

A comparative study of the antimicrobial properties and antioxidant enzyme activities of field-grown and *in vitro*-propagated plants of endemic *Digitalis trojana* Ivanina

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Abstract: The antimicrobial properties and antioxidant enzyme activities of field-grown and *in vitro*-propagated plants of *Digitalis trojana* Ivanina (Helen of Troy foxglove), a perennial endemic plant species of Turkey, were compared. The field work was carried out in May and July 2014, and plant samples of *D. trojana* were collected from Kazdağı (Turkey). Propagation of *D. trojana* was achieved by culturing leaf explants on MS medium supplemented with 13.3 µM 6-benzylaminopurine (BA) and 0.53 µM α-naphthaleneacetic acid (NAA). The antimicrobial activity, plant lipid peroxidation levels and antioxidant enzyme (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR)) activities were analyzed in 12- and 17-week-old *in vitro*-grown *Digitalis* plants collected in May and July from two different localities at 430 and 1173 m above sea level. Although the *in vitro*-propagated plants had very low antagonistic activities compared to field-grown plants, they exhibited remarkably similar antibacterial activities against *Escherichia coli* ATCC 11230, *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6633. There were no important differences between plants collected from the two localities (430 and 1173 m a.s.l.). Biochemical analysis showed that the antioxidant enzyme (SOD, APX, GR) activities of field-grown plants were higher than *in vitro*-grown plants. Also, the difference in altitude at which the plants were grown was apparently linked to decreases in antioxidant enzyme activities, except for POX in field-grown plants collected in July.

Key words: *Digitalis trojana*; *in vitro* propagation; field-grown; antimicrobial activity; antioxidant enzyme activities

INTRODUCTION

Digitalis trojana Ivanina is a member of the Plantaginaceae family, known by its common name Helen of Troy foxglove. The plant is a perennial endemic to Çanakkale and Balıkesir, northwestern Turkey [1]. According to the Red Data Book of Turkish Plants, the conservation status of this species is classified as vulnerable (VU) [2]. *Digitalis* species are a medicinally important group of plants because of their content of cardenolides or cardiac glycosides (CGs), which are used in human medicine. The cardenolides are effective drugs for treating several cardiac defects [3]. They also possess a broad spectrum of biological activities, including anticarcinogenic, acaricidal and antibacterial properties. Recent studies have focused on the anticarcinogenic effects of digoxin and digitoxin [4,5].

During the last two decades, there has been a tremendous rise in infectious diseases (hepatitis, malaria, tuberculosis, etc.) with fatal outcomes. This has been attributed to increased resistance of pathogenic microbes to antimicrobial agents/drugs and the indiscriminate use of synthetic antibiotics. Thus, there is a continual search for new antimicrobials, especially of natural origin [6]. In recent years, despite the progress of new technologies and medicine, it has become essential to develop and refine the multipurpose use of natural products. Antimicrobial activity studies with *Digitalis* species are very limited, as revealed by a literature survey [7,8]. Therefore, it is important to assess the antimicrobial activity of *in vitro*-propagated and field-grown *D. trojana*.

Plants are sessile organisms with complex protection mechanisms. During stress, reactive oxygen (O₂)

species (ROS) such as $^1\text{O}_2$, H_2O_2 , O_2^- and HO increase oxidative stress. During normal metabolism, nonenzymic components and antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), peroxiredoxin (PrxR) and superoxide dismutase (SOD) are essential for ROS detoxification [9]. Although ROS are toxic molecules, plants utilize these molecules to respond to changes in the environment and developmental dynamics [10]. The higher antioxidant levels in plants growing at higher altitudes enables them to deal with stressors [11,12].

There is no information about the potential link between antioxidant enzymes and the antimicrobial properties of *D. trojana* and their distribution in Kazdağı. The aims of this work were (i) to determine the antimicrobial and antioxidant enzyme activities of *D. trojana*, and (ii) to compare the antimicrobial and antioxidant enzyme activities of field-grown plants that were collected from different altitudes in May and July, with those of *in vitro*-propagated *D. trojana*.

