliform pileus covered with brownish fibrils and absence of stipe but *C. mollis* can easily be distinguished from *C. crocophyllus* by its elliptical spores. Like *C. crocophyllus, C. appalachianensis* Hesler & A.H. Sm., *C. subfibrillosus* Hesler & A.H. Sm. and *C. aureifolius* Hesler & A.H. Sm. have globose and punctate spores but the presence of the pleurocystidia are characteristic of these species [2,3].

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SYNTHESIS OF OPEN-CHAIN SUGAR DERIVATIVES AS ANTICANCER AND ANTIMICROBIAL AGENTS

İDRİS YAZGAN

ABSTRACT. In the present study, three sugar residues including mannose, galactose and lactose were modified with organic substituents via reductive amination reaction in order to get strong anticancer carbohydrate derivatives for A549 cell line and antibacterial agents targeting *E.coli* and *S.epidermidis*. The findings showed that carbohydrate residue along with substituted group play the major role for the observed activity of the carbohydrate ligands.

1. INTRODUCTION

Carbohydrates, the most common molecular group, are composed of simple sugars and complex polymeric structures [1]. Bioactive and bio-degradable sugars are of great interest in biochemistry due to their essential roles in molecular recognition [2]. The essential roles of carbohydrates are related to their interaction with lectins, which are proteins specific for carbohydrate recognition [3]. This is the reason behind the use carbohydrates as possible drugs and drug-precursors for different applications including carbohydrate-based antibiotic development [4]. For example, variety of monosaccharides and disaccharides have been developed and tested as antimicrobial and anticancer agents [5,6]. During the modification of carbohydrates, the groups attached to sugar residue bring tremendous difference for selectivity and sensitivity of the sugars for the lectins [7,8]. The observed sensitivity and selectivity between the sugars and lectins are similar to the outstanding selectivity observed for antigen-antibody interactions [4,9,10].

During cancer development, lectin composition of cell surface undergoes alteration; for example, new lectin groups can arise that result in the alteration of carbohydrate affinities towards the surface lectins. For instance, cancerous lung cells increase the number of galactose-binding lectins on their surfaces [11]. Similarly, mannose binding capacity of lung cells show increasing trend upon pathology development [12,13]. Particularly lactose-amine specific galectin-3 is increased during initial stages of lung cancer [14] while that takes place in metastasis of other cancer to lung [15]. Therefore, in this study mannose, galactose and lactose residues were used to develop carbohydrate ligands.

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2. MATERIAL AND METHOD

2.1. Materials

The following chemicals were purchased from Sigma-Aldrich (Ankara, Turkey), including Nutrient Broth, Nutrient Agar, NaCl, α-D-Mannose, α-D-Galactose, β-D-Lactose, 5-aminosalicylic acid, p-aminosalicylic acid, 4,4'-oxydianiline, 4-mercaptoanyline, acetic acid, borane dimethyl amine complex. 18.2 MΩ pure water was produced in our labs (Human Power 2 pure-water system).

2.2. Synthesis of Carbohydrate Derivatives

FIGURE 1: Synthesis of carbohydrate derivatives. R_1, R_2, R_3, R4 and R_4 can be –H, COOH, -OH and –SH while R group on the sugar residue was either –H or a monosaccharide.

Synthesis of sugar derivatives, as illustrated in Figure 1, were performed in 1:1 acetic acid/water mixture, for which purification was not needed to isolate imin from the media, where reducing agent boran dimethyl amine (DMA) was directly added to the mixture after fully completition of the imin formation [16]. Hydrophilic liquid interaction based (HILIC) approach was used to monitor reaction steps on thin layer chromatography.

2.3. Characterization of the carbohydrate-derivatives

^{1}H NMR and ESI-MS/MS based characterization techniques were used to characterize the synthesized sugar derivatives.

2.3.1 ^{1}H NMR Characterization

Each carbohydrate-derivative was dissolved in D_2O solvent at 5 mg/mL, followed by run in 400 MHz Jeol NMR. The results were analyzed in Delta NMR Software and Topsin 4.06 Software.

2.3.2. ESI-MS/MS Characterization

Molecular weight and fragmentation pattern of the sugar derivatives were characterized via Shimadzu LC-ESI-MS/MS-8030 Plus instruments.

2.4. Anticancer performance of the sugar ligands

Lung adenocarcinoma A549 cell line was used as target cell line while rat intestine epithelial non-cancerous immortalized IEC-6 cell line was used as control cell line. Cell culture studies for both cell lines were performed using Eagle modified medium (EMEM) supported with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 unit/ml bovine insulin, 10 % (v/v) fetal bovine serum and 4 mL/L of essential amino acids. The incubation was at 37 °C in 5% CO_2 incubator. Fresh 10^4 cells/mL in 100 µL were inoculated into 96-well plate, followed by 24 h incubation to provide cell attachment to the surface. Then after, sugar derivatives at 0.1 and 1 mM in 10 µL were added to the cells, where pure-water was used as the control. Cells were exposed to the carbohydrate derivatives for 24 h, followed by viability of the remaining cells were measured using Alamar-blue based fluorescent assay in a Microplate reader.

2.5. Antimicrobial performance of the sugar ligands

To characterize antibacterial activity of the carbohydrate derivatives, gram negative *Escherichia coli* and gram positive *S. epidermidis* were used as model organisms. Both cells were grown in Nutrient-Broth at 37 °C for overnight, followed by centrifugation at 3000 g. The collected cells were suspended in fresh Nutrient Broth. 1 mL samples of *E.coli* and *S.epidermidis* at 10^3 cfu/mL concentration were placed in 1.5 mL sterile polypropylene plastic tubes. Sugar derivatives at 1 mM in 50 µL were added to the bacteria samples to investigate the antimicrobial activity, where 16-h incubation at 37 °C was used as the minimum treatment period. After the incubation, turbidity of the medium was measured at 595 nm in PG Instruments T60 Spectrometer.

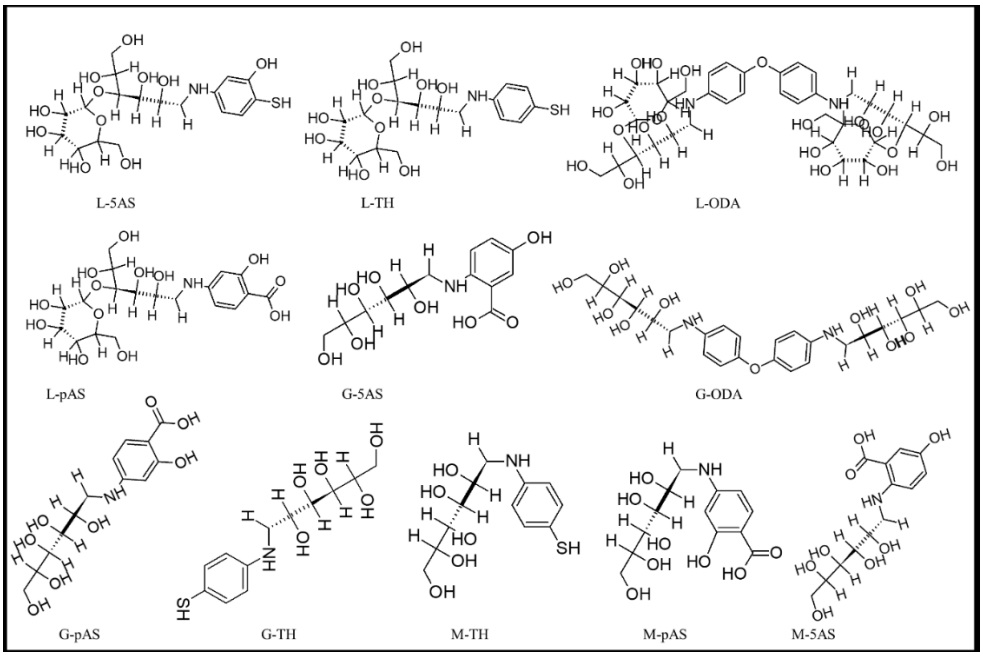

FIGURE 2: The carbohydrate derivatives synthesized in the study. Abbreviations: L-5AS: Lactose-5-aminosalicylic acid; L-pAS: Lactose-4-aminosalicylic acid; L-TH: Lactose-4-mercaptoaniline; L-ODA: Lactose-4,4'-oksidianiline; G-pAS: Galactose-4-aminosalicylic acid; G-5AS: Galactose-5-aminosalicylic acid; G-TH: Galactose-4-mercaptoaniline; G-ODA: Galactose-4,4'-oksidianiline; M-pAS: Mannose-4-aminosalicylic acid; M-TH: Mannose-4-mercaptoaniline; M-5AS: Mannose-5-aminosalicylic acid.

3. RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of the Sugar Derivatives

The straight forward synthesis of open chain sugar derivatives allows cheap, fast and high yield production of novel sugar derivatives that can possess unique properties, which advance sensitivity and selectivity of the inherent carbohydrate molecule towards the target lectin [17]. Purification of the carbohydrate products was performed via liquid-liquid separation technique thanks to the elevated hydrophilicity of carbohydrate derivatives that does not allow them to be dissolved in hydrophobic solvents including acetone, tetrahydrofuran and relatively less hydrophilic ethanol. During the reaction, organic

substituents were used 20 % more than that of used for sugar residue in order to increase the effectiveness of the liquid-liquid extraction. All of the synthesized sugar derivatives are shown in Figure 2. Purified products were then characterized with ^{1}H NMR and ESI-MS/MS, where only the results belong to characteristic carbohydrate derivatives are given with original spectrums.

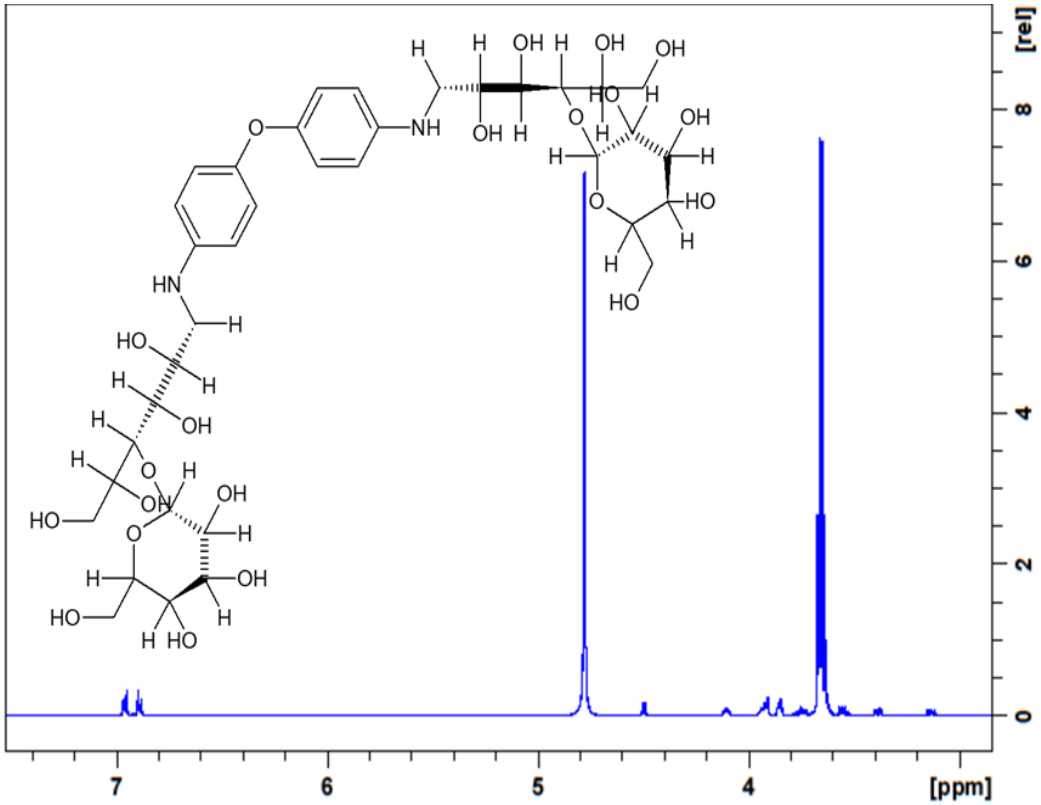

FIGURE 3a: ^{1}H NMR spectrum of Lactose-4,4'-oxydianiline.

ODA bound to lactose residue has only two characteristic microenvironment, so there are only two peaks at aromatic region (6.90 and 6.97 ppm) (Figure 3a). The lactose residue lost its shift at ~ 5.2 ppm related to α/β conversion of C_1-H, which revealed that the synthesis and purification was completed.

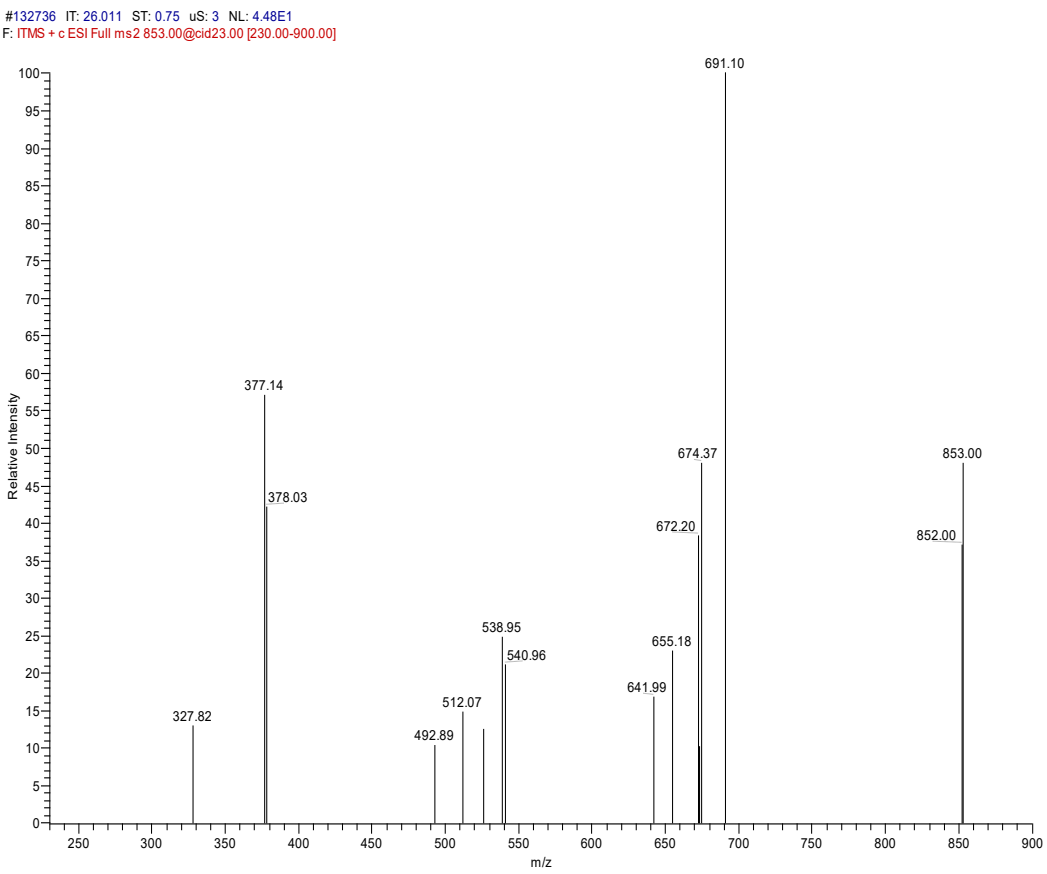

FIGURE 3b: MS/MS ms^2 pattern of Lactose-4,4'-oxydianiline.

As seen in Figure 3b, L-ODA gave 853 m/z; L-ODA originally has 848 Da, and +1 comes from positive EIS mode while the +4 comes from protonation of two amin groups within the molecule. The fragmentation pattern gave 691 m/z that resulted from loss of one deoxy-glucose from lactose while 674 m/z and 655 m/z values were resulted from loss of water molecules of the sugar residue.

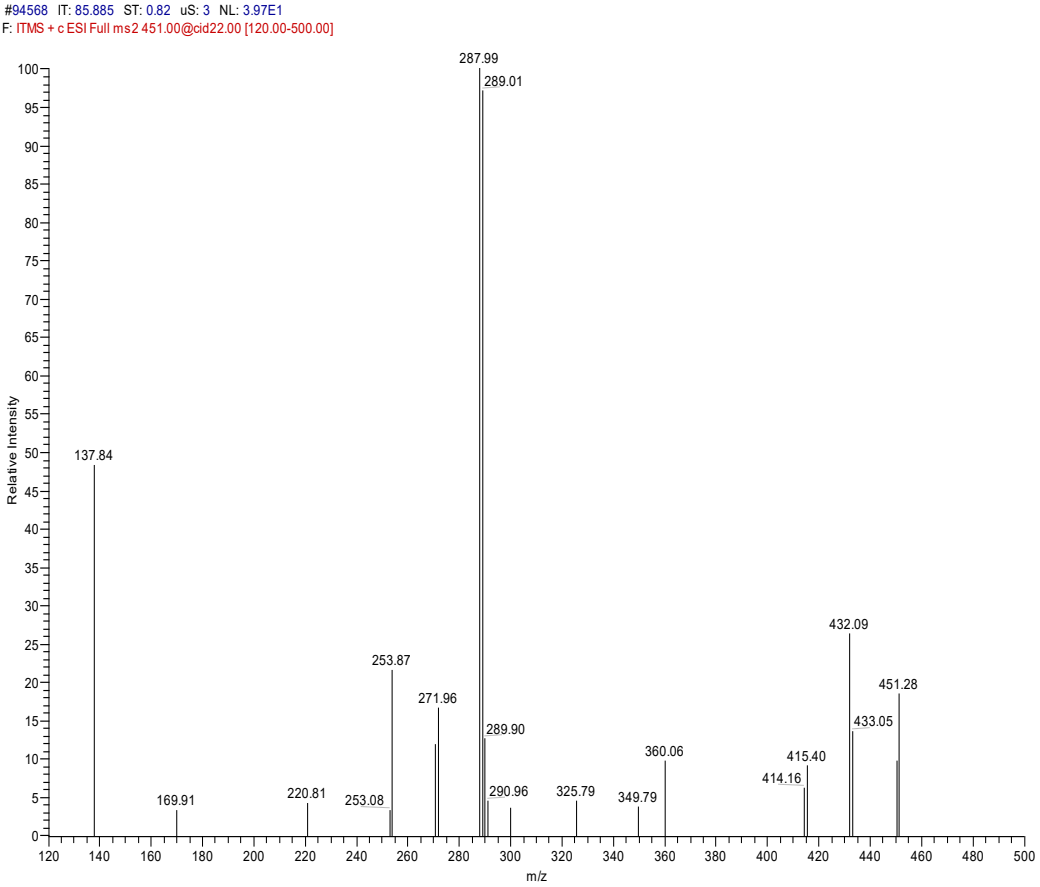

FIGURE 3c: MS/MS ms^2 pattern of Lactose-*p*-mercaptoanilin (L-TH).

As seen in Figure 3c, L-TH gave 451 m/z; L-TH originally has 449 Da, and +1 comes from positive EIS mode while the +1 comes from protonation of amin groups within the molecule. Amine groups found on different sugar resides tend to gain different proton because of the geometry of the molecule. 433 and 415 m/z values were resulted sequential elimination of water molecules from sugar residues of the L-TH. As observed for L-ODA, glucose residue of lactose within L-TH molecule ditched during fragmentation and 290 m/z value arose.

3.2. Anticancer Activity

Either anticancer activity of carbohydrate-based drugs is resulted from carbohydrate-lectin interaction, which can intervene signaling cascades responsible in gene expression controlling expression of such genes including apoptosis related genes, or the carbohydrates can interact with enzymes. Besides, a more complex mechanism can determine the carbohydrate mediated intervention of cellular mechanisms. As shown in Figure 4a, the carbohydrate derivatives showed dose-dependent toxicity on A549 cancerous cell line. Sugar residues substituted with same organic groups revealed dramatic toxicity differences, where the sugar residue played key role for the observed anticancer activity. For example, in the case of 4-mercaptoanilin modification, mannose derivative gave by far the most toxic activity for A549 cells while L-TH showed nearly no toxicity. Besides, G-TH derivative at 1 mM concentration gave lower toxicity than 0.1 mM of M-TH. Similarly, 4-aminosalicylic modified mannose revealed relatively higher toxicity than that of observed for lactose and galactose derivatives. Another important observation was that 5-aminosalicylic acid modified derivative of galactose gave higher toxicity than its 4-aminosalicyclic acid modified version, which situation was opposite for mannose and lactose derivatives. Therefore, it can be speculated that alteration in the position of functional group located on the substituent shows its potency depending on the sugar residue.