MATERIALS AND METHODS

Plant Material

Ten field-grown plants were collected in May and July 2014 from two different localities (430 and 1173 m a.s.l.) in Kazdağı National Park, Balıkesir, Turkey. Propagation of *D. trojana* was achieved using the optimized protocol as previously described [13]. For *in vitro* propagation via organogenesis, leaf explants were excised from seedlings grown under sterile conditions and cultured on MS medium [14] supplemented with 13.3 μM 6-benzylaminopurine (BA; B0904, Duchefa) and 0.53 μM α -naphthaleneacetic acid (NAA; N0903, Duchefa), 3% (w/v) sucrose, 0.7% (w/v) agar and 2 mg L^{-1} (w/v) polyvinylpyrrolidone (PVP; Sigma N0640). For shoot multiplication, shoots were subcultured for a period of two weeks and repeatedly subdivided. For rooting, shoots were separated individually and transferred to MS medium containing 0.1% (w/v) activated charcoal (AC). The cultures were maintained at $25\pm 2^\circ\text{C}$ under a 16-h photoperiod with $72 \mu\text{mol m}^{-2}\text{s}^{-1}$ in growth chambers.

Antimicrobial properties, lipid peroxidation level, total protein content and antioxidant enzyme activi-

ties were analyzed in 12- and 17-week-old *in vitro*-propagated plants and in field-grown plants in May and July, from two different localities in Kazdağı. The plant samples were harvested in three replicates and placed in liquid nitrogen. The samples were stored at -30°C until analysis.

Preparation of extracts for antimicrobial activity

The overhead parts of the each located *D. trojana* plant were dried at room temperature ($26\pm 3^\circ\text{C}$) in the dark and pulverized. Each dried, pulverized sample (15 g) was extracted with 150 mL of different solvents (ethanol, methanol, ethyl acetate and acetone) (Merck, Darmstadt, Germany) by Soxhlet equipment for 24 h [15]. For *in vitro* plant extraction, dried (at 50°C for 5 days) tissue powders (1 g) of each plant (12-17 weeks-old) were dissolved separately in 20 mL of 80% (v/v) ethanol overnight on a rotary shaker at room temperature. The extract was filtered using Whatman filter paper no. 1 and the extracts were stored in sterile screw-capped bottles at 4°C .

Test microorganisms

Gram-negative bacteria (*Salmonella typhimurium* CCM 5445, *Escherichia coli* ATCC 11230, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427), Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Enterococcus faecalis*) and yeast (*Candida albicans* ATCC 10231, *Kluyveromyces fragilis* NRRL 2415) were used for determining the antimicrobial activity of plants.

Antimicrobial activity screening

The antagonistic effects of each plant against bacteria and yeast samples were detected by measuring the diameter zones of inhibition (mm). Empty sterilized antibiotic disks were each imbrued with extract (50 μL). All the bacterial and yeast cultures were incubated at $35\pm 0.1^\circ\text{C}$ for 24 h and $25\pm 0.1^\circ\text{C}$ for 48 h by inoculation into nutrient broth and malt extract broth, respectively. An inoculum containing 10^6 bacterial cells or 10^8 yeast cells/mL was spread on Mueller-Hinton agar (Oxoid Lt., Hampshire, UK) plates (1 mL inoculum/plate) [16]. Inhibition zones formed on agar plates were evaluated in mm. Studies were performed

in triplicate. Treatments with penicillin (P10), ampicillin (AMP20) and nystatin (NYS30) served as positive controls, and treatments with ethanol, methanol, ethyl acetate and acetone without plant materials served as negative controls. For quantitative antimicrobial analyses, minimum inhibitory concentration (MIC) values of all samples were determined [17]. Streptomycin was used as a reference antibiotic for bacteria MIC values.

Lipid peroxidation level

The leaf lipid peroxidation level (500 mg) of 12- and 17-week-old *in vitro* plants and collected field-grown plants in Kazdağı was assayed by determining the level of malondialdehyde (MDA). The content of thiobarbituric acid reactive substance (TBARS) was assayed by MDA [18].

Antioxidant enzyme assay

A frozen plant sample (500 mg) was placed in liquid nitrogen and homogenized in 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA Na₂ for SOD, CAT and GR analyses. For the determination of APX, the extraction buffer contained 5 mM ascorbate. The analysis of POX required grinding a 500-mg sample with 50 mM of sodium acetate buffer (pH 6.5). All homogenates were centrifuged at 21000xg for 30 min at 4°C and the supernatants were used for determination of the enzyme activities and total protein content. The total protein content of the enzyme extract was determined according to Bradford [19]. SOD (EC 1.15.1.1) activity was measured as the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm [20-21]. CAT (EC 1.11.1.6) activity was measured by measuring the initial rate of disappearance of H₂O₂ in the reaction mixture at 240 nm [22]. The reaction mixture contained H₂O₂ and 0.1 mM EDTA in 0.05 M Na-phosphate buffer (pH 7) and the enzyme extract. GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell [23]. The reaction mixture contained Na-phosphate buffer (pH 7.8), glutathione disulfide (GSSG), NADPH.Na₄ and the extract. NADPH oxidation was determined at 340 nm. One unit of GR was defined as 1 mmol mL⁻¹ GSSG reduced per min. APX (EC 1.11.1.11) activity was measured according to Nakano and Asada [24]. One unit of APX was defined as 1 mmol mL⁻¹ ascorbate oxidized in the reac-

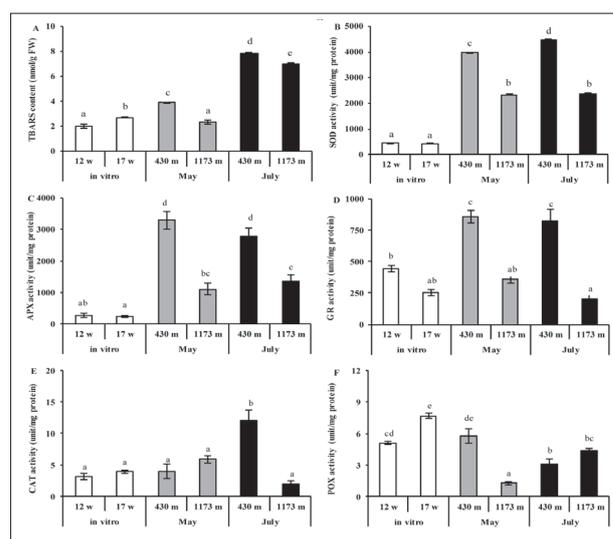


Fig 1. Alterations of TBARS content (A), the activities of superoxide dismutase (SOD) (B), ascorbate peroxidase (APX) (C), glutathione reductase (GR) (D), catalase (CAT) (E), and peroxidase (POX) (F) enzymes in *Digitalis trojana*. Vertical bars indicate means±standard error of five replicates. Means followed by the same letter were not significantly different at $p \leq 0.05$, as determined by Tukey's multiple range test (12 W – 12-week-old *in vitro* propagated plants; 17 W – 17-week-old *in vitro* propagated plants; 473 m – 473 m above sea level; 1173 m – 1173 m above sea level).

tion mixture containing 0.05 M Na-phosphate buffer (pH 7), 0.5 mM ascorbate, 0.1 mM EDTA.Na₂, 1.2 mM H₂O₂, and the enzyme extract at 290 nm per min. POX (EC 1.11.1.7) activity was measured according to Kanner and Kinsella [25]. The reaction mixture contained 50 mM Na-acetate buffer (pH 6.5), 90 mM H₂O₂, 100 mM pyrogallol and the enzyme extract. The activity was spectrophotometrically followed at 300 nm. A Mecasys Optizen Pop UV/Vis spectrophotometer was used for all spectrophotometric analyses.

Statistical analysis of antioxidant enzyme activities

Each assay was repeated two times independently. The vertical bars in the Fig. 1 indicate the means±standard error of five replicates. The data were subject to one way ANOVA according to randomized plot design in the *in vitro*-propagated plants and randomized split block design in the field-grown plants (months as the main-plot and altitudes as the sub-plot) using the Statistical Package for Social Sciences (SPSS) ver. 20.0. Mean comparison was conducted using Tukey's test at $p \leq 0.05$. (Means followed by the same letter are not significantly different).