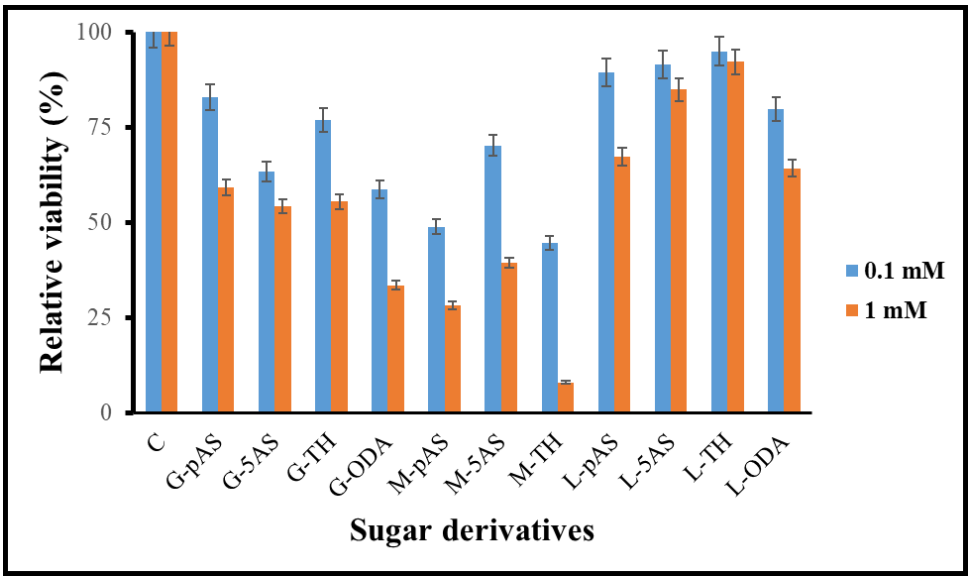

FIGURE 4a: Cytotoxicity of the carbohydrates on A549 cell line at 0.1 and 1 mM concentration.

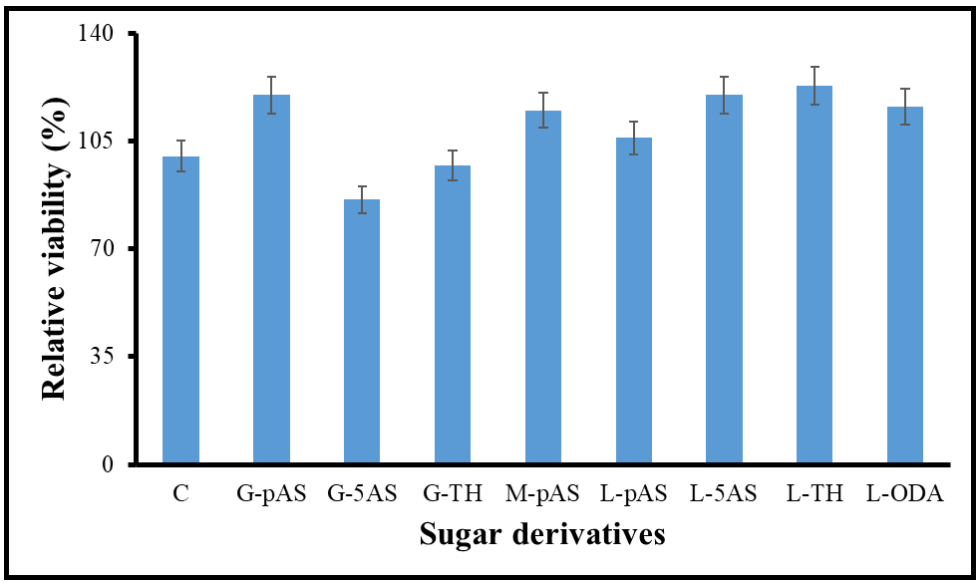

FIGURE 4b: Cytotoxicity of the carbohydrates on IEC-6 cell line at 1 mM concentration.

The sugar derivatives were then applied to non-cancerous IEC-6 cells to show their selectivity for the cancerous cell line. As seen Figure 4b, only G-5AS showed discernible toxicity (14%) while most of the derivatives behaved as either nutrient or signaling agent to enhance cellular growth in comparison to the control.

In carbohydrate-based drug development studies, the carbohydrates are modified in order to improve the interaction between the sugars and the carbohydrate-recognition domain of the lectin along with facilitating CRD's surrounding involvement to the recognition [3,9]. Another important phenomenon is that homo- and/or hetero-disaccharides in comparison to their monosaccharide versions can show similar or even higher affinities for the same lectin [17]. Therefore, the obtained differences can result from the differences in CRD regions along with its surroundings.

3.3. Antibacterial Results

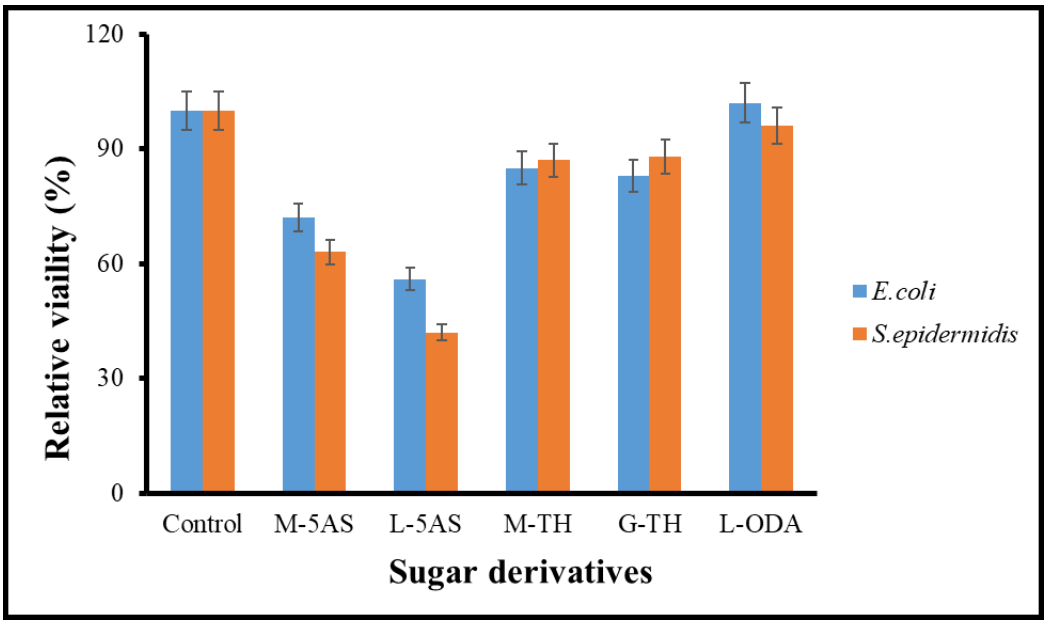

FIGURE 5: Antibacterial activity of selected carbohydrate derivatives.

As seen in Figure 5, L-5AS showed the highest toxicity towards *E.coli* and *S.epidermidis* while L-ODA did not show any toxicity for the tested bacteria. When the antibacterial capability of the sugar derivatices was compared with the anticancer capabilities, the carbohydrates can provide a good candidates as anticancer agents.

4. CONCLUSION

Carbohydrates play key roles in regulation of cell metabolism and cell-cell interaction that make them unique molecules, whose regulations strictly controlled by genes. Their modifications introduce novel properties for their sensitivity and selectivity towards the target mammalian and microbial cells. Therefore, in this study, mannose, galactose and lactose were modified with a variety of organic groups to advance the sensitivity towards cancerous A549 cell line and gram negative E.coli and gram positive S.epidermidis. The findings revealed that substituted organic group brought anticancer and antibacterial activity along with sugar residue selection. The findings can call new research on to understand how all these modified carbohydrates showed their unprecedented capabilities.

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INVESTIGATION OF MYXOMYCETES (MYXOMYCOTA) IN KIRIKHAN (HATAY PROVINCE)

HAYRİ BABA, ERDAL CENNET AND MUSTAFA SEVİNDİK

ABSTRACT. The present study was conducted with the samples collected in 10 different stations in Kırıkhan district and vicinity in 2012-2014. The samples were collected from tree barks, leaves, wood and other decayed plant material. The collected materials were used in Moist Chamber Culture to develop myxomycetes sporophores. Furthermore, myxomycetes were collected from their natural environment. In the field and laboratory studies, 45 taxa in 10 families and 22 genera were identified.

1. INTRODUCTION

Myxomycetes, known as plasmodial slime molds, true slime molds, or Mycetozoa are multinuclear fungus-like organisms without a cell wall that produce one or more spores and sporophores. Based on the current classification system, they are a member of the Mycetozoa group in Protista regnum and commonly found in terrestrial ecosystems. In the vegetative stage, they have a plasmodium, transparent adhesive sheath, and a pile of acellular protoplasm [1,2]. The plasmodium is a membrane-bound single cell that contains multiple nuclei. The generative stage includes four types of myxomycete fruiting bodies. The most common type is the sporangium. Fruiting bodies are usually composed of 6 parts: hypothallus, stalk, columella, peridium, capillitium and spores. In certain fruiting bodies, a pseudo-columella or a pseudo-capillitium may be present. Not all of these components are present in all fruiting body types. The columella is observed as an extension of the stalk into the spore mass, although it may not resemble the stalk. In a sessile fruiting body, the columella may be an area on the inside surface of the peridium where it contacts the substrate or may appear as a dome-shaped structure. A pseudo-columella is a columella that is not attached to the stalk. Capillitial elements may be attached to the columella or pseudo-columella. The capillitium includes thread like elements within the spore mass of a fruiting body. Several myxomycetes species have a capillitium, either as a single connected network,

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or as several free elements called elaters. Capillitial elements may be smooth, sculptured or spiny or they may appear to include several interwoven strands. Spores range in size between about 5 and 15 micrometers. Nearly all are round and most are ornamented to a certain degree. In fact, entirely smooth spores does not exist. Spore ornamentation could be reticulate, echinate, verrucose, or asperulate (with fine warts). Spore shape and size are very important in identification. Spores could be classified as either dark (found in the Stemonitales and Physarales) or light to brightly colored (all of the other orders) [3].

Known Myxomycetes count is 1017 globally [4], in Turkey the same figure is still 284 [5-16]. Turkey is located in a moderate climate and thus it has a very rich flora diversity and the number of available Myxomycetes is expected to be higher.

2. MATERIAL AND METHOD

2.1. Geographical location and vegetation

On the east, Kırıkhan is bordered by Syria and Kumlu, on the west, by Belen, on the north, by Hassa, and on the south by Antakya and Kumlu (Figure 1). Kırıkhan is located between 36-37 degrees north latitude and 36-37 degrees east longitude and the surface area of the district is 687.73 km². The Amik plateau region that extends in the north - south direction is an important passage between the Amanos and Kürd mountains. The most important passages include Yalangoz and Incirli to Syria in the east west direction, Gedik - Belen pass and Atik plateau in the west. The most important mountain in the district is Amanos mountains, which is an extension of the Taurus mountains. It extends to the southern Hatay province. The section in the Amanos where Ceyhan River crosses the mountain range is called Gâvur Mountains until the Belen Passage. The section that extends between the Belen Pass and Antakya is known as Kızıldağ. Gölbaşı Lake is the only lake in the district and exploited for aquaculture and irrigation. The Amik plain, formed by the dried Amik Lake, forms a part of the district. The Delibekir Stream, which passes through the town center, and Karasu that crosses the Amik plain, are the major streams in the district [17].

Forests are destroyed by irregular cutting, fire and grazing. Forests are usually found in mountainous areas. Vegetation on mountain slopes includes *Pinus* sp, *Quercus* sp, *Maquis* and wild olive trees in forests. The forests occupy a surface area of 9521 hectares. The largest green area in the district center is Vali Ürgen Landscaping with 168 hectares. Reeds and various marsh plants grow in the plain marshlands. Agricultural crops are cultivated on the entire plain. Nerium oleander is a poisonous plant that grows naturally. Kırıkhan residents mainly conduct cotton and wheat cultivation. Furthermore, olives, rice, pods, vines are grown. In addition, watermelon, melon, sesame, onion, pepper, tomato, eggplant, pumpkin, cucumber, spinach, lettuce, radish, okra, and beans are grown. The fruits grown in the region include figs, grapes, pears, apricots, pomegranates, plums, oranges, lemons, and mandarins. Fruit orchards are usually located in villages on the mountain slopes [17].

FIGURE 1. Research area

2.2. Climate of research area

The climate in the study area is Mediterranean. In the Mediterranean climate, daily and seasonal photoperiods are experienced, precipitation is usually during relatively cold seasons, and the dry season is summer. The climate is characterized by rains during the rainy season but the rainfall is sparse in other seasons. Most of the precipitation is in the form of a rain that runs on the soil and provides very little irrigation for the soil and plants. The average temperature is 7.31 degrees in winter and 32.3 degrees in summer [17].

2.3. Collection of samples

Samples were collected at 10 stations in Kırıkhan district and vicinity between 2012 and 2014, and the field trips were conducted in autumn, winter, spring and summer. Field study and sample collection locations and sample collection dates are presented in Table 1.

TABLE 1. Land trip dates and location information

Stations	**Dates**	**Altitude(m)**	**Coordinates**
Alan way	23.11.2013	1150	36° 36' 52" N; 36° 21' 27" E
Bektaşlı	20.04.2013	450	36° 65' 48" N; 36° 40' 72" E
Ceylanlı	02.02.2013/16.02.2014	280	36° 55' 92" N; 36° 38' 06" E
Delibekirli	02.03.2013/16.03.2013	525	36° 53' 91" N; 36° 31' 45" E
Karataş	15.12.2012/29.12.2012	300	36° 50' 15" N; 36° 33' 84" E
Kodallı	30.03.2013/06.04.2014	95	36° 54' 36" N; 36° 40' 45" E
Kurtlusoğuksu	18.11.2012/01.12.2012	85	36° 48' 66" N; 36° 29' 92" E
Saylak	04.04.2013	250	36° 62' 37" N; 36° 41' 07" E
Taşoluk	16.11.2013	450	36° 62' 66" N; 36° 38' 89" E
Telbizek	05.01.2013/19.01.2013	260	36° 54' 29" N; 36° 36' 69" E

Natural Myxomycete samples were collected from natural substrata, barks, woods, debris material. Samples were transferred to the laboratory in small carton boxes. Furthermore, of myxomycete fructifications were cultured in a moist chamber in the laboratory. All moist chamber cultures were prepared within a week after the field survey. Substrates were placed in petri dishes lined with filter paper. Distilled water was added to each petri dish and the samples were allowed to soak overnight. After 24 hours, excess water was removed. Cultures were maintained under diffuse light at room temperature (22-25°C) for approximately three months. All cultures were checked weekly for the presence of myxomycete plasmodia or fruiting bodies [7]. When myxomycete development was observed, the moist chamber was allowed to dry slowly and the myxomycetes were then dried further for one week. The samples were prepared as fungarium material and stored in the laboratory.

2.4. Identification of samples

Myxomycetes usually include 6 sections: hypothallus, stalk, columella, peridium, capillitium and spores. For the identification of the samples, a stereomicroscope and a high-resolution light microscope were used. Stereomicroscope examines the general structure, fructification

type, shape, color, macroscopic measurements, the presence or absence of lime or the color and shape of samples. With light microscopy, it is possible to observe whether the capillitium, pseudo-capillitium and columella or pseudo-columella were present, the shape, size and form of the capillitium, the branching form, whether the columella is free or attached to the stem, the characteristics of pseudo-capillitium, the shape, color, size and ornamentation of the spores in detail.

The Myxomycetes specimens were identified based on relevant references such as Martin and Alexopoulos [18], Farr [19], Thind [20], Farr [3], Martin et al. [21], Neubert et al. [22], Stephenson and Stempen [1], Alexopoulos et al. [23], Lado and Pando [24], Sesli et al. [12].

3. RESULTS AND DISCUSSION

Between 2012 and 2014, 377 samples were collected at 10 different stations in Kırıkhan and processed in the laboratory, revealing 208 myxomycetes. Identification of myxomycete samples collected in the natural environment and grown in moist chamber culture revealed a total of 45 species in 5 groups, 10 families, and 22 genera. Twenty-two samples were collected in the natural environment, 186 samples were obtained in the moist chamber culture, 11 samples were obtained in both the natural environment and the moist chamber culture. The water pH was measured before the sample water was discharged during the application of moist chamber technique and it was determined that the sample pH values were generally neutral. This finding was consistent with the findings reported by Härkönen and Uotila [25].

The relative abundance of fruit bodies of each species was determined by the categorization based on a modification of the method proposed by Stephenson et al. [26]. For this purpose, species represented in more than 3.0% of the collections were considered abundant (A), those that were represented between 1.5 % and 3.0 % were considered common (C), those that were represented between 1.5 % and 0.5 % were considered occasional (O), and those that were represented in less than 0.5 % were considered rare (R) [26]. The mean number of species per genus (S/G) was calculated based on the collected datasets in the study area. In the present study, it was determined that 9 species were abundant (A), 4 species were common (C), 7 species were occasional (O), and 25 species were rare (R) (Table 2). The mean number of species per genus (S/G) was calculated with the data collected in the study area and it was determined that the species/genus ratio (S/G) was 2.04. The Myxomycete biodiversity in Antakya was 3.64, and 2.3 in Kuseyr mountain [7]. This value was significant when compared to other study findings. For example, in a North American study, it was observed that S/G ratio in Mountain Lake was 3.65 and in Cheat Mountain, it was 2.24. Another study calculated S/G ratios in northwestern India and southern India as 3.04 and 4.13, respectively [26]. A low S/G reflects a higher overall diversity when compared to a high S/G rate.

TABLE 2. Myxomycetes name and occurrence

No	Species	Occurrence	No	Species	Occurrence
1	*Echinostelium minutum*	O	24	*P. oblatum*	R
2	*Cribraria cancellata*	R	25	*P. robustum*	C
3	*C. intricata*	R	26	*Arcyria cinerea*	A
4	*Licea castanea*	R	27	*A. globosa*	O
5	*L. kleistobolus*	R	28	*A. incarnata*	O
6	*L. minima*	R	29	*A. minuta*	A
7	*L. pedicellata*	R	30	*A. pomiformis*	R
8	*Lycogala epidendrum*	R	31	*Perichaena vermicularis*	R
9	*Dictydiaethalium plumbeum*	R	32	*Trichia contorta*	R
10	*Diderma hemisphaericum*	R	33	*T. munda*	R
11	*Didymium bahiense*	A	34	*Collaria lurida*	R
12	*D. difforme*	C	35	*Comatricha ellae*	A
13	*D. megalosporum*	C	36	*C. laxa*	O
14	*D. melanospermum*	R	37	*C. nigra*	A
15	*D. squamulosum*	A	38	*C. pulchella*	R
16	*Badhamia macrocarpa*	R	39	*Enerthenema papillatum*	R
17	*B. panicea*	O	40	*Lamproderma arcyrioides*	R
18	*Craterium leucocephalum*	R	41	*Paradiacheopsis longipes*	R
19	*Leocarpus fragilis*	R	42	*Stemonitis fusca*	A
20	*Physarum album*	A	43	*Stemonitopsis amoena*	A
21	*P. contextum*	O	44	*S. typhina*	C
22	*P. leucopheum*	O	45	*Symphytocarpus*	R
23	*P. notabile*	R		*sp.*	

In the literature, myxomycete samples were mostly observed on decayed Gymnosperm woods, leaves and debris [1,3,11,23], and the number of field studies on myxomycetes have increased. However, myxomycete spores were collected and processed on different material that might have been infected. Liceales, Trichiales and Stemonitales are generally known to be present in coniferous forests [18, 27,28]. The majority of the specimens were identified on Gymnosperm rashes.

If myxomycete distribution is based on the substrate on which it was developed, corticolous myxomycetes develop on plant barks, lignicolous myxomycetes develop on rotten wood and barks, foliiaceous myxomycetes develop on leaves, fimicolous myxomycetes develop on animal manure, nivicolous myxomycetes are those with special needs for development [29]. In our study area, samples were collected only from bark and rotten wood.

Natural samples increased in winter in our study area, however myxomycetes species obtained with the moist chamber technique increased in the autumn. The best months for finding Plasmodial slime molds in our research area is winter and spring. Due to rain, relative humidity is apparently at an optimum level and temperatures are mild. Primary characteristics of these months in our research area include alternate rainy and sunny periods. These provide favorable conditions for adequate moisture levels and suitable temperatures that allow

Plasmodial slime molds complete their life cycle. The present study findings were consistent with previous studies conducted in Hatay, Turkey and other locations based on the seasonal distribution of myxomycetes.

The distribution of the species determined in our study area demonstrated that there were four families (*Stemonitidaceae*, *Physaraceae*, *Didymiaceae* and *Arcyriaceae*) that included 35 species. This figure constituted 76% of the species collected in our study area. This rate was determined as % 71 by Yağız [30], as 72.4% by Baba [31], as 70% by Baba et al., [11], and these findings were similar to our and several other studies conducted in Turkey.

The genera and species determined in our study area included 6 *Physarum*, 5 *Arcyria*, 5 *Didymium*, 4 *Comatricha*, 4 *Licea*, 2 *Badhamia*, 2 *Cribraria*, 2 *Stemonitopsis*, 2 *Trichia*, 1 *Collaria*, 1 *Craterium*, 1 *Dictydiaethalium*, 1 *Diderma*, 1 *Echinostelium*, 1 *Enerthenema*, 1 *Lamproderma*, 1 *Leocarpus*, 1 *Lycogala*, 1 *Paradiacheopsis*, 1 *Perichaena*, 1 *Stemonitis*, and 1 *Symphytocarpus* species.

Analysis of the distribution of myxomycete species demonstrated that *E. minutum, A. cinerea, A. denudata* and *S. fusca* were the most common species in the present study. Most myxomycetes species were cosmopolitan, the humidity and temperature were the main factors in diversity and abundance of these species. In most studies, it was observed that these species were commonly distributed across several substrates [1]. *A. pomiformis, A. cinerea, C. ellae* and *C. nigra* were identified in almost all stations. *Stemonitopsis amoena* is detected in 6 out of 10 stations. Most myxomycetes species grow globally and, in most studies, these species were observed to be prevalent in several substrates [30, 32-34].

Altitude is an important factor for various myxomycete families. According to Rojas and Stephenson [35], as the altitude increases, the type and number of myxomycetes significantly decrease. In high altitudes, substrate pH decerases. In the present study area, *Liceaceae* was most prevalent near sea level. Physaraceae prevalence exhibited a rapid increase above 750 m. Stemonitaceae were prevalent at almost every altitude. The highest Didymiaceae prevalence was observed at low altitudes. It was not possible to identify Arcyriaceae at low altitudes. It grows at altitudes between 20 and 750 m. Thus, it was found that Didymiaceae and Liceaceae were more prevalent at low altitudes and their prevalence decreased as altitude increased. Arcyriaceae was identified mostly in mid altitudes. Stemonitaceae could adapt to all altitudes except the coastline. However, although Physaraceae were observed at all altitudes, it could be suggested that the highest prevalence was observed at high altitudes. The sizes of *A. cinerea, P. corticalis, P. album* and *S. amoena* change at high altitudes. Furthermore, all studied species were highly prevalent at an altitude of 100-400 m. In contrast, certain Mycetozoa species prefer certain altitudes or are only present at these altitudes. *Didymium difforme* was prevalent between 50-100 m, *L. castanea* was prevalent between 20-300 m [36].

In the present study, most prevalent ornamented spore was verruculose or verrucose, followed by spinulose. The least common types of ornamentation were smooth, reticulate and echinulate. It was suggested that the net and thorn type ornaments should be more prevalent in wider surfaces, improving their attachment to the surface. Since flying ornamented spores attach to the surface better, this improves their susceptibility to germination. Comparison of the study samples based on sporophore types demonstrated that fructification types of the collected species were generally sporangium, 61% of the species were sporangium with stem. Pseudoaethalium, aethalium, and plasmodiocarpic fructification rates were almost identical.

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4. CONCLUSION

Middle Amanos mountains are located on one side of the Kırıkhan district and Amik plain on the other, and the district has cosmopolitan geographical, climatic and surface shapes, rich biodiversity and rich myxobiota. In the study area, climatic conditions and vegetation are suitable for myxomycetes, as demonstrated in the present study findings, and the present study added a total of 45 species to the regional and Turkish myxobiota.

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NOMENCLATURE

Current myxomycetes names were checked from nomen eumycetozoa
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PUTRESCINE AS A PROTECTIVE MOLECULE ON DNA DAMAGE AND DNA METHYLATION CHANGES IN WHEAT UNDER DROUGHT

ESRA ARSLAN, GÜLERAY AĞAR and MURAT AYDIN

ABSTRACT. The world suffers with the agricultural drought stress which leading to decreasing crop production, and also adversely affecting cereals on morphological, physiological, biochemical and molecular levels. However, exogenous treatment of some osmotically active materials like putrescine has been regarded as a good preventive against these harmful effects of drought. But there is a lack of information on putrescine has any effects on DNA damage and DNA methylation in crops. The current study was goal to determine DNA damage levels and DNA methylation changes in *Triticum aestivum* cv. Karasu 90 subjected to different concentrations of drought (-2, -4, -6 bar PEG) and whether putrescine (0.01, 0.1, 1 mM) has any ameliorative effect on these changes is determined with RAPDs and CRED-RAs techniques. In addition, total oxidant status (TOS) and total antioxidant status (TAS) values were investigated based on drought and putrescine treatments. The findings showed that drought stress caused DNA damage and DNA methylation changes. However, these effects decreased after putrescine treatments. Putrescine has been shown to decrease oxidative damage caused by drought via increasing antioxidant status in drought stress. According to results, it was concluded that putrescine could be preferred for its force to protect wheat DNA from the damaging effects of drought and the demethylation positively contributed to drought stress tolerance.

1. INTRODUCTION

Drought, which is a major abiotic stress globally, brings on extensive limits on crop productivity due to its unsuitable influences on plant morphology, physiology and also biochemistry, preventing growth and development [1]. Moreover, long-term drought induces oxidative stress by increasing the production of reactive oxygen species (ROS). ROS are constantly synthesized as byproducts in the chloroplast, mitochondria and peroxisome parts of the plant under normal conditions but increasing in stress conditions and they can damage the phospholipids of cell membranes, chlorophyll, proteins and nucleic acids [2]. In particular, irreparable oxidative stress-related damages to the DNA strand give rise to instability in the genome [3]. Plants have antioxidant defense organization to prevent oxidative damage caused by ROS. Antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathion peroxidase (GPX), catalase (CAT), etc., play a role in the direct removal of ROS and inhibit uncontrolled oxidation steps [4].

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Drought also alters gene expression via epigenetic modifications like DNA methylation and histone modifications [5-7]. It has been presented that water stress induces cytosine methylation in crops like wheat [8], pea [9], rice [10] etc. in many researches. Considering the worse effects of drought especially on the wheat which is the world's most grown and consumed crop, it has been inevitable to investigate the impact of DNA methylation on wheat. Furthermore, various DNA methylation patterns indicated in tolerant and sensitive wheat genotypes under drought stress [8].

Plants improve some strategies that are at morphological, anatomical, biochemical and molecular levels to avoid or tolerate the stresses which allow them to adapt and defense themselves from stress so as to cope up all these stresses [11]. One of them is phytohormones. Plant hormones play an important role in the regulation of plant responses to the environment [12]. Many researchers reported that plant hormones regulate plant responses to oxidative stress elicited by different stress factors [13, 14]. One can understood from these papers that osmotic, cold and drought stress caused to increase of ABA, salicylic acid and polyamine levels. Polyamines (putrescine, spermidine, spermine and cadaverine) are important growth regulating molecules known to participate in a wide variety of developmental events, including flowering, senescence, root development, organogenesis and embryogenesis [15, 16]. Plants exposed to abiotic stress raise polyamine levels to help regulate themselves tolerance to stress. Polyamines provide tolerance to stress as bounding to RNA and DNA guard DNA from enzymatic degradation, oxidative damages, mechanical shearing. Moreover, Polyamines stabilize RNA, to counteract of ribosomal dispersion [17]. It was the first indicate by Ruiz-Herrera et al. (1995) [18] that the impact of polyamines on cytosine-DNA methyltranferases was quite selective and this effect related to both the binding and activity of the methylases by polyamines. However, the protective effect of polyamines against DNA damage and DNA methylation changes in plants subjected to drought stress has not been elucidated.

The main of present study was to see whether putrescine has any protective effect against genetic and DNA methylation variations in *Triticum aestivum* cv Karasu 90 in drought stress. We used RAPDs to investigate the genetic damage and CRED-RAs to access the differences in methylation level and changes of pattern of DNA methylation. Also, total oxidant status (TOS) and total antioxidant status (TAS) were determined in drought stress and putrescine treatments.

2. MATERIAL AND METHOD

2.1. Plant material and treatment conditions

Karasu 90 (*Triticum aestivum* L.), which is a drought-sensitive cultivar, was used as plant material in this study. The equal seeds were surface-sterilized with 0.5% sodium

hypochlorite solution a 5 minutes and afterward rinsed several times with sterile distilled water. Sterilized seeds were soaked in various doses of putrescine [0 (distilled water), 0.01, 0.1 and 1 mM] (Sigma, 51799) for 24 h at 25±1 °C in darkness as pretreatment. The solutions were then carefully removed and the seeds were dried for 1h in laminar flow cabinet (Esco Airsystem, Singapur). Replicates of 25 seeds were sown in 12 cm diameter sterile petri dish with two layers of filter paper saturated with solution of different osmotic potentials (0, -2, -4 and -6 bar) which were created with PEG 6000 (Sigma Aldrich, USA) according to Michel and Kaufmann's equation [19]. The dishes were kept at 25 ± 1°C in 16 h photoperiod. Each treatment was replicated three times. Afterwards 10 days of germination, young leaves were harvested randomly from ten plants for each treatment and snap frozen in liquid nitrogen.

2.2. Genomic DNA isolation

The genomic DNA was obtained from young leaves using the method specified by Taspinar et al. (2017) [20] and stored at -20 ºC for later on use. The quality and quantity of isolated DNA were measured using a Nano-Drop (Qiagen, Qiaxpert Instrument, Germany) spectrophotometer and 1% (w/v) agarose gel with ethidium bromide staining.

2.3. RAPD and CRED-RA procedures

13 oligonucleotide primers (Sentegen Biotechnology, Türkiye) (OPA-4, OPA-12, OPH-16, OPH-18, OPH-19, OPB-10, OPY-1, OPY-7, OPY-13, OPW-4, OPW-6, OPW-13 and OPW-18) amplified polymorphic amplicons and used in RAPD-PCR reactions. For CRED-RA analysis, genomic DNA sample from each treatment were separately digested with HpaII (New England Biolabs, USA) and MspI (New England Biolabs, USA) endonucleases according to manufacturer’s instruction. Digestion was checked on 1% (w/v) agarose gel and after 1µl of each digestion product were amplified with 8 RAPD primers (OPA-4, OPB-10, OPH-18, OPY-1, OPY-13, OPY-15, OPW-4 and OPW-13). PCR amplifications (SensoQuest GmbH, Germany), electrophoresis (Bio-Rad, USA) and procedures for each technique were carried out according to Taspinar et al. (2017) [20].

2.4. Determination of TOS and TAS

TOS and TAS values for treatments were measured with Rel Assay brand commercial kits (Rel Assay Kit Diagnostics, Turkey).

2.5. Analysis

Molecular analysis (RAPD and CRED-RA) were carried out with Total Lab TL120 computer software. Genomic template stability (GTS, %) for RAPD and the average of polymorphisms

(%) for CRED-RA were obtained according to Taspinar et al. (2017) [20]. To determine Polymorphism Information Content (PIC) and Discriminating Power (D) values, Botstein et al. (1980) [21] and Prevost and Wilkinson (1999)'s [22] articles were used. A data matrix was created from RAPD gels by assigning 1 to present bands and 0 to absent bands. The data matrix was used to compute pairwise Jaccard similarity coefficients among all the drought and putrescine treatments (NTSYS-pc, ver. 1.8). Cluster analysis (UPGMA,SAHN in NTSYS) was performed on the matrix of Jaccard coefficients [23]. All data obtained from TAS and TOS parameters were analyzed by one way ANOVA using SAS PROC GLM (SAS version 9.4, SAS Institute Inc., Cary, NC). Treatment means were compared using the Fisher's least significant difference (LSD) at $p<0.05$.

3. RESULTS

3.1. RAPD

3.1.1 Levels of GTS

Totally, 32 oligonucleotide primers with %60-70 GC content were tested with untreated DNA (0 mM putrescine + 0 bar PEG6000) and only thirteen gave specific and stable results (TABLE 1). A total of 92 bands were obtained in control treatment. Among these 10 bands were occurred in OPH-19 (FIGURE 1) and 5 bands in OPH-16. Each primer produced 25 (OPH-19)– 2 (OPB-10) polymorphic bands in all treatments out of control. Molecular sizes of bands ranged from 2432 (OPH-16) to 57 (OPW-13). Compared to control, putrescine and/or PEG6000 treatments led to prominent variations in RAPD patterns. These changes reveal as loss of bands available in control or appearance of new bands. GTS was used for comparing the changes in RAPD profiles. GTS values tended to decrease with increasing concentration of PEG6000 treatments. The values were calculated as 33% in -2 bar, 28.6% in -4 bar and 19.1% in -6 bar PEG6000 treatments. Besides, putrescine treatments had very high GTS values compared to stress treatments. 75.4% was in 1 mM put, 68.6% in 0.1 mM put and 64.6% in 0.01 put were determined. Also in combined treatments the lowest value was 43.5% in -6 bar PEG6000 + 0.01 mM putrescine treatment and the highest value was 60.3% in -2 bar PEG6000 + 1 mM putrescine treatment (Table 1).

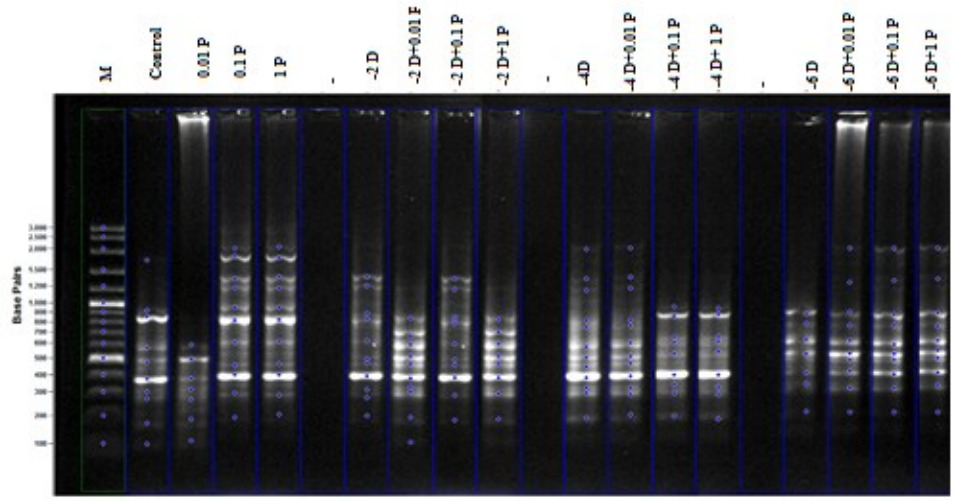

FIGURE 1. RAPD profiles of genomic DNA from *Triticum aestivum* Karasu 90 exposed to varying putrescine and/or PEG6000 concentrations with primer OPH-19 *M: marker, P: putrescine, D: drought.

3.1.2 Numerical analysis

PIC values of all primers varied between 0.284 and 0.360 and average became 0.321. While the primer OPW-18 was the highest PIC value, the primer OPH-19 was the lowest had (TABLE 2). D values of primers had been in 0.831-0.970 and average was 0.918. The primer OPW-18, which has both the discriminating power and the highest polymorphic band content, was determined as the most distinctive primer (TABLE 2). Similarity index of all treatments varied between 0.506 and 0.849. While the closest similarity coefficients to control was determined in -2 bar PEG6000 + 0.01 mM putrescine treatment as 0.645 ratio, the furthest similarity to control was in -6 bar PEG6000 + 1 mM putrescine treatment as 0.506 ratio (TABLE 3). The dendrogram (FIGURE 2) grouped all treatments into two main clusters. First cluster is untreated sample. Two cluster was divided into two main subclusters. The first subcluster was consisted of putrescine doses alone and combination with putrescine and -2 bar PEG6000 treatments while the second cluster was consisted of putrescine and -4 and -6 bar PEG6000 doses.

TABLE 1. Molecular sizes of bands (+: appearance / -: disappearance) and the average GTS values in RAPD profiles *P: primers, C: control

P	C	+/-	0 mM Putrescine / Drought (bar)			1 mM Putrescine / Drought (bar)				0.1 mM Putrescine / Drought (bar)				0.01 mM Putrescine / Drought (bar)			
			-2	-4	-6	0	-2	-4	-6	0	-2	-4	-6	0	-2	-4	-6
OPA-4	6	+	-	524	524	-	447 407	-	400	-	757	506 368	-	-	769	358	-
		-	980 537 443 388	980 537 443 388	980 537 443 388	980 740 443	-	-	556 378	980 740	800	1047 635	612 524 431	-	800	1047	556 431 378
OPA-12	6	+	579	392	500 362	613 423	763	-	-	-	-	-	987	-	-	1145 684	-
		-	745	1208 919	1208 566	1208 500 140	1500 1574	-	1075 362	1208 1102	1500 945 457 159	530 392 138	112	1208 1102 919 745 500	1500 945 457 159	530	745 112
OPH-16	5	+	1820 1470 1332 964 800 289	1322 892 509 292 207	1035 576 465 236	325	-	-	690	2303 1490 582	-	-	9135 09	2432 1720 311	-	473 176	-
		-	-	-	-	745 651	964 749 289	-	1085	631	964 749 800 611	728 509	6672 36	725	1132 800	738 207	964 236
OPH-18	7	+	6883 79	2202	836	1458	1237	836 465	993 600	628	1165	579	1526 991	1458 688	1200	400	15269 83 863
		-	1654 1237 723	1654 1373 1237	1654 1373 1237 723	1654 1373	1303	-	-	1654 1373 517	1303	2202 943	-	1654 1373 723	1303	2202 756 635	-
OPH-19	10	+	1352 881 463	1319 1315 746 503	1980 782 629	-	586 104	-	489	2000 1174	590 458	-	586 489	2062 1363 1166 465	-	649	500
		-	1684 918 97	918 97	1684 918 300 172 97	1684 918	1352 881	-	-	561 97	1200	1980 1319 382	629	97	1352 1200 881 392	746 503 382	-
OPB-10	6	+	-	619	-	-	-	-	-	-	-	-	633	-	-	-	633
		-	1017 836 727 491	563 491	1017 836 727 491	1017 836 727 491	593 370	984 823 504	1017 963 383	1017 836 727	593 370	504	581	1017 836 727 491	593 370	823	581

OPY-1	7	+	1123	446 191	955 500 191	500	506	245	430 322	-	452	274	446 311	-	204	-	12658 41
		-	1083 353 231 70	353; 231 70	888 353 231 70	353	452	1109 191	269	353	518	1109 191	269	1083 888 353	-	-	955 667
OPY-7	6	+	864	2046 1544 834	1940 1544 888	709	337 136	653 186	-	-	727 165	625 170	2454 993 181	-	-	1028 935	-
		-	917 681 337	1065 979 200	979 337 200	476 200	864 286	900	1940 1544 888 625	476 200	864 586	900	-	917 681 476 337	-	979	19401 544 888 625 315
OPY-13	6	+	496	676 541	844 473	1500	526	488 300 123	791	1691	213	187 127	-	823	890	1500 700	181 109
		-	507 200 115	754 115	904 507 115	115	496	1231 918 515	1030 844	115	-	1231 918	-	1152 754 115	942	541	10308 44
OPW-4	9	+	813	864	841	1526	930 605	520	583 492 209	1053	900 583	1354 684 503	-	1022	2000	2098 442	11215 68 500
		-	2439 1279 775 516 169	2439 1279 775 516 169	1761 1279 775 516 389 237 169	237	334 225	362 229	2023	237	225	362 229	-	237	-	362	20232 02
OPW-6	8	+	-	900 543	718 432	-	547	-	-	-	-	-	-	-	-	-	-
		-	1829 1555 500 400 218	1829 1555 500 400 218	1829 1555 757 500	1829 1555 400 218	-	1077 900 543	718 432 207	1829 1555 400 218	777	1077 900 543	718 432 207	1829 1555 400 218	777	770	-
OPW-13	9	+	2254 544	570 437	576	469	469 53	1458 770	63	469	50	1426	66	-	647 447 57	764 673 127	783 338 70
		-	1973 1567 500 63	1973 1567 920 714 138	1973 1567 714 507 138 63	1973 1567	2254 1275 770 155	570 330	-	1973 1567 714	2254 770 345 155	570 507 330	-	1973 1567 1280 920 714	2254 155	437	-
OPW-18	7	+	-	-	-	-	709	1448	1448	-	437 396	1225	416	-	-	1983 448	-
		-	1248 726 420 372 205	621 420 372 205	621 420 372 205	1479 1248 726 420	-	-	-	1479 1248 726 420	-	407	1448	1479 1248 726 420	1420 1329		-
GTS %	**100**		**33**	**28.6**	**19.1**	**75.4**	**60.3**	**57.4**	**56.6**	**68.6**	**59.8**	**45**	**43.9**	**64.6**	**50.3**	**44.3**	**43.5**

3.1.3 TOS and TAS

TOS and TAS values for treatments were presented in TABLE 4. TOS levels showed significant difference between the control and putrescine doses ($p<0.05$). The TOS value compared to the control decreased depending on the increase in putrescine doses (from 6.353 umol/L to 2.580 umol/L) whereas it increased due to increasing in drought stress doses (from 16.357 umol/L to 23.783 umol/L). When the effects of putrescine doses on the amount of TOS under drought stress were investigated, all putrescine doses applied under all doses of PEG6000 caused remarkable reductions in TOS value. A significant decrease in TAS value was occurred in drought stress and the difference between control and drought doses was significant ($p<0.05$). Furthermore, putrescine application caused a significant increase in TAS value compared to the control. On the other hand, putrescine applied in drought stress caused increase in TAS level compared to drought stress doses applied alone.