RESULTS

Antimicrobial activity

The antibacterial and antifungal activities of extracts obtained from field- and *in vitro*-grown 12- and 17-week-old *D. trojana* plants against the test microorganisms were assessed by the presence and diameters of the inhibition zones, MIC and MBC (minimal bactericidal concentration) (Tables 1 and 2). There was no inhibition zone in the negative control. All the field-grown and *in vitro*-propagated plants exhibited antibacterial activity against *E. coli*, *Ps. aeruginosa* (except 2J), *S. aureus*, *E. faecalis*. *Ps. aeruginosa* in particular was more susceptible to all extracts of both field- and *in vitro*-grown plants as compared to all standard antibacterial antibiotics (except 2J).

The extracts exhibited antifungal activity against *C. albicans* and *K. fragilis* test fungi, however, the antifungal activity was lower than the activity of the standard antifungal agent NYS. When the activities of extracts of the field-grown plants were compared in terms of different sampling times and locations (May vs. July 2014; 430 vs. 1173 m), we did not observe any significant differences between the two groups of plants. However, the antifungal activity of the field-grown plants was higher than that of plants grown *in vitro*. Further, the *in vitro* plants produced no significant antagonistic effect against *St. typhimurium*, *S. aureus*, *E. faecalis*, *C. albicans* and *K. fragilis*. The microdilution method was used for all plant sample extracts to determine the MICs and MBCs. The extracts had weak effects against the test microorganisms, with MICs and MBCs ranging from 1024 (1000) to 64 (128) µg/mL. These values were considerably lower than for the standard antibiotics.

Lipid peroxidation level, total protein content and antioxidant enzyme activities

A statistically significant change in the total protein content of *in vitro*-propagated 12- and 17-week-old plants was not observed (Table 3). There were no statistically significant changes in SOD, APX and CAT activities (Fig. 1B, C and E, respectively), although GR and POX activities significantly changed during the growth of plants *in vitro* (Fig. 1D and F, respec-

Table 1. The antibacterial and antifungal activities of extracts obtained from field-grown and *in vitro* propagated 12- and 17-week-old *D. trojana* against test microorganisms assessed by measuring the diameter zones of inhibition (mm).

Source of plant material	Solvent	Type of Microorganisms									
		S.t.	E.c.	Ps. a	P.v.	B.s.	S.a.	E.f.	C.a	K.f.	
1M	Ethanol	15.0	12.0	15.0	15.0	12.0	10.0	15.0	12.0	16.0	
	Methanol	-	9.0	10.0	16.0	10.0	12.0	10.0	10.0	13.0	
	Ethyl acetate	9.0	9.0	13.0	16.0	10.0	9.0	10.0	13.0	12.0	
	Acetone	9.0	9.0	9.0	12.0	10.0	9.0	10.0	8.0	13.0	
1J	Ethanol	15.0	11.0	14.0	12.0	12.0	12.0	12.0	11.0	15.0	
	Methanol	14.0	13.0	15.0	13.0	15.0	13.0	13.0	12.0	16.0	
	Ethyl acetate	10.0	13.0	15.0	11.0	16.0	10.0	11.0	12.0	12.0	
	Acetone	12.0	11.0	14.0	12.0	14.0	10.0	14.0	11.0	12.0	
2M	Ethanol	9.0	13.0	13.0	-	-	11.0	16.0	15.0	15.0	
	Methanol	13.0	12.0	15.0	12.0	16.0	14.0	11.0	10.0	13.0	
	Ethyl acetate	10.0	13.0	10.0	10.0	14.0	10.0	14.0	10.0	9.0	
	Acetone	11.0	14.0	10.0	12.0	15.0	12.0	13.0	13.0	-	
2J	Ethanol	10.0	14.0	16.0	-	-	11.0	16.0	15.0	-	
	Methanol	13.0	14.0	13.0	14.0	11.0	15.0	9.0	11.0	14.0	
	Ethyl acetate	12.0	14.0	8.0	-	10.0	9.0	11.0	10.0	11.0	
	Acetone	8.0	14.0	-	-	8.0	9.0	13.0	10.0	9.0	
III	Ethanol	-	10.0	14.2	10.0	12.4	.5	7.2	-	7.0	
IV	Ethanol	-	10.0	14.4	7.0	13.2	7.5	7.0	7.0	7.0	
Standard antibiotics		S.t.	E.c.	Ps. a	P.v.	B.s.	S.a.	E.f.	C.a	K.f.	
P10		13.0	16.0	8.0	10.0	14.0	15.2	14.0	NT	NT	
AMP20		13.0	14.0	10.0	16.0	12.0	23.0	16.0	NT	NT	
NYS30		NT	NT	NT	NT	NT	NT	NT	18.0	18.0	