TABLE 2. Polymorphism Information Content (PIC) and Discriminating Power (D) of primers used in RAPD

Primers	PIC[a]	D[b]
OPA-4	0.327	0.933
OPA-12	0.346	0.956
OPH-16	0.324	0.927
OPH-18	0.314	0.912
OPH-19	0.284	0.831
OPB-10	0.298	0.879
OPY-1	0.321	0.924
OPY-7	0.319	0.920
OPY-13	0.314	0.912
OPW-4	0.340	0.949
OPW-6	0.304	0.893
OPW-13	0.328	0.933
OPW-18	0.360	0.970
Average	**0.321**	**0.918**

a: Botstein et al. (1980); b: Prevost and Wilkinson (1999)

TABLE 3. Jackard similarity index of treatments

	C	0.01 Put	0.1 Put	1 Put	-2 D**	-4 D	-6 D	-2 D + 0.01 Put	-2 D + 0.1 Put	-2 D + 1 Put	-4 D + 0.01 Put	-4 D + 0.1 Put	-4 D + 1 Put	-6 D + 0.01 Put	-6 D + 0.1 Put
0.01 Put	0.620														
0.1 Put	0.629	0.731													
1 Put	0.551	0.661	0.767												
-2 D	0.576	0.678	0.629	0.649											
-4 D	0.592	0.629	0.645	0.608	0.649										
-6 D	0.543	0.637	0.547	0.584	0.624	0.624									
-2 D + 0.01 Put	0.645	0.690	0.673	0.694	0.808	0.686	0.637								
-2 D + 0.1 Put	0.604	0.665	0.665	0.710	0.767	0.620	0.620	0.804							
-2 D + 1 Put	0.608	0.678	0.669	0.739	0.755	0.665	0.641	0.784	0.849						
-4 D + 0.01 Put	0.514	0.592	0.608	0.653	0.629	0.784	0.694	0.641	0.616	0.620					
-4 D + 0.1 Put	0.555	0.641	0.592	0.555	0.620	0.661	0.743	0.624	0.657	0.653	0.657				
-4 D + 1 Put	0.571	0.641	0.576	0.555	0.596	0.661	0.751	0.649	0.616	0.637	0.624	0.771			
-6 D + 0.01 Put	0.551	0.637	0.604	0.576	0.600	0.600	0.755	0.604	0.637	0.624	0.653	0.686	0.678		
-6 D + 0.1 Put	0.514	0.616	0.567	0.596	0.588	0.653	0.759	0.616	0.649	0.645	0.690	0.739	0.698	0.718	
-6 D + 1 Put	0.506	0.673	0.624	0.612	0.661	0.604	0.751	0.649	0.673	0.669	0.649	0.682	0.657	0.751	0.804

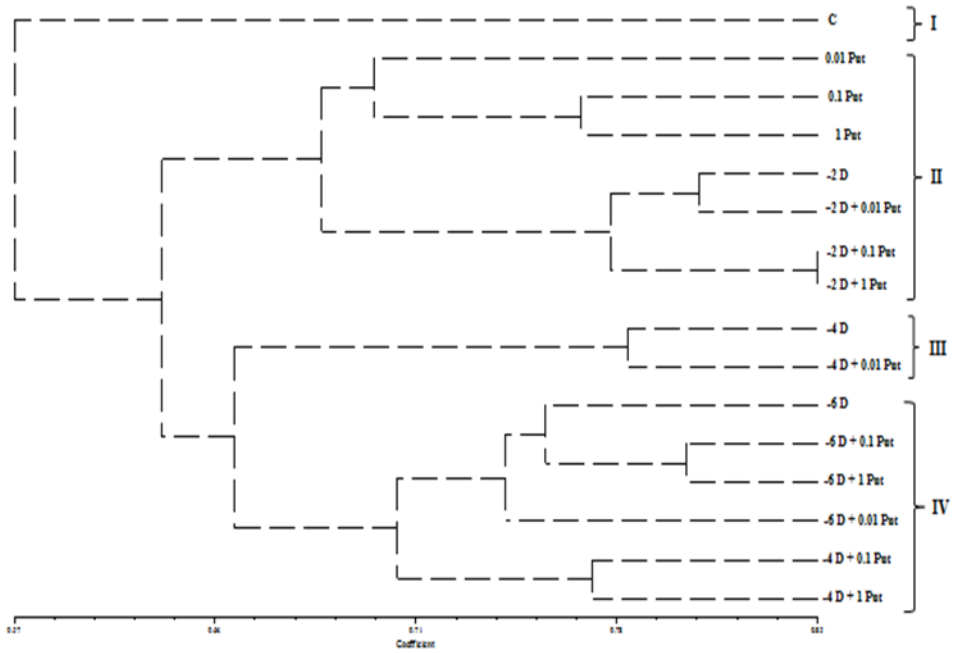

FIGURE 2. UGPMA dendrogram of the genetic similarity among putrescine and/or PEG6000 treatments inferred from a matrix of Jaccard coefficient

3.1.4 CRED-RA

Eight oligonucleotide primers which gave specific and sTABLE results used in RAPD analyzing were selected for CRED-RA analysis (TABLE 5). Compared to the PCR products obtained from the DNA of control treatment, putrescine and/or PEG6000 treatments resulted in certain changes in CRED-RA patterns. HpaII polymorphism values were higher than MspI polymorphism values for the most part of the whole treatments, since HpaII polymorphism ranged from 9.7% to 41.1% and MspI polymorphism ranged from 4.1% to 37% (TABLE 5). DNA methylation was emerged with all of doses of two treatments. The highest methylation value was 72.9% and the lowest was 47.3% in stress treatments. The highest methylation value was 18.5% and the lowest was 4.1% in putrescine treatments. The DNA methylation values changed in combined treatments according to dose variabilities. While MspI polymorphism was 20.7% in 1 mM put and -6 bar PEG6000, this value decreased as 16.3% in 1 mM put and -2 bar PEG6000 (TABLE 5).

TABLE 4. Comparison of TOS and TAS values based on the experimental treatments

Treatment	TOS (umol/L)	TAS (mmol/L)
Control	7.513^{k}	0.567^{f}
0.01 Put	6.353^{l}	0.664^{e}
0.1 Put	4.413^{m}	0.945^{b}
1 Put	2.580^{n}	1.567^{a}
-2 D	16.357^{c}	0.456^{gh}
-2 D + 0.01 Put	12.317^{h}	0.444g^{h}
-2 D + 0.1 Put	10.403^{i}	0.571^{f}
-2 D + 1 Put	8.447^{j}	0.888^{c}
-4 D	20.320^{b}	0.378^{i}
-4 D + 0.01 Put	18.713^{c}	0.436^{h}
-4 D + 0.1 Put	14.390^{f}	0.555^{f}
-4 D + 1 Put	10.767^{i}	0.738^{d}
-6 D	23.783^{a}	0.264^{j}
-6 D + 0.01 Put	20.737^{b}	0.435^{h}
-6 D + 0.1 Put	18.140^{d}	0.498^{g}
-6 D + 1 Put	13.340^{g}	0.661^{e}
Means	**13.036**	**0.629**
F value (Treatment)	1208.36**	255.71**
LSD$_{(0.05)}$ (Treatment)	0.520	0.055
Coefficient of variation (%)	2.40	5.30

TABLE 5. Percentage polymorphisms of studied CRED-RA amplicons

Primers	Drought (bar)	0 mM Putrescine		1 mM Putrescine		0.1 mM putrescine		0.01 mM putrescine	
		H	M	H	M	H	M	H	M
OPA-4	0	-	-	0	0	0	0	0	14.2
	-2	28.5	37.5	25	0	25	14.2	44.4	44.4
	-4	100	100	0	20	20	20	66.6	66.6
	-6	66.6	100	50	40	80	60	75	80
OPB-10	0	-	-	0	0	0	0	0	14.2
	-2	62.5	100	50	33.3	75	66.6	33.3	66.6
	-4	100	100	14.2	14.2	75	25	80	42.8
	-6	75	100	11.1	12.5	0	25	14.2	50
OPH-18	0	-	-	25	16.6	40	20	40	20
	-2	25	33.3	20	33.3	14.2	14.2	16.6	14.4
	-4	40	60	0	25	0	66.6	0	0
	-6	40	80	33.3	40	33.3	16.6	50	16.6
OPY-1	0	-	-	20	16.6	50	16.6	60	33.3
	-2	20	16.6	25	16.6	40	20	40	40
	-4	50	16.6	50	16.6	0	16.6	0	16.6
	-6	60	33.3	33.3	40	33.3	16.6	20	16.6
OPY-13	0	-	-	0	0	20	16.6	50	16.6
	-2	33.3	66.6	0	0	0	0	0	0
	-4	66.6	50	20	0	20	0	50	0
	-6	100	50	0	0	66.6	33.3	66.6	50
OPY-15	0	-	-	33.3	0	25	0	50	33.3
	-2	60	25	20	14.2	33.3	16.6	33.3	16.6
	-4	25	40	33.3	25	66.6	16.6	28.5	40
	-6	80	60	50	16.6	0	16.6	20	16.6
OPW-4	0	-	-	0	0	0	0	0	0
	-2	66.6	66.6	0	0	0	0	0	0
	-4	100	66.6	25	25	33.3	25	66.6	66.6
	-6	66.6	80	0	0	33.3	16.6	33.3	16.6
OPW-13	0	-	-	0	0	0	0	0	16.6
	-2	100	33.3	0	33.3	14.2	42.8	16.6	14.2
	-4	57.1	100	14.2	16.6	33.3	33.3	28.5	37.5
	-6	100	80	0	16.6	33.3	40	50	50
Average	**0**	**-**	**-**	**9.7**	**4.1**	**16.8**	**6.6**	**25**	**18.5**
	-2	**49.4**	**47.3**	**17.5**	**16.3**	**17.5**	**21.8**	**23**	**24.5**
	-4	**67.3**	**66.6**	**19.5**	**17.8**	**31**	**25.3**	**40**	**33.7**
	-6	**73.5**	**72.9**	**22.2**	**20.7**	**34.9**	**28**	**41.1**	**37**

4. DISCUSSION

In the current study, we investigated both genetic and DNA methylation changes in *Triticum aestivum* seedlings under drought stress conditions using RAPD and CRED-RA assays respectively, and effects of putrescine under these changes. The changes in the RAPD patterns generated by drought stress and putrescine included disappearance of normal bands and appearance of new bands when compared with control, as seen in TABLE 1. These changes differed from primer to primer among thirteen primers. According to PIC and D values the primer OPW-18 were the most distinctive primer in our study (TABLE 2). Also, we carried out the cluster analysis to determine the differences between all the treatments (TABLE 3, FIGURE 2). There was close relationship among putrescine and -2 bar PEG6000 treated groups. The other subcluster was shown that -4 and -6 bar PEG6000 treated groups were close to each other. It was thought that -2 bar PEG6000 had a separate effect in comparison with -4 and -6 bar PEG6000 groups.

As seen in TABLE 1, drought stress doses caused an enormous decrease on GTS value by comparison with other treatments (19.1 28.6 and 33%, respectively). These changes caused by drought were clearly dependent on extensive DNA damages [24-27]. Although many studies have proved that abiotic stresses induce DNA damage in different plants [2, 28], the molecular mechanism responsible for genotoxicity remains unclear even today. It was recommended that abiotic stress could stimulate the release of free radicals and ROS [29, 30]. In point of fact, we proved that TOS levels were gradually increased according to PEG600 doses (TABLE 4). Many ROS don't appear to interact with DNA but they are precursors for OH• radical. The reaction of OH• radical with DNA generates a multitude of products, since it assaults sugar, pyrimidines and purines, containing guanine residues to form 8-hydroxydeoxyguanosine (8-OHdG). In addition, 8-OHdG mostly produces transversion mutation (G to T). To limit ROS resulting damage, plants produce a wide range of antioxidants. After ROS has been occurs, detoxification mechanisms are effectively activated to minimize ROS-induced damage [4]. Antioxidant defense systems protect plant cells from oxidative damage by controlling the signaling pathways that lead to uncontrolled oxidations by scavenging ROS [2].

By the way, we determined that putrescine treatments caused an increase of GTS values against drought stress. According to results, especially 1 mM concentration of putrescine has increased GTS value and showed the most perfect effect in all stress treatments (TABLE 1). The defensive effects of polyamines contrary to DNA damage are related to its ability to bind to nucleic acid. Previous studies have shown the protective effect of polyamines against environmental stress in different plants [31-33]. It has been assumed that polyamines exhibit multiple functions by binding to negatively charged macromolecules due to basic net charge. Miyamoto et al. (1993) [17] have reported that total spermidine is bound to RNA, DNA and membrane lipids and protect DNA from enzymatic degradation, X-ray irradiation and mechanical shearing in Escherichia coli.

Therewithal, in this study we determined that TOS levels were quite low in dose-dependent of putrescine, while TAS levels were at the highest (TABLE 4). At this point, we are thinking of putrescine could be stimulate antioxidants and activate tolerance mechanisms in plant. These findings are consistent with Shi et al. (2013) [34] who reported that nucleoside diphosphate kinase (NDPK) and three antioxidant enzymes [2- Cys POD, ascorbate peroxidase (APX), Cu/Zn SOD] were generally regulated by polyamines (putrescine, spermidine and spermine) in bermuda grass. Similarly, Shi and Chan (2014) [35] found that the increased NDPK2 protein level by polyamine treatment is directly related with activities of antioxidant enzymes. Likewise, it was determined that overexpressing AtNDPK2 in Arabidopsis plants conferred enhanced tolerance to multiple environmental stresses that elicited ROS accumulation through interacting with oxidative stress-activated MPK3 and MPK6 and modulated the antioxidant enzyme activities such as APX, CAT and POD [36].

When plants are exposed to environmental stress, they activate mechanisms in biochemical, physiological and molecular levels induced DNA methylation and histon modification. DNA methylation is a well-characterized model to explain the epigenetically changes in gene expression. It is known that hypermethylation is associated with gene silencing while hypomethylation is linked with active transcription [37] and also known that hypermethylation and demethylation was periodic in nucleosomes. These status of methylation changes may be attributed to stress, kinds of plants and also tissue specificity. DNA demethylation was detected in salt stress in cotton [38], cold treated maize roots [39], heavy metal treated white clover [40], while hypermethylation was determined in chromium-exposed rapeseed [41], in pea exposed drought stress [9]. Our results well agreed with the outcomes of the earlier studies. We achieved the highest value of polymorphism (72.9%) in the -6 bar PEG6000 dose, so DNA methylation was showed quite a high rate of change (TABLE 5). Some researchers have emphasized that polyamines can inhibit direct DNA methylation by inhibition both the binding and activity of cytosine-DNA methylases [18, 42, 43]. Inhibition activity of cytosine-DNA methylases is non-competitive. It suggested that polyamines have an indirect effect on methylation as a mechanism for the antitrypanosomal effect of the ornithine decarboxylase inhibitor DFMO [44]. Other research provide that polyamines are capable of binding to A and B DNA, in A-DNA, binding occurs mainly to major groove, whereas in B-DNA putrescine and cadavarine bind to both sugar-phosphate backbone and major and minor grooves [33, 45, 46] Also experiment with B-DNA differing in the guanine to cytosine ratio showed that polyamines interacted mainly with phosphate groups and did not affect a native secondary structure DNA, thus providing for normal transcription of stress induced genes. So, polyamines could inhibit DNA methylation, which permits expression of specific genes responsible for the synthesis of stress protein. As would be expected, our results demonstrate that putrescine decrease cytosine DNA methylation (TABLE 5). Cleary, more information on molecular mechanism of the protective role of polyamines against DNA methylation in plants are needed.

5. CONCLUSION

As a conclusion we could state that putrescine is a protective material in drought stress conditions the points of DNA damage and DNA methylation alterations in wheat. RAPD and CRED-RA are used as accurate and reliable techniques as well as antioxidant and oxidant enzyme measurements confirm this opinion. In order to clarify the molecular mechanism of these applications it is necessary to measure the expression values of antioxidant enzyme genes in future studies.

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REDISCOVERY OF *BUNIUM MICROCARPUM* SUBSP. *LONGIRADIATUM* (APIACEAE) ENDEMIC TO CUDİ MOUNTAIN/ŞIRNAK (TURKEY)

MEHMET FIRAT

ABSTRACT. *Bunium microcarpum* (Boiss.) Freyn & Bornm subsp. *longiradiatum* Hedge & Lamond is restricted endemic to Cudi Mountain/Şırnak (Turkey). First collected in 1966 from one locality by Davis from Cudi Mountain (Silopi), but did not collected until 2019. Recently it was recollected for the second time from its type locality. The revised description and mature fruit sizes of *B. microcarpum* subsp. *longiradiatum* are given for the first time. In addition, habitus and habitat photographes and distribution map of this subspecies has been given.

1. INTRODUCTION

SW Asia and especially Turkey are diversity centers of the Apiaceae. Two endemic genera and more than 169 endemic species and subspecies are distributed in Turkey. The family Apiaceae comprises 100 genera and 477 species (505 taxa) in Turkey and 167 taxa are endemic [1]. The genus *Bunium* (Apiaceae) comprises about 50 species of geophytes with tuberiform storage roots, distributed in the arid and subarid SW and central Asia, Europe, and North Africa [2]. The center of diversity of *Bunium* is in the Mediterranean region. In Turkey, there are sixteen known species, including four endemics [3]. The most recent classification of *Bunium* was elaborated by Kljuykov [4] who based his treatment on the chromosome number, embryo features and other characters. Six sections and 15 subsections were recognized, and they were subsequently partly supported by the molecular phylogenetic analysis [2].