1M – field grown plants collected from 430 m in May; 1J – field-grown plants collected from 430 m in July; 2M – field-grown plants collected from 1173 m in May; 2J – field-grown plants collected from 1173 m in July; III – 12-week-old *in vitro*-propagated plants; IV – 17-week-old *in vitro*-propagated plants.

S. t. – *S. typhimurium* CCM 5445; E.c. – *E. coli* ATCC 11230; Ps. A. – *Ps. aeruginosa* ATCC 27853; P.v. – *P. vulgaris* ATCC 8427; B.s. – *B. subtilis* ATCC 6633; S.a. – *S. aureus* ATCC 6538P; E.f. – *E. faecalis*; C.a. – *C. albicans* ATCC 10231; K.f. – *K. fragilis* NRRL 2415; Nt – Not tested; P – Penicillin (10 µg/disc); AMP – Ampicillin (20 µg/disc); NYS – Nystatin disks (30 µg/disk)

tively). POX activity in *in vitro*-propagated 17-week-old plants was significantly increased (52.48%) as compared to *in vitro*-propagated 12-week-old plants (Fig. 1F), while GR activity decreased (42.94%; Fig. 1D). The TBARS content in field-grown plants was significantly decreased (40.19%) in May and July (11.19%; Fig. 1A) with increased altitude. The total protein content was significantly increased (70.75%) in May and in July (94.28%; Table 3). At higher-altitude SOD activity was significantly decreased (41.48% and 46.96%), as was that of APX (66.54% and 50.87%)

Table 2. Minimum inhibitory concentration (MIC) of the extracts of field-grown and *in vitro* (12- and 17-week-old) plants of *D. trojana*.

Source of plant material	Type of Microorganisms [MIC (MBC) ($\mu\text{g/mL}$)]								
	S.t.	E.c.	Ps. a	P.v.	B.s.	S.a.	E.f.	C.a	K.f.
1M	128 (256)	1024 (1024)	128 (256)	128 (256)	1024 (1024)	1024 (1024)	128 (256)	1024 (1024)	64 (128)
1M	-	1024 (1024)	1024 (1024)	64 (128)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	512 (1024)
1M	1024 (1024)	1024 (1024)	512 (1024)	64 (128)	1024 (1024)	1024 (1024)	1024 (1024)	512 (1024)	1024 (1024)
1M	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	512 (1024)
1J	128 (256)	1024 (1024)	256 (512)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	128 (256)
1J	256 (512)	512 (1024)	256 (512)	512 (1024)	256 (512)	512 (1024)	512 (1024)	1024 (1024)	64 (128)
1J	1024 (1024)	512 (1024)	256 (512)	1024 (1024)	64 (128)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)
1J	1024 (1024)	1024 (1024)	256 (512)	1024 (1024)	256 (512)	1024 (1024)	256 (512)	1024 (1024)	1024 (1024)
2M	1024 (1024)	512 (1024)	512 (1024)	-	-	1024 (1024)	64 (128)	128 (256)	128 (256)
2M	512 (1024)	1024 (1024)	128 (256)	1024 (1024)	64 (128)	256 (512)	256 (512)	1024 (1024)	512 (1024)
2M	1024 (1024)	512 (1024)	1024 (1024)	1024 (1024)	256 (512)	1024(1024)	1024 (1024)	1024 (1024)	1024 (1024)
2M	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	128 (256)	1024 (1024)	512 (1024)	512 (1024)	-
2J	1024 (1024)	512 (1024)	128 (256)	-	-	1024 (1024)	64 (128)	128 (256)	-
2J	512 (1024)	512 (1024)	512 (1024)	256 (512)	1024 (1024)	128 (256)	1024 (1024)	1024 (1024)	256 (512)
2J	1024 (1024)	512 (1024)	1024 (1024)	-	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)
2J	1024 (1024)	512 (1024)	-	-	1024 (1024)	1024 (1024)	512 (1024)	1024 (1024)	1024 (1024)
III	-	1024 (1024)	256 (512)	1024 (1024)	512 (1024)	1024 (1024)	1024 (1024)	-	-
IV	-	1024 (1024)	256 (512)	1024 (1024)	512 (512)	1024 (1024)	1024 (1024)	1024 (1024)	1024 (1024)
Standard antibiotics	S.t.	E.c.	Ps. a	P.v.	B.s.	S.a.	E.f.	C.a	K.f.
ST	16(32)	4.0(4.0)	1.0(1.0)	8.0(8.0)	0.5(0.5)	2.0(4.0)	2.0(4.0)	NT	NT
AMP20	1.0(4.0)	32(64)	16(32)	0.5(0.5)	0.5(2.0)	<0.25(0.35)	1.0(4.0)	NT	NT
NYS30	NT	NT	NT	NT	NT	NT	NT	8.0(16.0)	16.0 (16.0)