During floristic surveys at Cudi Mountain (Silopi/Cizre) (Figure 1), from may to July 2017, some interesting *Bunium* specimens were collected. To determine those specimens, a wide range of literatures [e.g. 5-7] were used. Finally, collected *Bunium* matterials were identified as *Bunium microcarpum* (Boiss.) Freyn & Bornm subsp. *longiradiatum* Hedge & Lamond which was firstly described from Cudi Mountain (Şilopi/Cizre).

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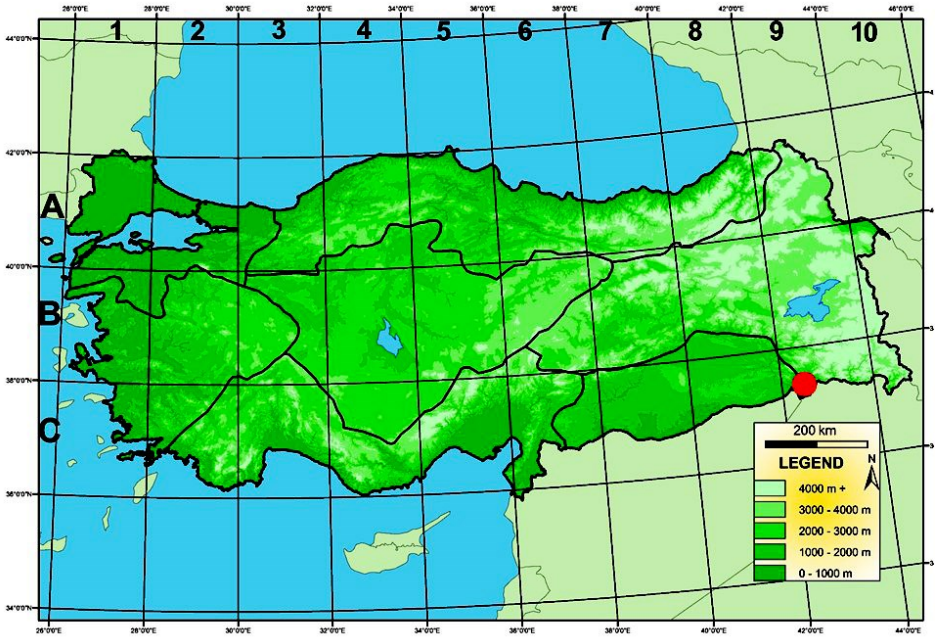

FIGURE 1. Geographical distribution of *Bunium microcarpum* subsp. *longiradiatum* (●) in Turkey.

2. MATERIAL AND METHOD

Photos of the living material were taken with a Sony DSCR1 digital camera. Accessions were georeferenced using a Magellan eXplorist 710 GPS. A total of 10 herbarium specimens of the species were collected from one adjacent localities and deposited in the herbaria VANF [8] and in the personal herbarium of the author (private Herbarium of Mehmet Fırat).

3. RESULTS AND DISCUSSION

Taxonomy

Bunium microcarpum (Boiss.) Freyn & Bornm subsp. ***longiradiatum*** Hedge & Lamond (Figures 2–4)

Type specimens: Turkey C9 Mardin, Cudi Da. above Hessana, 1200-1400 m, Davis 42840 (holo. E, Photo: Virtual herbarium E00000459!) (Figure 2 A).

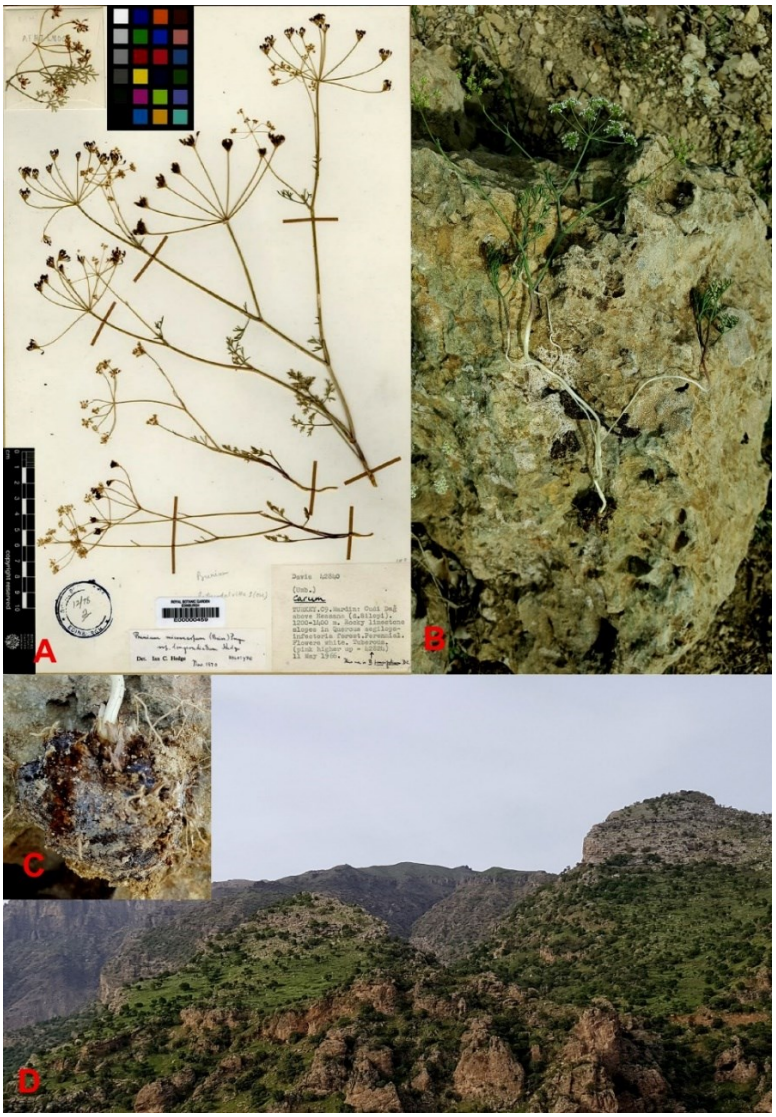

FIGURE 2. *Bunium microcarpum* subsp. *longiradiatum;* **A–** holotype (E, Photo: Virtual herbarium E00000459!), **B–** Habit, **C–** Tuber, **D**– Habitat

Emended description: Prostrate, ascending or erect perennial, glabrous throughout, from tuber-like thickened root; tubers globose, c.1cm in diam. Stems, terete, finely ridged, 1–2 mm in diam. at base, branched from base; 12–30 cm. Basal leaves with thin and long petioles, to 12 cm long; leaf blades bipinnatisect, to 3 cm long, 2 cm broad, triangular in outline; ultimate segments linear, 2–6 x 0.5–1.5 mm, acute. Lower cauline leaves with sheaths 0.5–1 cm long, narrowly white–membranous at margin, 2-pinnate, 4–9 x 1–3 cm (inc. petioles). Upper cauline leaves few, 1-2-pinnate, with prominent sheathing petioles or reduced to lanceolate-linear sheaths. Rays 7-10, spreading, ascending to erect, unequal, 3–6 cm. Bracts 0–4, white–membranous at margin, 1–4 x 0.5–1.5 mm. Pedicels 10–16 per umbellule, unequal, 2–5 mm. Bracteoles 2–6, 1–3 x 0.4–0.6 mm. Petals white, apex incurved, c. 0.5 mm long. Fruits oblong to oblong-elliptic, glabrous, 2.5–3.7 x 1–2 mm; styles 0.5–0.8 mm, spreading to deflexed: stylopodium conical. Vallecular vittae 1; commissural 2.

Key to Flora of Turkey *Bunium microcarpum* subspecies

1. Rays of fruiting umbels 4-5.5 cm; ultimate lobes of middle cauline leaves c. 5 mm……...……..……**subsp. longiradiatum**

1. Rays of fruiting umbels up to 3 cm; ultimate lobes of cauline leaves more than 5mm

2. Fruits oblong or oblong-linear, 3–3.5 mm…….……**subsp. bourgaei**

2. Fruits ovoid, c. 2.5 mm……..……..**subsp. microcarpum**

Phenology: Flowering time May and fruiting from June to July

Distribution: *Bunium microcarpum* subsp. *longiradiatum* is only distributed from Cudi mountain (Silopi/Şırnak) Turkey. According to the grid system Davis the species, falls specifically within the C9 square. It is growing type locality Cudi mountain (Silopi/Şırnak) Turkey.

Habitat and ecology: *Bunium microcarpum* subsp. *longiradiatum* grows in *Quercus* sp. and *Juniperus* sp Limestone slopes, at c. 1100–1400 m. It is associate with other plants such as; *Juniperus* sp., *Galium* sp., *Bromus* sp. and *Poa* sp. *Bunium paucifolium* var. *paucifolium*

Vernacular name: *Bunium microcarpum* subsp. *longiradiatum* is called Kurdish name “Xîlxîloşk” by the local people of the Silopi province. The other *Bunium* species are known by the local people under many names in Kurdish; e.g. "Çavreşok", "Kimî", "Kizbelok", "Kurdek", "Şemok", "Şelîlok", "Xîlok","Xumxumik" "Poşmê", and in Turkish; e.g. "Tavşan kapağı" [9].

FIGURE 3. *Bunium microcarpum* subsp. *longiradiatum;* **A, B–** Habit, **C–** Upper cauline leaves, **D**– Basal leaves

Examined material: *Bunium microcarpum* subsp. *longiradiatum.* Turkey. C9 Şırnak, Silopi district, Cudi mountain, Above Hessena, Limestone slopes, *Juniperus* sp. and *Quercus* sp. forest, 971 m, 08.05.2015, *M. Fırat 33745* [(VANF, Herb. M. Fırat), (in flower)]; ibid. 16.07.2015, *Fırat 33893* [(VANF, Herb. M. Fırat), (in fruit)].

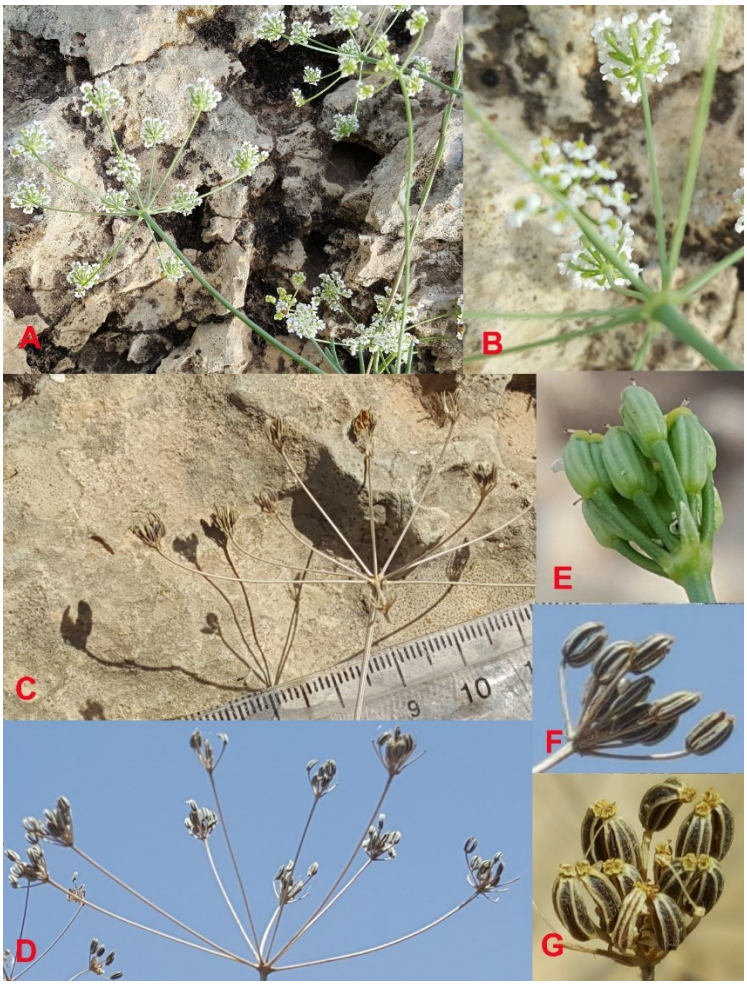

FIGURE 4. *Bunium microcarpum* subsp. *longiradiatum;* **A, B–** Umbellule at flower, **C, D–** Umbellule at mature fruits, **E**– Detail young fresh fruits, **F, G–** Detail mature fruits

COMMENTS

Bunium microcarpum subsp. *longiradiatum* is an endemic species that is known from just type localities. In addition to the revised description, mature fruit sizes are given for the first time (mature fruits oblong to oblong-elliptic, glabrous, 2.5–3.7 x 1-2 mm; styles 0.5-0.8 mm, spreading to deflexed: stylopodium conical. Vallecular vittae 1; commissural 2). Despite the investigations until today, there was found no trace of this species in the nature, and thus it has been evaluated as Endangered (EN) according to IUCN (Red List Categories and Criteria) [10]. With this study, the species was recollected 53 years later and photos of this species were taken for the first time in their habitat.

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ROLE OF SODIUM NITROPRUSSIDE ON MITIGATION OF SALT STRESS IN SWEET CORN

FATEME MANSHOORI, MOHAMMAD ARMIN AND HAMID MARVI

ABSTRACT.To evaluate the effect of foliar application of sodium nitroprusside (SNP) on physiological characteristics of sweet corn under salt stress a factorial experiment in completely randomized design with three replications was conducted in the research greenhouse of Islamic Azad University of Sabzevar.Factors were application time of SNP at the concentrations of 200 ppm (vegetative, reproductive and vegetative + reproductive) and salinity (0,1.5, 3 and 4.5 dS m^{-1}).The results showed that increasing salinity levels from 0 to 4.5 dSm^{-1} decreased the height by 31.81%, plant dry weight by 34.34%,the number of ear by 46.34%, chlorophyll a content by 30.54%, chlorophyll b content by 55.81%, carotenoid content by 37.40%, total chlorophyll content and the amount of potassium by 54.86% and increased the amount of sodium by 63.86%. Application of sodium nitroprusside twice in vegetative and reproductive stage resulted in maximum height, plant dry weight,chlorophyll a, chlorophyll b, total chlorophyll and foliar application had higher levels of carotenoids. Overall the results indicated that sweet corn is sensitive to salinity and cannot tolerate salinity more than 3 dS m^{-1}. At low salinity condition SNP foliar application at vegetative + reproductive stage and in high salinity level at the vegetative stage can reduce the effects of salinity.

1. INTRODUCTION

Salinity stress, especially in arid and semi-arid conditions, is an important limitation to crop production. Salt stress directly or indirectly affects biochemical, morphological and anatomical characteristics of crop species including germination [1], growth [2], cell division [3], photosynthesis [4], nutrient metabolic and uptake [5], crop development and yield [6] and so on. Different methods are used to reduce the inevitable effects of salinity in plants such as planting of tolerant cultivar [2], nutrient management [7-9], agronomy practice [9] and nowadays foliar application of osmo-protectants or compatible solutesas well as glycine and betaine [10], salicylic acid [11], proline [12], ascorbic acid, 24-epibrassinolide and sodium nitroprusside [13]. Sodium nitroprusside (SNP) is a nitric oxide releasing compound (NO), whose role in plants has been the subject of many research studies [13-16]. Nitric oxide is itself an active nitrogen species, which is thought to be able to mediate as a messenger molecule in adaptive responses to biological and non-biological stresses in plants, and to collect ROS as an antioxidant agent and eliminate it [17]. Although NO is less well known in plant functions, recent advances in research have shownthat it has a major role in regulating

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many plant growth functions, growth and development, response to environmental conditions that affects morphology, signal transduction, seed germination, root growth, the formation of shoots, the maturity of fruits[18]. However, the protective role of NO in plants depends on NO concentration, tissue type, age and plant species, and stress type[13]. Regarding the effect of NO on reducing the effects of salinity, it has been observed that the use of sodium nitroprusside as a NO component which reduces the adverse effects of salinity[19]. Protective role of NO against oxidative damage and increase tolerance to osmotic stress was reported by application of 0.2 mM in rice seedling [20]and 1 mM in barely seedling[21]. Also, in 8-day old rice seedlings, pretreatment of 1 mM SNP over two days increased the salt tolerance to sodium chloride[19].Fan et al., (2007) reported that application of NO under salt stress in Cucumber increased antioxidant enzyme activities and also chlorophyll and proline content that resulted the enhacement of seedling growth.

Sweet corn (*Zea mays* L. var. *saccharata*) is a corn with a mutation on the locus Su (Sugary) on chromosome number 4. The genetic variation causes the accumulation of soluble sugars and polysaccharides in the endosperm of seeds. Sweet corn is one of the tropical plants known to be the third most widely consumed cereal after wheat and rye, and during its growth period it requires a lot of heat and is sensitive to frost. Also, high and low temperatures can damaged (temperatures above 35 °C and less than 10 °C[22].Unlike corn, which is one of the cereals used to feed livestock or the production of flour, sweet corn is used as a vegetable and fresh food for human. Now sweet corn is one of the most popular vegetables in different parts of the world and its consumption is increasing due to its delectability and rich vitamin content. The value of this crop for processing (canning and freezing) and as a fresh vegetable is the second and fourth respectively [23]. In 2003, the area under sweet corn cultivation in the world was 1,019,698 hectares with an average production of 8602 kg per hectare which 8.772.112 tons of corn were produced. About 27 percent of corn acreage and 46 percent of the world belongs to the United States and the largest producers of corn are America, Nigeria, France, Hungary, Peru, South Africa and Japan respectively [24].

Salinity is the most common environmental stresses throughout the world, including Iran[25]. There is a lack of research on the effects of salinity on sweet corn, however, it is believed that sweet corn is semi-sensitive to salinity[26].This plant is resistant to salinity during germination, but increased levels of salinity delay germination. High soil salinity and low temperature in sweet corn delay the emergence of leaves and the formation of the first internodes and reduce the green cover. Continuing stress in subsequent growth stages reduces plant height, and the number of seeds. Salinity stress also causes tissue hydration, ion toxicity, food insecurity, and so on. Studies on sweet maize hybrids have shown that they are the same as salt stress, although hybrids show the same response to germination of salt stress seeds, but root length, stem length, fresh and dry weights and stems of the roots have decreased with increasing salinity. Also, salinity increases the amount of malondialdehyde, proline and H_2O_2in the seedling [27].

We hypothesized that SNP application improves the physiological traits of sweet corn grown under salt conditions. Thus, the objective the present study is to explore up to what extent foliar-applied SNP could alter chlorophyll content and ion content of sweet corn grown under salt conditions.

2. MATERIAL AND METHOD

This research was carried out as a factorial experiment based on a completely randomized design with three replications in greenhouse of Islamic Azad University in 2015.The experimental factors included salinity in four levels (0, 1.5, 3, 4.5 dS m-1) and nitroprusside application at 200 ppm in three growth stage of sweet corn (vegetative, reproductive and vegetative+reproductive). 4-5 leaves and tassel observation in 50% of plant were considered as vegetative and reproductive stage, respectively. Salt treatments were conducted by the addition of NaCl and $CaCl_2$ in the equivalent proportion of 1:1 in tap water (ECi = 0.3 dS m-1).

Sweet corn (Gold seed kSC_40_3cultivar) was planted in pots with a diameter of 25 cm at a depth of 5 cm. After ensuring of complete and optimal development of plants, 5 plants per pot were maintained, and the rest of the plants are excluded. Soil contain 50% sand and 50% field soil. Soil was kept moist during whole duration of experiment. Pot soil moisture content was maintained in a range of 70 to 100% of field capacity. According to the results of soil analysis, the required fertilizers (300 milligrams of urea, 150 milligrams Ca $(H_2PO_4)_2.H_2O$, 100 milligrams of K_2SO_4, 40 milligrams of $FeSO_4.7H_2O$, 20 milligrams of $MnSO_4.H_2O$, 20 milligrams of $ZnSO_4.7H_2O$, 10 milligrams of $CuSO_4.5H_2O$ and 5 milligrams H_3BO_3 per kg of soil) were added to the soil before planting and mixed well. Urea fertilizer was consumed in three stages (pre-cultivating, 3leaves stage and stem elongation). The plants were maintained in a greenhouse under environmental conditions (27 (±5)°C, 65 (±10)% RH and a 14 h light, 10h dark photoperiod.