Nt: Not tested

Table 3. Total protein content in field-grown and *in vitro*-propagated *Digitalis trojana* plants.

	<i>In vitro</i>	May	July			
	12-week-old	17-week-old	430 m	1173 m	430 m	1173 m
Total protein content (mg/g)	0.23 \pm 0.002 ^{de}	0.22 \pm 0.003 ^{cd}	0.14 \pm 0.001 ^b	0.23 \pm 0.001 ^e	0.11 \pm 0.001 ^a	0.22 \pm 0.001 ^c

The values indicate means \pm standard error of five replicates. Means followed by the same letter were not significantly different at $p\leq 0.05$ as determined by Tukey's multiple range test. Bovine serum albumin (BSA) served as a standard (430 m above sea level; 1173 m above sea level).

and GR (58.28% and 74.98%). The decreases in CAT and POX activities were nonsignificant (84.32% and 84.32%, respectively) in May and July (Fig. 1B-F). In field-grown plants, the increase in seasonal temperature affected the total protein and TBARS contents and antioxidant enzyme activities. The average TBARS content in July was 136.94% higher than in May (Fig. 1A). In contrast to TBARS, the average total protein content in July was 7.78% lower than in May (Table 3). Compared to the mean enzyme activities in May and July, SOD, APX and GR activities decreased by 8.2%, 11.74%, and 5.75%, respectively (Fig. 1B-D). How-

ever, the average CAT and POX activities increased by 40.65% and 6.09%, respectively (Fig. 1E and F).

The average total protein contents of field-grown plants in May and July were, respectively, 17.79% and 26.25% lower than *in vitro*-propagated plants (Table 3). Also, the average POX activities were, respectively, 46.19% and 42.91% lower (Fig. 1F). The average TBARS contents in May and July were 33.29% and 216.10% higher, respectively, when compared to *in vitro*-propagated plants (Fig. 1A). Interestingly, the average SOD and APX activities of field-grown plants

were 6- to 8-fold higher than in *in vitro*-propagated plants (Fig. 1B and C). Compared to *in vitro*-propagated plants, in field-grown plants in May and July, (i) the average SOD activity was 604.51% and 662.25% higher, respectively (Fig. 1B); (ii) the average APX activity was 812.75% and 705.58% higher, respectively (Fig. 1C); (iii) GR and CAT activities were higher, with the average GR activity 67.11% and 57.51% higher, respectively (Fig. 1D), and CAT activity 38.86% and 95.22% higher, respectively (Fig. 1E).

DISCUSSION

In vitro culture technologies are becoming increasingly important to reveal the antimicrobial and antioxidant enzyme activities of economically important plant species. In plant tissue cultures, the biological activities of natural products do not undergo seasonal variations (limitations) [26,27]. In this research, we found that the antimicrobial activity of *in vitro*-grown plants was lower than in field-grown plants. However, all the plants exhibited antagonistic activity against *Ps. aeruginosa*. There is no information in the literature about the antimicrobial activity of *D. trojana*. Benli et al. [8] used extracts from leaves and flowers of *D. lamarkii* and observed strong growth inhibition against *B. subtilis*, *S. aureus*, *L. monocytogenes* and *Shigella* sp. bacteria cultures. However, they did not observe any antibacterial effect against *Ps. aeruginosa*, *E. coli* and *E. faecalis*. The difference in the results presented in our study indicates that the two species of *Digitalis* sp. possess different antimicrobial substances. This suggests that various species of *Digitalis* sp., including plants that are potentially important sources of medicinal substances, have yet to be identified. Detailed phytochemical investigations are required to determine the types of substances responsible for the antagonistic activities of these medical plants. Ethanol was reported to be the best solvent for extracting antibacterial or antifungal compounds [28]. The issues raised in this investigation with ethanol are analogous to those reported in the aforementioned research. It is important that the concentration of extract used in the test can be correlated with high activities of its chemical compounds. We found that the results obtained with the ethanol extract of field-grown plants were higher than the results obtained for the other solvents.

Thus, only ethanol was used for the extraction of *in vitro*-propagated plants.

Cultivated and *in vitro*-cultured plants are not as medically potent as wild-grown plants [29,30]. Our antimicrobial activity results showed that *in vitro*-propagated plants had lower antimicrobial activity than field-grown plants. It is thought that the production of secondary metabolites in *in vitro*-propagated plants is lower than that in field-grown plants. Bioactivity testing of *in vitro*-cultured plants is required to preserve naturally-grown plant populations and to support their use in traditional medicine [31].

Environmental changes such as seasonal variation, light and temperature affect antioxidant enzyme activities in *in vitro*-propagated plants [32-34]. Stress conditions inhibit the plant growth rate and antioxidant enzyme activities. We detected a decrease in the TBARS content in both May and July because of higher SOD, CAT, APX and GR activities in field-grown *D. trojana* plants as compared to *in vitro*-propagated plants. These results indicate that the antioxidant enzymes of *D. trojana* were effective at different altitudes. The observed decreases in activities of the antioxidant enzymes (SOD, APX, GR in both months, CAT in July and POX in May) were influenced by the lower atmospheric oxygen concentration at the higher altitude. While there is no research about the antioxidant enzyme activities of *D. trojana*, many studies on different plants from mountainous regions have shown that the activities of antioxidant enzymes are lower in leaves the higher the altitude. The activities of CAT and Cu/ZnSOD in *Pinus mugo* leaves collected from 1590 and 1920 m a.s.l. were lower than in samples collected at lower altitudes (1420 m) in the Tatra Mountains, Poland [35]. Also, the activities of SOD and POX declined significantly in the leaves of *Plantago major* with an increase in altitude from 1600 to 3300 m on Mahan Mountain, China [36]. On Tianshan Mountain (China), SOD, CAT and GR activities in *Polygonum viviparum* increased with the increase in altitude (from 2200 to 3900 m) [37]. Similarly, in the western part of the French Alps at 2400 m, *Soldanella alpina* had high activities of SOD, CAT and GR [12]. SOD activity in the alpine plant *Daphne oleoides* was higher than in *Teucrium polium* from the steppes, in Turkey (Northwest Anatolian Mountains and Central Anatolian steppes) [38]. These results are compatible

with our results obtained for field-grown plants. This may be one of the reasons why *D. trojana* is not distributed at altitudes higher than 1173 m.

In conclusion, this study revealed that field-grown *D. trojana* has higher antimicrobial and antioxidant enzyme activities than *in vitro*-propagated plants. To our knowledge, this is the first report on the antimicrobial and antioxidant enzyme activities of *D. trojana*. Our results suggest that field- and *in vitro*-grown *D. trojana* can be considered in the development of new antibiotics, and that the antioxidant enzyme activities are affected by increasing altitude and seasonal changes.

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