At the beginning of the reproductive stage, plants were completely removed from the pots and length of three randomly selected plants in each pot was measured for determination of plant height by the meter.

One gram of finely cut fresh leaves were taken and ground with 20 – 40ml of 80% acetone. It was then centrifuged at 5000 –10000 rpm for 5mins. The supernatant was transferred and the procedure was repeated till the residue becomes colorless. The absorbance of the solution was read at 470nm, 645 nm and 663nm against the solvent (acetone) blank [28].The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation:

Total Chlorophyll: 20.2 (A645) + 8.02 (A663)

Chlorophyll a: 12.7 (A663) – 2.69 (A645)

Chlorophyll b: 22.9 (A645) – 4.68 (A663)

Carotenoids: 100 (A470) - 3.27 (mg chl. a) – 104 (mg chl. b) / 227

Hamada and El-Enany[29]method was used to measure sodium and potassium elements. For this purpose, 0.5 g dry matter of leaves washed and then 10 ml of concentrated nitric acid was added and kept at room temperature for 48 hours. In order to remove all vapors, the specimens were placed on a heated oven thermostat for 2 hours. After leaving acidic vapors and viewing a colorless solution, 100 ml of distilled water was added to each sample. Using Whatman filter paper, the samples were get smooth and sodium and potassium values were measured by photometric photometry [29].

The data were analyzed using SAS software and the averages were compared by Duncan multiple range test. Tables and graphs were drawn in Excel software.

3. RESULTS AND DISCUSSION

The final plant height, plant dry weight, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, leaf chlorophyll index, leaf sodium and potassium content were affected by SNP application and salinity, whereas the interaction effect of SNP
application and salinity for chlorophyll B, total chlorophyll and leaf chlorophyll index were significant (Table 1).

TABLE 1: Analysis of variance for plant height, plant dry weight, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids content, sodium content and potassium content.

SOV	df	Plant height	Plant dry weight	Chloro-phyll a	Chloro -phyll b	Total chlorophyll	Carotenoids content	Sodium content	Potassium content
Time (A)	2	623**	21.74**	0.75 **	0.31 **	1.75 **	0.04 **	2.90 **	3.80 **
Salinity (B)	3	3154**	10.01**	0.98 **	1.62 **	5.12 **	0.09 **	12.64 **	29.52 **
A×B	6	62.24 ^{ns}	1.12 ^{ns}	0.11 ^{ns}	0.16 **	0.48 **	0.006 ^{ns}	0.19 ^{ns}	3.86 **
Error	24	98.84	1.09	0.09	0.04	0.05	0.008	1.38	0.66
CV		14.43	24.78	11.64	16.51	6.74	17.28	20.63	16.39

ns: not significant; (*) and (**) represent significant difference over control at $P < 0.05$ and $P < 0.01$, respectively.

3.1. Plant height

The highest plant height was observed when SNP used in vegetative+reproductive (115.33 cm) and the lowest in vegetative time (101.375 cm, Table 2). It seems that low plant height at SNP application in vegetative stage was due to low absorption of SNP due to less leaves per plant. In the vegetative stage, the number or surface area of the leaves has not been sufficient so that SNP application has not shown its beneficial effects due to decreased absorption, while at the beginning of the reproductive stage, although the vegetative growth process has to be cut off, SNP application has increased the plant height by increasing the length of the thistle. It was reported that SNP application could increase plant growth in saline conditions by raising the activity of antioxidant enzymes that protects the plant from damage caused by free oxygen radicals [30]. In wheat, SNP in saline conditions increases tolerance by raising the amount of proline in the leaf[18]. In cotton that SNP consumption not only promotes plant growth but also augments stem and root lengths. It also rises osmotic pressure of the cell and improves cytoplasmic viscosity that leads to elongate stem length. On the other hand high levels of SNP had a negative effect on stem elongation[31].

TABLE 2: Effect of SNP application time on plant height, plant dry weight, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, sodium and potassium contents.

SNP application time	Plant height	Plant dry weight	Chlorophyll a	Chlorophyll B	Total chlorophyll	Carotenoids content	Sodium content	Potassium content
	Cm	g. plant^{-1}	mg. g^{-1} FW				mg. g^{-1}	
Vegetative	101 b	1.38 c	1.97 b	1.08 b	3.05 c	0.45 b	5.77a	4.89 b
Reproductive	111 a	1.85 b	1.99 b	1.32 b	3.32 b	0.57 a	6.15a	4.45 ab
Vegetative + Reproductive	115 a	2.2 a	2.41 a	1.39 a	3.81 a	0.51 ab	5.17a	5.87 a

Values followed by the same letter within the same columns do not differ significantly at p = 5% based on Duncan.

The highest plant height was observed in the control (129 cm) and the lowest was in 4.5 dSm-1 (88.17 cm, Table 3). There was no significant difference between salinity treatments at 1.5 dSm-1 levels and control. There are several reasons for the reduction of plant height with increasing salinity stress. For example, in high salt levels cause plants to absorb water difficultly which reduces available water and cell division depending on turgor pressure. On the other hand, under stress conditions, photosynthesis rate of the plant is also affected. Reducing photosynthesis lessen the contribution of photosynthetic metabolites to growth, which will also reduce theplant height. Reduction and disruption of nutrient uptake are also due to decreasing of plant height. Reduction in plant growth under salt stress occur due to decreased water absorption and metabolic activity, sodium and chloride toxicity along with food deficiency[32-35].

3.2.Plant dry weight

SNP foliar application at vegetative + reproductive stages revealed maximum dry weight (0.022 g.plant-1) and at vegetative stage the lowest value was observed (0.014 g.plant-1, Table 2). Spraying at the reproductive stage in comparison with the vegetative stage increased 35.71% of plant dry weight. The higher dry weight with SNP application at reproductive stage in comparison to the vegetative stage may be due to the fact that in the vegetative stage, the level and number of leaves were less than the reproductive stage, therefore, at this stage, lower absorption of SNP was carried out by the plant. On the other hand, SNP application in the reproductive phase might have delayed or decreased the leaf loss, which also increased the dry weight of the plant.

TABLE 3: Effect of salt stress on plant height, plant dry weight, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, sodium and potassium contents.

Salt stress ($dS.m^{1}$)	Plant height	Plant dry weight	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids content	Sodium content	Potassium content
	Cm	g. $plant^{-1}$	mg. g^{-1} FW				mg. g^{-1}	
0	129 a	2.22 a	2.54 a	1.75 a	4.31 a	0.61 a	4.34c	6.95 a
1.5	120 a	1.97 ab	2.21 b	1.44 b	3.65 b	0.58 a	5.23bc	5.91 b
3	99 b	1.66bc	1.99 bc	1.09 c	3.08 c	0.48 b	6.11ab	4.14 c
4.5	88 c	1.44 c	1.77 c	0.77 d	2.54 d	0.38 c	7.11a	2.88 d

Values followed by the same letter within the same columns do not differ significantly at *p* = 5% based on Duncan.

Salt stress significantly reduced dry weight. Results revealed that the maximum dry weight (0.22 $g.plant^{-1}$) was produced in the control, while the lowest dry weight was recorded at 4.5 $dS.m^{-1}$ (0.01 $g.plant^{-1}$). There were significant differences between treatments (Table 3). Increasing salinity to 4.5 $dS.m^{-1}$ reduced 45.95% of plant dry weight. Reducing dry weight with increasing salt stress may be due to usage of a part of photosynthesis or growth metabolites for the production of secondary metabolites in order to cope with salinity, which reduces the photosynthesis assimilate for the other parts. On the other hand, the decrease in height, number of leaves and disruption in absorption and transfer of nutrient in high salinity are the main reasons of dry weight loss of the plant at high salinity conditions.

3.3.Chlorophyll a

The highest amount of chlorophyll a was observed when SNP was used at vegetative + reproductive (2.15 $mg.g^{-1}$) and it has the lowest at vegetative stage (1.98 $mg.g^{-1}$, Table 2). The main reason for increasing the amount of chlorophyll a when SNP applied twice compared to once might be due to the production of more pre-structures of chlorophyll production. It has been reported that the use of SNP by preventing the activity of ROE reduces oxidative damage in photosynthetic pigments, which increases the total chlorophyll content of the leaf [36]. Similar results have been reported on the increase of photosynthetic pigmentation due to SNP consumption in chickpea[37] and sunflower[38].

Results revealed that, salt stress dramatically reduced leaf chlorophyll a content (Table 3). The main reason for the reduction of photosynthetic pigments in high salinity may be due to prevent the absorbtion of Mg^{+2} due to high amounts of sodium, which leads to the inhibition of chlorophyll synthesis. Previous studies suggest that high levels of sodium inhibit protein synthesis and weaken the binding of chlorophyll and chloroplast in which leading to chlorophyll degradation[39]. Lin and Shi (2010) showed that, with increase salinity up to 10 dSm^{-1}, net photosynthesis rate, stomatal conductance and chlorophyll a content of sunflower had a decreasing trend[39]. Reduction of chlorophyll content in salt stress conditions may be due to the activity of chlorophyllase enzymes. Some regulators, such as absisic acid and ethylene, stimulate the activity of this enzymes[40].

3.4.Chlorophyll b

Chlorophyll b content was higher in twice SNP application at vegetative + reproductive stages than in other treatments (Table 2). There was no significant difference between SNP application at reproductive stage and vegetative+ reproductive. Spraying at the reproductive stage increased chlorophyll b content 56.67 % when compared with the application at vegetative stage. The amount of chlorophyll depends on the type of leaf and the time of sampling. Therefore, it seems that no significant difference between the amount of chlorophyll b in the reproductive and vegetative was due to selected leaf sample. As shown in table 3, the maximum chlorophyll b content (12.19 cm) was measured in control. There were significant differences among treatments. Increase of salinity to 10 $ds.m^{-1}$ in sunflower decreased chlorophyll content, stomatal conductance and chlorophyll content [39].

Delay in the SNP application produced a higher chlorophyll b content at low salinity stress whereas, at high salinity, spraying in early growth stage produced more chlorophyll b than spraying at the end of growth stage (Figure 1). This suggests that in high salinity levels, the tolerance of sweet corn to salinity is low so delay in SNP application until reproductive stage could not induce mitigation of salinity. Salinity can damaged by the membrane tissue and might increasedchlorophyllase enzymes activity, causing a large part of chlorophyll to be degraded[41].

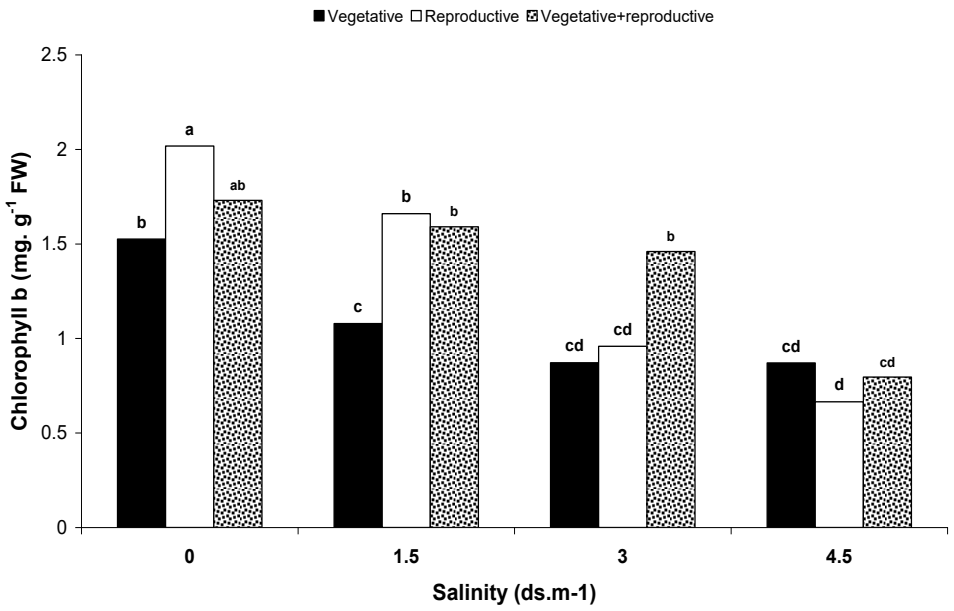

FIGURE 1: Interaction between SNP application time and salinity on chlorophyll b content.

3.4. Total chlorophyll content

The highest total chlorophyll content of the leaves was observed when SNP was used twice at vegetative + reproductive (3.81 mg.g^{-1}) and lowest at vegetative stage (3.058 mg.g^{-1}, Table 2). Since total chlorophyll content is the sum of chlorophyll a and b, both of them were less at vegetative stage than the other stages, so the total amount of chlorophyll is the lowest. The higher total chlorophyll content by SNP spraying in vegetative + reproductive stages due to higher levels of chlorophyll a and b in these two stages. In cotton, it was reported that SNP application reduces the damage caused by salt stress to chlorophyll [42].

With increasing salinity stress, the total chlorophyll content decreased linearly, so that the control treatment had the highest chlorophyll content and 4.5 dS m^{-1} treatment had the lowest total chlorophyll (Table 3). As shown in figure 2, at the 0 and 1.5 dS m^{-1}, salinity levels, delay in SNP sprays induce the production of more total chlorophyll while at higher salinity levels early SNP spraying resulted in higher chlorophyll content. In the studies about the effects of

salinity on the physiological and morphological characteristics of grape varieties, it has been shown that salinity induce the decrease of chlorophyll index in leaves significantly [43].

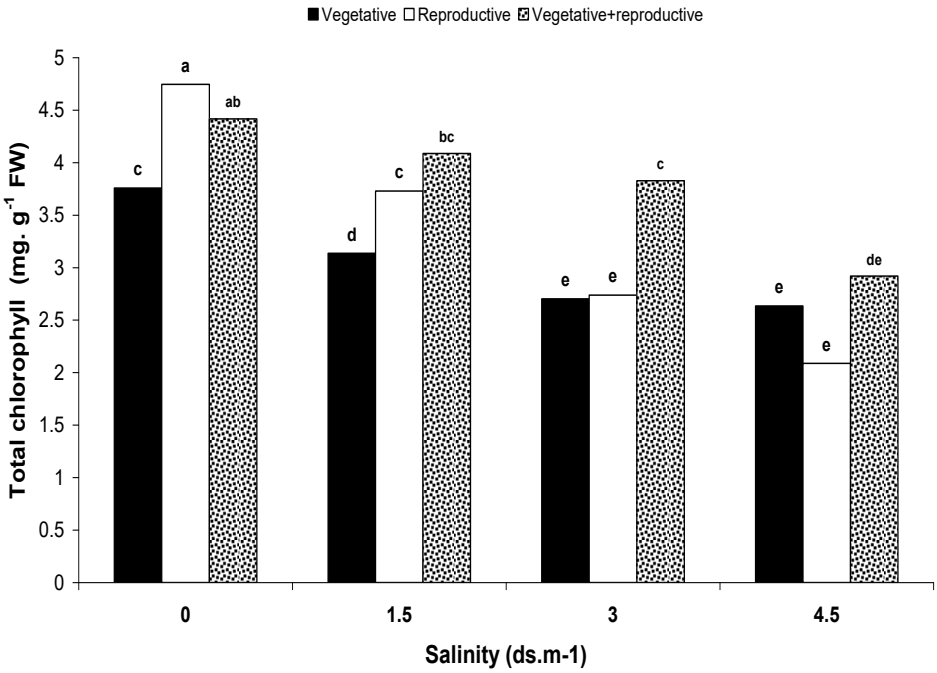

FIGURE 2: Interaction between SNP application time and salinity on total chlorophyll content.

3.5. Carotenoids content

The highest level of carotenoids in the leaf was observed when SNP was used at reproductive stage (0.55 mg.g^{-1}) and its lowest during vegetative stage (0.445 mg. g^{-1}, Table 2).There was no significant difference among treatment. A previous research on tomato showed that pre-treatment of SNP had no significant effect on carotenoids in this plant [5].

The highest amount of leaf carotenoids was obtained in the control (0.61 mg.g^{-1}) and the lowest at the salinity level of 4.5 dS m^{-1}, (0.38mg.g^{-1}), which showed a significant difference between treatments. There was not significantly different between control and 1.5 dS m^{-1}(Table 3). Contrary to the above results, previous sties suggest that the carotenoids have an antioxidant role, therefore, in salinity stress conditions, their amounts increase [15].

3.6. Leaf sodium content

Results display that, at 4.5dS m^{-1} leaf sodium content was maximum and control had the minimum of sodium. No significant difference was observed between control and 1.5 $ds.m^{-1}$ salinity level (Table 3). Increasing salinity levels to 1.5, 3 and 4.5 dS m^{-1} dramatically increased sodium content 15, 35 and 40%, respectively. It was also reported in sugar beet that salt stress has induced a significant increase in the concentrations of Na and Cl ions [44].

3.7. Leaf Potassium content

The highest amount of potassium was observed when SNP was used twice at reproductive + vegetative (5.75 $mg.g^{-1}$) and the lowest was measured at reproductive stage (4.45 $mg.g^{-1}$). The use of the solution at the vegetative stage (4.108 $mg.g^{-1}$) showed a higher effect on the amount of potassium in the leaf compared to reproductive stage (4.458 $mg.g^{-1}$, Table 2). KoohiFaeq et al. (2011) showed a significant decrease in potassium content in leaves and roots with increasing salinity.

The highest amount of potassium in leaf was in control (6.96 $mg.g^{-1)}$ and lowest in 4.5 ds.m-1 (2.89 $mg.g^{-1}$), which the values were significantly different from each other. (Table 3). The reduction of potassium in salinity conditions can be due to sodium competition for binding to plasma membrane carriers and potassium leakage due to instability of the plasma membrane [45]. Previous studies have reported that the concentration of potassium ion in sugar beet decreases in salinity stress conditions, which is consistent with the results of the current study on sweet corn. It has also been reported that wheat growth decreases with decreasing potassium ion in salinity conditions[46].
As shown in figure 3, in high salinity conditions, delay in SNP spray application could not reduce the effects of salinity stress, and the increase in the frequency of spraying due to increased concentrations of SNP had inhibitory effects on potassium uptake, which may be due to reducing root activity to potassium absorption. While in the control treatment, and low salinity levels (1.5 $dS.m^{-1}$), increasing the amount of spraying (spraying at the vegetative+reproductive stage) increased the content of potassium in sweet corn leaf (Figure 3), which also indicates that SNP can have both inhibitory and stimulatory effects on potassium levels. It has been reported that in salt stress conditions, sodium absorbtion increases calcium and potassium decreases. In such a situation, the addition of appropriate amounts of SNP reduces sodium uptake and increases the absorption of potassium, magnesium and calcium, which may be the effect of SNP in hormone signaling that might be implicated in salt tolerance. As the ratio of potassium to sodium increases, the activity of H+ ATPase enzyme also increases. In addition, the protective effects of SNP in salt stress conditions may be associated with increased osmotic regulation associated with salt discharging.

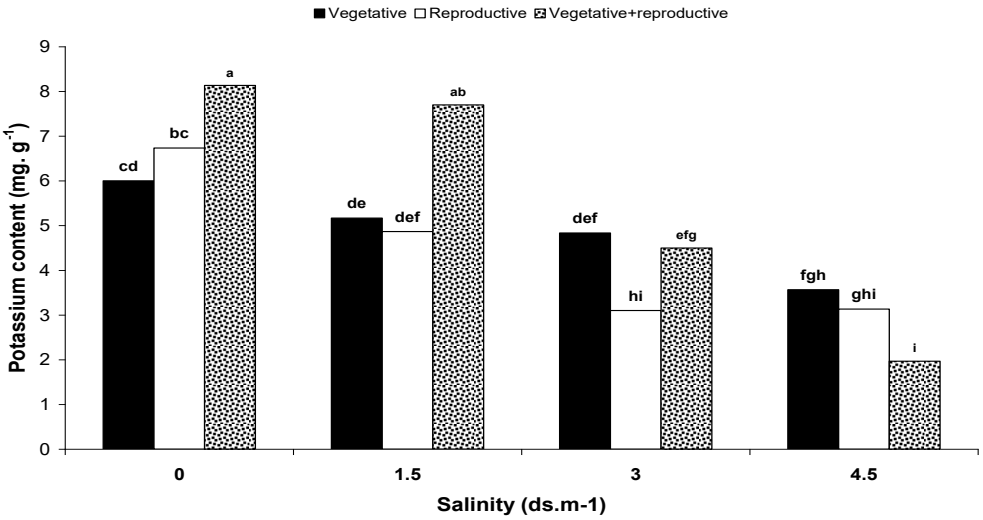

FIGURE 3: Interaction between SNP application time and salinity on potassium content.

4. CONCLUSION

In conclusion, the foliar spraying of SNP in salt-sensitive sweet corn was an effective way to stimulate physiological and morphological traits when plants were exposed to salt stress. The time of exogenous application of SNP to mitigation of salinity in Sweet corn depends on salinity levels. At low salinity condition sodium nitroprusside foliar application in vegetative + reproductive stage and in high salinity level once in the vegetative stage can reduce the effects of salinity.

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INVESTIGATION OF THE ANTIMICROBIAL ACTIVITY OF *Vitis vinifera* L. BOĞAZKERE

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ABSTRACT. In this study, extracts from Boğazkere cultivar of *Vitis vinifera* L. at 10 µL, 50 µL and 100 µL concentrations were tested against 18 different bacterial and fungi strains using disc diffusion (DD) method along with minimum inhibitory concentration (MIC) and minimum bactericidal/fungisidal concentration (MBC/MFC) tests to reveal possible antimicrobial properties. Then, the obtained results were compared with 18 known antibiotics. The results revealed that 7.33-19.66 mm inhibition zones were obtained for 15-different microorganisms at 100 µL concentrations while those obtained 7.33-12.33 mm inhibition zones for 12 microorganims at 50 µL volume, where no inhibition zone was observed at 10 µL volume addition. The extracts of Boğazkere for the tested concentrations showed no antimicrobial capability against *Salmonella kentucky, Enterococcus durans, Salmonella typhimurium* and *Candida ablicans*. MIC tests showed that the extract at 0.039-20 mg/100 mL concentration range was bacteriostatic for the entire tested microorganism. Bactericidal effects of the extract were obtained for *Listeria innocua* at 10 mg /100 mL while that was 20 mg /100 mL for *Pseudomonas fluorescens, Pseudomonas aeruginosa, Enterococcus faecium* and *Staphylococcus aureus*. The findings show that Boğazkere grape species has antibiotic character, what makes them possible preservatives for food products.

1. INTRODUCTION

Humankind has accumulated the knowledge of healing potentials of the plants from the early times of humanity, and has revealed it to the next generations. Phenolic compounds are the basis of curing potentials of the plants. Plants inherently use the phenolic compounds for defense, and those give smell, flavor and color. Antimicrobial properties of the plants derived from the chemicals that have been in use of curing microorganisms-mediated diseases for ages [1].

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There are two characteristic mechanisms that microorganism use to develop resistance for antibiotics, which are natural and acquired resistance. In natural resistance, the microorganisms do not possess compound or system targeted by the antibiotics while the acquired resistance refers to that the microorganism develop a mechanism (e.g. discarding the antibiotic via newly developed proteins) to eliminate antimicrobial agent's effect that inherently kills the microorganism [1, 2].

Antimicrobial agents can trigger a variety of reactions in living organisms. They can either act on cellular membrane or target elements within the cytosol; for example inhibition of nucleic acid synthesis, and cause organelles malfunction and cell wall metabolism problems. Besides, they can cause troubles at organ levels including cardiovascular and urinary system defects [3].

Plants within Vitaceae family are among the oldest plants cultivated worldwide owing to their strong adaptation for different climate and soil types. There are about 1200 cultivated grape types belong to *Vitis vinifera* species. They are used to produce wine, juice, molasses, dried fruit roll-up and as ingredient of cosmetic stuffs, in addition to that they are consumed as dried and fresh fruits. Besides high sugar and vitamin contents, grape carries high amount of antioxidants. Boğazkere grape is one of the most valuable grapes of Anatolia, which is mostly cultivated in Diyarbakır. They are mostly produced in red soils possess gravel, clay and calcareous character. Beads of Boğazkere are dark red colored and mid-sized [4-8].

Baydar et al. (2006) tested the extracts of seeds from Hasandede, Emir and Kalecik Karası grapes (extraction was performed in water/acetone/acetic acid solvent system) on 15 different microorganisms using agar-disc diffusion methods for the concentrations of 1%, 2.5%, 5% and 10%. Among the grape types, Hasandede revealed suppression capacity for all the bacteria at the tested concentrations [4].

Baydar et al. (2004) performed ethyl acetate/methanol/water mediated extraction of grape seeds, whose lipid content was removed beforehand. The extracts were tested for 15 microorganisms using disc diffusion method, where the highest antibacterial activity was observed for *Listeria monocytogenes* while no activity was obtained for *Enterobacter aerogenes* [9].

Anastasiadi et al. (2009) analysed chemical contents belong to pomace of four *Vitis vinifera* types and tested anti-*L. monocytogenes* capability using MIC test approach. The findings revealed that the extracts from plant stem and seeds could be introduced to food samples as preservatives [10].

Ege (2015) carried out acetone/water/acetic acid/methanol mediated extraction from seeds of Müşküle (white), Kara dimrit (blue-black) and Öküzgözü grape types, whose lipid content was removed beforehand. Antimicrobial activity of the extracts was tested for 4 fungi and 6

bacteria strains using MIC test. The findings revealed that all the extracts did not show antimicrobial capability against 4 fungi and 4 bacteria strains [11].

Abtahi et al. (2011) extracted dried white, red and black grape samples in 70% alcohol, then which were tested on *Escherichia coli* PTCC1330*, Staphylococcus aureus* PTCC 1431*, Salmonella typhimurium* PTCC1639 and *Pseudomonas aeruginosa* PTCC1310 strains using MIC tests. The findings showed that the extracts gave positive results for all the tested bacteria with showing its highest activity for *S. aureus* strain [12].

Waqar et al. (2014) tested the antimicrobial activity of the extracts from leaves of *V. vinifera* on *E. coli*, *P. aeruginosa, S. aureus* and *Enterococcus faecalis* strains using disc diffusion method. The inhibition zones belong to *S. aureus* as 30 mm, *E. faecalis* as 28.9 mm, *E. coli* as 28 mm and *P. aeruginosa* as 23.7 mm were obtained [13].

Yadav et al. (2015) tested the antibacterial capability of water-, ethanol-, acetone-, and methanol- mediated extracts of grape-peel at three different concentrations for the antibiotic resistant *S. aureus, E. faecalis, Enterobacter aerogenes, Salmonella typhimurium* and *E. coli* using disc diffusion method. The findings revealed the highest antibacterial capacity for methanol mediated extracts. *S. typhimurium* and *E. coli* showed resistance for the tested concentrations. The extract gave the inhibition zones for *S. aureus* as 22 mm, *E. faecalis* 18 mm and *E. aerogenes* as 21 mm [14].

In the present work, antimicrobial activity of Boğazkere belong to *Vitis vinifera* species performed along with evaluating its possible preservative role for food samples.

2. MATERIAL AND METHODS

2.1 Plant Samples and Extraction

Samples of Boğazkere grapes, collected in Kırşehir Toklumen vineyards of Kavaklıdere Company in September of 2017. The collected samples, protected under proper conditions until they reached the laboratory. The samples rinsed thoroughly, followed by the beads were detached from the stems using clean blade. The beads were then grinded in mortar, followed by liquid part, removed with clean cheesecloth. The obtained pomace was mixed with liquid nitrogen, and then grinded into fine particles in mortar. The particles were added to the liquid part. The mixture, was mixed with 96% ethanol solution at 1:1 ratio, which then underwent mixing on orbital shaker at 100 rpm for three days. Followed by the extraction, filtration was performed using whatman paper, where alcohol and water content was eliminated using Rotary evaporator (run in water bath and under vacuum). Samples used for disc diffusion

method was prepared by dissolving 2 gr of dried sample in 8 mL etanol: 2 mL pure water solvent system. In MIC test, 2 gr of dried sample was dissolved in 10 mLof water, followed by filtration through 0.2 µm sterile filter. The prepared stocks were kept under proper conditions until further usage.

2.2 Test Microorganisms

The prepared extracts of Boğazkere were tested for the following standart strains or isolated microorganisms: *Enterobacter aerogenes* (ATCC 13048), *Salmonella infantis, Listeria monocytogenes, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (DSMZ 50071), *Pseudomonas fluorescens, Salmonella kentucky*, *Enterococcus faecalis* (ATCC 29212), *Listeria innocua, Salmonella enteritidis* (ATCC 13075), *Enterococcus durans, Salmonella typhimurium, Candida ablicans* (DSMZ 1386), *Enterococcus faecium*, *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (DSMZ20044)*, Bacillus subtilis* (DSMZ 1971), and *Escherichia coli* (ATCC 25922), *Saratia marrescens* (ATCC 13048).

2.3. Preparation of Inoculation

All the tested microorganisms were grown in Nutrient agar, where the identical colonies were collected using a sterile disposable swabing tool and placed into 10 mL of 0.9 % steril NaCl solution. Based on 0.5 McFarland turbiditiy standard, bacterial colonies were prepared at 10^8 cfu.mL^{-1} while that was 10^7 cfu.mL^{-1} for *C. albicans* [15-17].

2.4. Loading Extract to Empty Disks

In disc diffusion method, sterile empty antibiogram discs were used to evaluate antimicrobial activity of the extracts.

The stock Boğazkere extracts at 10 µL, 50 µL and 100 µL volumes, placed on the empty discs, followed by incubated at 30 ℃for overnight under sterile condition. The dried samples, kept at +4 ℃until further usage.

2.5 Disc Diffusion Method (DD)

0.1 mL of inoculum was evenly spread over the Mueller Hinton Agar (MHA) using swab stick. Antimicrobial vulnerability test was performed in accordance with Bauer-Kirby method [18].

For each MHA, empty, 10 µL, 50 µL and 100 µL extract impregnated discs were added onto the surface of MHA. Bacterial samples and fungus sample were incubated at 37 °C and 27 °C for 24 h. Right after 24 h incubation period, the inhibition zones were measured in millimeter (mm). The tests were performed in three parallel.

2.6. Determination of minimum inhibitory concentration (MIC)

All the microorganisms showed vulnerability for the Boğazkere extracts from the disc diffusion tests were included into MIC test. MIC test was performed in sterile 96-well plate. MIC value was accepted as the concentration at which bacterial growth was not visually observed [19-20].

100 µL of sterile Muellller Hinton Broth was added to each well of 12-well microplate. 100 µL from the stock Boğazkere extract was added to the first well of the 12-well microplate (Number 1 well). 2-times serial dilution was then applied to dilute the extract from number well 1 to well 10 (Number 10 well). 100 µL solution was then discarded from number 10 well. 10 µL from each of the selected microorganisms were added from Number 1-Number 11 wells. Wells from 1-10 were used to evaluate MIC of Boğazkere while Number 11 well was used for microbial positive control and Number 12 well was used as system control. Bacterial samples and fungi sample were incubated at 37°C and 27°C for 24 h to explore MIC values for each microbial strain. The tests were performed in three parallel.

2.7. Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

Minimum bactericidal/bacteriostatic concentration (MBC) test is to find out whether any bacterial development takes place in the wells where no bacterial growth was observed in MIC test. The findings of this test clarifies whether the MIC concentrations are bactericidal or bacteriostatic.

Followed by the MIC test, samples from the wells (where no growth was visually observed) were transferred onto a fresh Nutrient Agar, followed by incubated at 37°C for overnight. In

the case of no growth on the fresh agar, the MIC concentration was accepted as *minimum bactericidal concentration* while that was accepted, as *minimum bacteriostatic concentration* in the case growth was clear. The tests were performed in three parallel.

2.8. Controls

In disc diffusion method, sterile empty discs were used as negative control while 18-standard antibiotic discs were used for 19 microorganisms as positive control.

3. RESULTS AND DISCUSSIONS

Antimicrobial activity of the extract from *Vitis vinifera* cv. Boğazkere was tested for 18 bacterial strains and one fungus species using disc diffusion method. The findings based on triple parallel examination with inhibition zone diameters are given in Table 1. The findings revealed that Boğazkere extract possessed antimicrobial activity for 15 microorganisms by giving inhibition zones between 7.33 and 19.66 mm.

Treatment with 10 µL of the preprared stock extract did not show any antimicrobial activity for the tested microorganisms while at 50 µL treatment antibacterial activity was observed for *S. infantis, L. monocytogenes, K. pneumoniae, P. fluorescens, P. aeruginosa, E. faecalis, L. innocua, E. faecium, S. aeureus, S. epidermidis, B. subtilis* and *E. coli* with causing inhibition zones in the range of 7.33-12.33 mm.

Treatment with 100 µL of the stock antibacterial activity for *E. aerogenes*, *S. infantis*, *L. monocytogenes*, *K. pneumonia*, *P. fluorescens*, *P. aeruginosa*, *E. faecalis, L. innocua, S. enteritidis, E. faecium, S. aeureus, S. epidermidis, B. subtilis, E. coli* and *S. marcescens* was observed with causing inhibition zones between 7.33 and 19.66 mm diameters. However, *S. kentucky, E. durans, S. typhimurium* and *C. ablicans* did not show any vulnerability towards the extract at the tested concentrations.

Followed by the disc diffusion method, MIC tests were performed for all the microbial strains that showed vulnerability for the extract. Microbial resistance gradually increased upon decreases in the applied extract concentration. The extract between 0.039 and 20 mg/100 mL concentrations gave MIC values for the selected bacterial strains, which was given in Table 2. Further studies on MIC tests revealed that most of the MIC values were bacteriostatic while such high concentrations were more of bactericidal doses (Table 2).

TABLE 1. Antimicrobial acitivity of Boğazkere extract based on disc diffusion method (Negative test result equal to empty disk diameter = 6 mm).

Mikroorganisms	Zone Diameter (mm)											
	10 µL				50 µL				100 µL			
	A	B	C	Mean	A	B	C	Mean	A	B	C	Mean
B. subtilis	-	-	-	-	10	9	10	**9.66**	12	12	12	**12**
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-	-	-	7	7	8	**7.33**
E. coli	-	-	-	-	9	7	10	**8.66**	10	9	10	**9.66**
E. durans	-	-	-	-	-	-	-	-	-	-	-	-
E. faecalis	-	-	-	-	12	12	14	**12.66**	12	17	17	**15.33**
E. faecium	-	-	-	-	12	12	13	**12.33**	20	19	20	**19.66**
K. pneumoniae	-	-	-	-	7	8	7	**7.33**	10	10	10	**10**
L. innocua	-	-	-	-	12	10	13	**11.66**	16	15	16	**15.66**
L. monocytogenes	-	-	-	-	9	10	11	**10**	12	14	16	**14**
P. aeruginosa	-	-	-	-	7	7	8	**7.33**	9	8	9	**8.66**
P. fluorescens	-	-	-	-	10	14	14	**11.33**	14	15	17	**15.33**
S. aeureus	-	-	-	-	11	7	10	**9.33**	13	10	12	**11.66**
S. enteritidis	-	-	-	-	-	-	-	-	8	8	10	**8.66**
S. epidermidis	-	-	-	-	7	7	8	**7.33**	8	8	7	**7.66**
S. infantis	-	-	-	-	9	7	7	**7.66**	10	12	12	**11.33**
S. kentucky	-	-	-	-	-	-	-	-	-	-	-	-
S. marcescens	-	-	-	-	-	-	-	-	9	9	10	**9.33**
S. typhimurium	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 2. Results for Minimum inhibition concentration (MIC) test and Minimun Bactericidal Concentration (MBC). *(*Bcd: Bactericidal effect, Bst: Bacteriostatic effect*)*. (Initial concertation of the extract was 10 mg/100 µL).

BACTERIA	MIC	MMC (Bcd)	MBC (Bst)
E. aerogenes	1.25	-	1.25
S. infantis	5	-	5
L. monocytogenes	10	-	10
K. pneumoniae	20	-	20
P. fluorescens	10	20	10
P. aeruginosa	0.625	20	0.625
S. kentucky	-	-	-
E. faecalis	20	-	20
L. innocua	0.625	10	0.625
S. enteritidis	0.039	-	0.039
E. durans	-	-	-
S. typhimurium	-	-	-
C. albicans	-	-	-
E. faecium	10	20	10
S. aeureus	0.625	20	0.625
S. epidermidis	2.5	-	2.5
B. subtilis	5	-	5
E. coli	10	-	10
S. marrescens	5	-	5

Antibiogram tests revealed that Lincomycin (L2) posed antibacterial activity only for *P. aeruginosa*, *S. aereus*, *B. subtilis* among the tested 18 different bacterial strains with 9 mm, 25 mm and 16 mm diameter zone inhibitions. Meropenem (MEM 10), Gentamicin (CN10), Neomycin (N30) and Ciprofloxacin (CIP5) showed antibacterial activity nearly all of the tested bacteria. The highest activities were observed for *B. subtilis* (46 mm zone diameter) and *S. marcescens* (43 mm zone diameter) with Ampicillin 10 mcg and Ciprofloxacin 5mcg treatment. Similarly Meropenem at 10 mcg. Caused 40 mm zone diameter formation for *B. subtilis* (Table 3).

Yadav et al. (2015) tested the antibacterial capability of water-, ethanol-, acetone-, and methanol- mediated extracts of grape-peel at three different concentrations (i.e. 260 mg/TAE/ml, 540 mg/TAE/ml and 1080 mg/TAE/ml) for the antibiotic resistant *S. aureus, E. faecalis, E. aerogenes, S. typhimurium* and *E. coli* using disc diffusion method. The findings revealed the highest antibacterial capacity was from the methanol-mediated extracts. *S. typhimurium* and *E. coli* showed resistance for the tested concentrations while the rest gave vulnerability for all the extracts performed in different solvents ($P < 0.05$). The extract gave the inhibition zones for *S. aureus* as 22 mm, *E. faecalis* 18 mm and *E. aerogenes* as 21 mm [15], for which zone inhibitions were obtained as 11.66 mm, 15.33 mm and 7.33 respectively upon treatment with Boğazkere extract in the present work. The difference in the findings might be related to the difference of the plant species and chemistry of the extraction solvent.

TABLE 3. Disc diffusion test results of positive control antibiotics

BACTERIA	L2	OFX5	ME M10	TE30	CZ30	VA 30	AM 10	K 30	CN 10	S 10	S 300	NA 30	SH 100	SXT 25	N30	IP 5	AM C30	C30
E. aerogenes	-	27	28	18	13	-	10	24	25	-	23	24	30	28	20	32	10	30
S. infantis	-	24	37	9	15	-	20	-	20	10	-	-	12	-	10	30	21	30
L. monocytogenes	-	19	25	-	18	-	22	-	20	11	-	-	12	25	10	25	25	27
K .pneumoniae	-	30	30	17	-	-	-	25	24	20	25	24	18	-	20	35	11	30
P. fluorescens	-	23	25	18	-	-	-	-	20	13	16	-	17	-	12	33	-	-
P. aeruginosa	9	19	14	20	-	21	30	12	15	-	-	-	20	25	12	24	30	23
S. kentucky	-	32	33	15	-	-	25	23	14	11	-	23	-	26	21	32	26	30
E. faecalis	-	20	21	10	-	22	30	18	15	-	20	-	19	28	17	23	30	25
L. innocua	-	17	26	22	-	20	28	25	25	26	35	17	21	26	12	22	30	24
S. enteritidis	-	32	32	20	19	-	23	22	21	20	15	25	24	25	18	30	25	28
E. durans	-	17	27	21	12	-	-	25	20	22	22	24	24	26	18	21	10	30
S. typhimurium	-	32	32	15	15	-	25	26	27	-	-	25	33	23	22	35	30	33
E. faecium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aereus	25	28	35	25	-	21	40	24	25	20	17	-	21	28	22	30	37	25
S. epidermidis	-	30	32	15	10	-	-	25	24	18	24	28	30	30	21	37	10	33
B. subtilis	16	28	40	33	9	24	46	28	30	20	35	23	30	35	34	35	10	33
E. coli	-	-	36	-	-	-	-	20	25	21	23	-	30	16	22	-	18	25
S. marcescens	-	38	38	18	24	-	-	30	27	25	25	39	33	30	22	43	10	32

(-) No effect, Lincomycin: L2, Ofloxacin: OFX 5, Meropenem: MEM 10, Tetracycline: TE 30, Ceftazidime: CAZ 30, Vancomycin: VA 30, Ampıcillin: AM10 Kanomycin: K 30, Gentamicin: CN 10, Streptomycin: S10, Compound Sulphonamides: S 3 300, Nalidixic acid: NA 30, Spectonomycine: SH 100 Sulphamethoxazole trimethaprim: SXT 25, Chloramphenicol: C 30, Neomycin: N 30, Cıprofloxacin: CIP 5, Amoxycillın clavulanic acıd: AMC30

Baydar et al. (2006) tested the extracts of seeds from Hasandede, Emir and Kalecik Karası grapes (extraction was performed in water:acetone:acetic acid solvent system, 90:9.5:0.5) on *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* FMC 19, *Enterobacter aerogenes* CCM 2531, *Enterococcus faecalis* ATCC 15753, *Escherichia coli* DM, *E. coli* O157:H7 KUEN 1461, *Klebsiella pneumoniae* FMC 5, *Mycobacterium smegmatis* RUT, *Proteus vulgaris*

FMC 1, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* EU, *Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus* Cowan 1 and *Yersinia enterocolitica* EU using agar-disc diffusion methods for the concentrations of 1%, 2.5%, 5% and 10%. Among the tested grape types, Hasandede revealed suppression capacity on all the bacteria at the tested concentrations [4]. Hasandede extract at 10% concentration gave the highest antibacterial activity for *Aeromonas hydrophila* ATCC 7965 with 30.67 mm inhibition zone. Extracts from all the tested grape types at 0.5% and 1% concentrations gave bacteriostatic effect for *Escherichia coli* DM and *E. coli* O157:H7 KUEN 1461 strains while all the grape types showed bacteriostatic effect for *S. aureus* Cowan 1 strain. In our study, extracts of Boğazkere caused 9.33-11.66 mm inhibition zone formations for *S.aureus* while it was between 12.66 and 15.33 mm for *E. faecalis*. However, the extract did not show any inhibitory effect on *E. aerogenes* for 10 µL and 50 µL treatment while only for 100 µL treatment 7.33 mm zone inhibition was observed. Similar to the Baydar et al. study, our MIC findings were more of bacteriostatic.

Baydar et al. (2004) performed ethyl acetate/methanol/water mediated extraction of grape seeds, whose lipid content was removed beforehand. The extracts were tested for *Aeromonas hydrophila, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Enterobacter aerogenes, Enterococcus faecalis Escherichia coli, Klebsiella pneumoniae Listeria monocytogenes, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus* using disc diffusion method [9].

The findings revealed that the highest antibacterial activity of methanol: water: asetic acid mediated extracts was for *L. monocytogenes* with 33.5 mm zone inhibition diameter at 20 % concentration. Similarly for acetone: acetic acid: water mediated extract gave the highest antibacterial capability for *L. monocytogenes* strain at 4 % concentration. Ethyl acetate: methanol: water mediated extract did not show any activity towards *E. aerogenes* at 4% concentration. However, certain extracts showed antibacterial activity for such bacterial strains even at 4% concentrations. In our study, the Boğazkere extract caused 9.33-11.66 mm zone inhibition for *S. aureus* strain at 50 µL and 100 µL volume application while at 10 µL concentration no activity was observed. Similarly, the extract caused 12.66-15.33 mm zone inhibition for *E. faecalis* strain at 50 µL and 100 µL volume application while at 10 µL concentration no activity was obtained. However, the extract gave anti-*E. aerogenes* activity at 100 µL treatment with 7.33 mm zone diameter.

Anastasiadi et al. (2009) analysed chemical contents belong to fruit, seed, stem and pomace of four *Vitis vinifera* types (i.e. Mandilaria, Voidomato, Asyrtiko and Aidani) and tested anti-*L. monocytogenes* capability using MIC test approach. The findings revealed that extracts from plant stem and seeds were very effective, and they seem a possible preservative could be introduced to food samples [10]. In our study, the Boğazkere extract gave anti- *L.*

monocytogenes activity at 50 µL and 100 µL volume treatment with causing 10.00 and 14.00 mm inhibition zone formation while at 10 µL no activity was observed.

Ege (2015) carried out extraction from seeds of Müşküle (white), Kara dimrit (blue-black) and Öküzgözü grape types, whose lipid content was removed beforehand. The extractions, performed in dedicated solvents systems of acetone, water, acetic acid and methanol. The extract stocks were prepared at 65.536 mg/mL concentration. Antimicrobial activity of the extracts was tested *Alternaria alternata, Aspergillus niger, Botrytis cinerea, Penicillium expansum, Escherichia coli 35218, Pseudomonas aeruginosa 27853, Klebsiella pneumonia 700603, Enterococcus faecalis 51299, Streptococccus pneumonia 49616* and *Staphylococcus aureus 44300* using MIC test. The findings revealed that all the extracts did not show antimicrobial capability against Alternaria *alternata, Aspergillus niger, Botrytis cinerea, Penicillium expansum, Escherichia coli 35218, Pseudomonas aeruginosa 27853, Klebsiella pneumoniae 700603* and *Enterococcus faecalis 51299*. In contrasto to this, Müşküle exract at 32.768 mg/mL and Öküzgözü at 65.536 mg/mL concentraions showed anti *S. aureus* activity. Similarly, Kara dimrit, Müşküle and Öküzgözü posssed anti- *S. pneumonia* activity at 2.048 mg/mL, 4.096 mg/mL and 32.768 mg/mL concentrations [11]. In our study, the Boğazkere extract did not show antimicrobial activity against *K. pneumonia, E. aerogenes, Candida ablicans, L. monocytogenes*, *P. fluorescens* ve *S. marcescens*. However, the extract at the range of 0.039 and 20 mg/100 µL gave antibacterial activity against *S. infentis, P. aeruginosa, S. Kentucky, E. faecalis, E. coli, L. innocua, S. enteritidis, S. typhimurium, E. faecium, S. aeureus, S. epidermidis* and *B. subtilis* strains. The difference between our study and the mentioned literature could be related to the different extraction solvent usage.

Abtahi et al. (2011) extracted dried white, red and black grape samples in 70% alcohol, which were then tested on *E. coli* PTCC1330*, S. aureus* PTCC 1431*, S. typhimurium* PTCC1639 and *P. aeruginosa* PTCC1310 strains using MIC tests. The findings showed that the extracts gave positive results for all the tested bacteria with showing the highest activity for *S. aureus* strain [12]. The obtained MIC values for *E. coli, S. aureus, S. typhimurium* and *P. aeruginosa* strains were 125, 32, 125 and 250 µg/mL, respectively. In our study, the Boğazkere extract gave 10 mg/100 µL and 0,625 µg/mL MIC values for *E. coli* and *S. aureus*, respectively. In contrast to this, it did not give any effect on *S. typhimurium*. In the present work, Boğazkere extract showed antimicrobial effect against other microorganisms with MIC values range between 0.039 and 20 mg/µL. As it was seen from the results, different grape types have different effect on the same microorganisms.

Waqar et al. (2014) tested the antimicrobial activity of the extracts from leaves of *V. vinifera* on *E. coli*, *P. aeruginosa, S. aureus* and *E. faecalis* strains using disc diffusion method. In the study, 5 mg of leaf extract was dissolved in 70% ethanol, followed by 3 discs were treated with 3 mg/0.1 mL of the dissolved extract. The inhibition zones belong to *S. aureus* as 30 mm, *E. faecalis* as 28.9 mm, *E. coli* as 28 mm and *P. aeruginosa* as 23.7 mm were obtained [13]. In our study, the Boğazkere extract gave antibacterial capability against

S. aureus, *E. faecalis*, *E. coli* and *P. aeruginosa* with 9.33-11.66 mm, 12.66-15.33 mm, 8.66-9.66 mm and 7.33-8.66 mm zone inhibitions, respectively. However, the extract did not show any effect on these bacteria at 10 µL concentration. The obtained difference between the two studies came from utilization of different grapes and different parts of the plant.

The findings of this study along with the literature provide strong insight into that leaves, fruits and seeds of *Vitis vinifera* L. can provide antibacterial activity depending on the extraction method. Even at low concentrations of *Vitis vinifera* L. Boğazkere extracts can pose antimicrobial effect for the tested bacterial strains. Our findings are endorsing the literature revealing that the plant extracts can be alternative and viable tools to fight against microbial development, which has been under investigation for a long time.

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SKULL SEXUAL DIMORPHISM APPEARS IN TOY RABBITS

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ABSTRACT. The aim of this paper was to use geometric morphometrics to study the skull sexual dimorphism of toy rabbits, which present paedomorphic (babyness) traits comparing data with those from agriotype (ancestor), *Oryctolagus cuniculus*. For this purpose, we examined 43 post-weaned corpses belonging to wild rabbit (n=22, 7 ♂ and 15 ♀, 1295.6 ± 333.8 kg of body weight and 88.0 ± 1.12 cm of ear length) and toy type (n=21, 4 ♂ and 17 ♀, short and upright ears, 1031.3 ± 644.13 kg of body weight and 6.2 ± 1.08 cm of ear length). Heads were radiographed using a Potro® machine on a latero-lateral projection and 7 landmarks were located on the skull and studied by means of geometric morphometric procedures. Size and shape between genders appeared statistically different only for toys, mainly focused on splanchnocranium (face) for shape. Detected sexual dimorphism could be attributed to selection arising from differential mating success, or sexual selection, due to human management. Moreover, the inconsistency with Rensch's hypothesis – which establishes that males in larger species will tend to be larger relative to females than in smaller species- allows us to suggest that Rensch's hypothesis is not necessarily followed in artificial selection experiencing miniaturization in body shape. It must be outlined the opportunities to tackle paedomorphic questions via geometric morphometrics methods in toy rabbits.

1. INTRODUCTION

Toy rabbits are a type of the wild rabbit (*Oryctolagus cuniculus*) selected by their paedomorphic traits. Toys are typical for *babyness* traits [1]: relatively big skull, shortened rostrum, and short ears, which render them especially attractive to owners [2]. Under development of a trait relatively to the ontogenetic course of this trait in the ancestor correspond to paedomorphosis [3,4]. Toys underdevelop some traits, giving them a clear infantile aspect. There is some evidence that there are functional constraints represented mainly by miniaturization of size in small toys [2]. Breeders point out that toy females may suffer from complicated parturition. Modern lineages of these types of companion rabbit present a unique opportunity to test hypothesis about paedomorphosis.

The aim of this paper was to use geometric morphometrics to study the skull sexual dimorphism of toy rabbits, comparing data with those from agriotype (ancestor), the wild rabbit, for which, at least from Iberian Peninsula, no dimorphism has been described for

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males and females [5]. Sexual dimorphism for toys and wilds are studied both as size as shape difference.

2. MATERIAL AND METHOD

2.1. Sampling procedure and data collection

The present study examines 43 post-weaned animals belonging to wild rabbit (n=22, 7 ♂ and 15 ♀, 1295.6 ± 333.8 kg of body weight and 88.0 ± 1.12 cm of ear length) and Toy type (n=21, 4 ♂ and 17 ♀, short and upright ears, 1031.3 ± 644.13 kg of body weight and 6.2 ± 1.08 cm of ear length). Fresh corpses of toys were collected from a breeding farm, and wilds were supplied from pest control campaigns. Then, they were beheaded, and ear length was obtained with a calliper. Sampled specimens were sexed whenever possible.

2.2. Data acquisition

Then heads were then radiographed using a Potro® machine on a latero-lateral projection. Exposure values ranged from 40 to 60 kV and 3.2-5 mAs. Pictures (each approximately 1.1 MB) were then saved in jpeg format and transferred to a computer.

2.3. Size and shape analysis

We firstly digitized 7 landmarks (LMs) by TpsDig 2.16 [6] to obtain the *x-y* coordinates of all points (Figure 1). The landmarks included in this study are chosen to correspond to those commonly used in both traditional [7] and GM. For the same 45 individuals, all images had a double digitalization of all landmarks for assessing the measurement error. First author (PMPC) was responsible of this preliminary study.

To perform the study, LMs were converted to shape coordinates by the generalized least square (GLS) Procrustes superimposition (GPA). GPA preserves all information about shape differences among specimens removing information about location, orientation and rotation from the raw coordinates and standardizes each specimen to unit centroid size (CS, a dimensionless size-measure computed as the square root of the summed squared Euclidean distances from each landmark to the specimen centroid) [8]. The information about the shape variation was extracted from the Procrustes superimposition [9,10]. Then we extracted the covariance matrix, generated by the Procrustes coordinates, and that includes the measures of the association between Procrustes coordinates themselves (that are the *X* and *Y* coordinates of each landmark after the Procrustes superimposition) [9,10]. The covariance matrix was used as a base for the subsequent analyses. A Mann-Whitney test was done to analyse CS differences between sexes, while a NPMANOVA using the Euclidean distance was used to study shape differences between types. A *deformation grid was* used to capture

the morphological shape differences and changes. Geometric procedures were performed in MorphoJ version 1.06c [11] and the rest of analysis with PAST version 2.17c softwares [12].

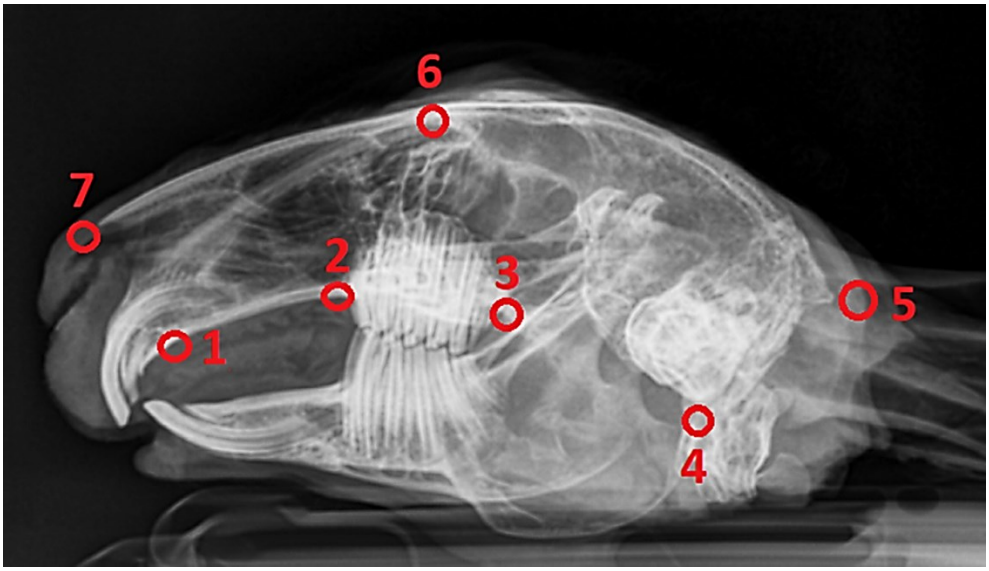

FIGURE 1. Location of the 7 skull landmarks used in the analysis: 1: base of inner upper incisor teeth; 2: most basal oral point of maxillary premolar teeth series (1st pM); 3: most basal caudal point of maxillary molar teeth series (3rd M); 4: ventral point of tympanic bulla; 5: external occipital protuberance; 6: dorsal projection of anterior cranial fossae; 7: most oral point of nasal bone. Latero-lateral projection.

3. RESULTS

Step 1 - Error evaluation

The evaluation of measurement error by the Procrustes analysis of variance (ANOVA) showed that error was negligible (F=0.04, p=1). Thus, all ulterior analyses went on using the averaged two replicas.

Step 2 - Analysis of size

Mean male and female CS were shown by Mann-Whitney test to be no significantly different for wilds (U=52, p=1.0, average of 617.84 (s.d. 44.33)), while for toys there appeared

significant differences (U=10, p=0.034, average of ♂ 476.6 (s.d. 72.99) and ♀ 577.4 (s.d. 80.9)). This indicates skull size sexual dimorphism only in toy rabbits.

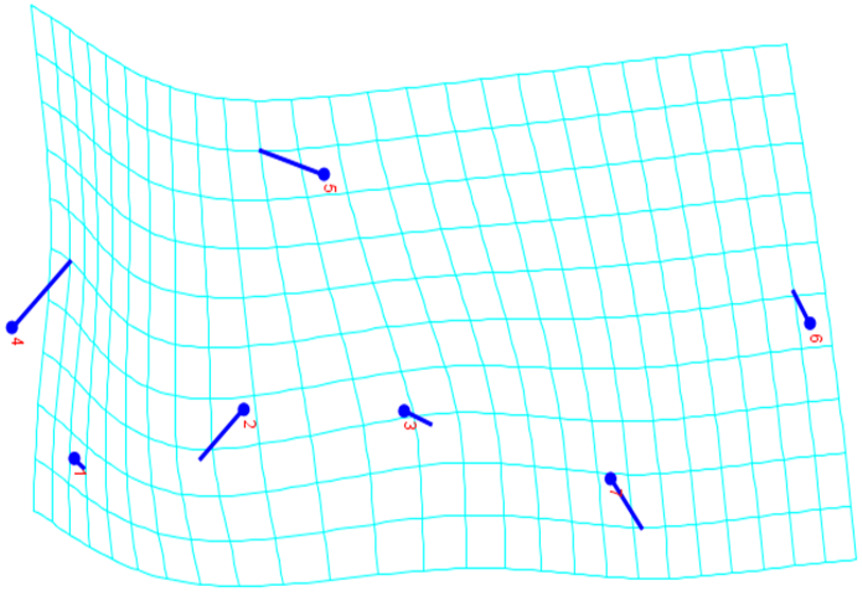

FIGURE 2. The differences were focused on splanchnocranium (face) (7 landmarks; see Figure 1).

Step 3 - Analysis of shape

The NPMANOVA test (Euclidean distances) indicated that the Procrustes distance between sex means was significantly different for skulls of toys (F=192.3, p=0.036) but not for wilds (F=0.950 p=0.464). This indicates skull shape sexual dimorphism only in toys skulls. In terms of geometric morphometrics the differences were focused on splanchnocranium (face) (Figure 2).

4. DISCUSSION

Geometric morphometrics provide the opportunity to get new insights in the variety of morphological characteristics and morphs of wild and domestic rabbits. The technique not constrained by focusing on particular shape features a priori, so that it was possible to detect differences in any direction of shape space. Such shape differences among groups can be easily visualized through deformation grids. The positioning of landmarks can be

individually adapted to particular research questions, so that geometric morphometric methods can be broadly applied for a wide variety of morphological questions. Traditional comparative morphological approaches are often based on selected measurements, and results are somewhat restricted to those few variables.

Toy rabbits, exhibiting paedomorphy did not present head sexual dimorphism, while the agriotype (ancestor wild species,) did [5,13]. Probably it reinforces Gould's conviction that fairly simple epigenetic perturbations often underlie complex morphological evolutionary changes [14]. Rensch's hypothesis establishes that males in larger species tend to be larger relative to females than are males in smaller species [15,16], but this was not the case, as toys were smaller than ancestors.

The difference in consistency with Rensch's hypothesis between wild rabbit and toys allows us to suggest that Rensch's hypothesis is not necessarily followed in artificial selection towards a miniaturization. Sexual dimorphism patterns not consistent with Rensch's hypothesis have been demonstrated in domestic chicken breed, too [17], and in fact this is logical if we keep in mind that artificial selection and formation of breeds (or varieties, or lineages) in domesticated animals is a different process involving for instance different genetic changes than speciation [18,19]

The link between developmental processes which suggests that developmental polymorphisms could affect variation in sexual size dimorphism [20] could reinforce this hypothesis. Thus, there are several promising trajectories to address important morphological questions on paedomorphy among domestic mammals, so that there is no doubt that this field will evolve further rapidly.

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[2] Kelley, J. L., General Topology, Van Nostrand, 1970.

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