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Increasing the production of some specific cytotoxic triterpenoids and evaluation of the morpho-physiological response associated with *in vitro* salt stress in *Pistacia khinjuk* Stocks.

Emine Ayaz-Tilkat *

Batman University, Faculty of Science and Literature, Department of Biology,

Batı Raman Campus, 72100, Batman, Türkiye

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Abstract: Triterpenoids are a class of secondary metabolites that are known to exhibit and possess various biological activities. Using plant biotechnological tools, a novel approach for producing these compounds offers opportunities not only to produce such bioactive secondary metabolites but also to increase the quantities of these essential phytochemicals. Therefore, this study was aimed to elucidate the biosynthesis of some cytotoxic triterpenoids through *in vitro* micropropagation of *Pistacia khinjuk* Stocks with the application of different NaCl concentrations (0 to 250 mM) and to evaluate the triterpenoids content as well as synchronize the morpho-physiological properties of plant materials during the salt application. The effects of these salt concentrations were investigated on *P. khinjuk* seedlings grown for four weeks by using the MS medium supplemented with 100 mg/L L-ascorbic acid and NaCl of given concentrations. LC-MS/MS results showed that the amount of all triterpenoid types from different plant parts was increased while root-shoot lengths, RWC, total Chl, Chl-a/b, and carotenoid values were decreased significantly at high salinity concentrations (150 mM). The reduction of the quantitative and qualitative morpho-physiological parameters was due to the production of an increased amount of secondary compounds, i.e., triterpenoids rather than primary compounds in response to the increased salt concentrations.

Keywords: *Pistacia khinjuk* Stocks; LC-MS/MS; salt stress; triterpenoid; *in vitro*; plant physiology.

1. Introduction

Many higher plants accumulate a variety of secondary metabolites which play the vital roles in many essential defense mechanisms such as defenses against salinity, drought, and ultraviolet radiation stress along with microorganisms and herbivores and allelopathy [1]. Plant secondary metabolites are highly variable in their chemical construction. Plant-derived secondary metabolites display far-going therapeutic and pharmaceutical properties [2]. Among all the plant secondary metabolites, terpenoids (-mono, -di, tri etc.) represent the most diverse and major category of metabolites widely distributed in almost all plant groups [3,4]. Many medicinal activities like antimicrobial, antifungal, antiparasitic, antiviral potentials of these triterpenoids have been discovered by numerous studies [5, 6]. Moreover, they have also been utilized for preventing cancer and other several diseases due to their anti-inflammatory, antihyperglycemic, antispasmodic, anti-allergenic, and immunomodulatory properties [7]. Secondary phytochemicals can be obtained naturally by conventional agricultural methods which

* Corresponding author e-mail: eayaztilkat@gmail.com

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eventually depend on season, climate and geographical location as well as the growth conditions of plants. Therefore, production of secondary metabolites through *in vitro* micropropagation could be a fruitful approach since approximately 50,000 defined secondary metabolites, and more than 30,000 bioactive chemical compounds have been produced through plant tissue cultures [8]. The capacity of producing secondary metabolites was found to be higher in differentiated tissues such as root, embryo, and shoot cultures compared to undifferentiated callus and cell suspensions [9-11]. Besides, it was observed that micropropagated plant parts might produce a higher amount of substances than the plants in nature [12]. Furthermore, biotechnological methods with the use of elicitors or precursor compounds such as biotic and abiotic stress sources can influence the higher accumulation of secondary metabolite [13]. However, type of stress or elicitors, the genotype and developing stage of a plant, structure of the tissue or organ, and the duration of exposure to the stress play crucial roles in tolerance of plants to stress factors. Many studies have been reported on the production of secondary metabolites by *in vitro* propagation of plants under abiotic stress conditions [14-17].

Nowadays, a significant amount of agronomy area in the world is affected by salinity [18]. Salt stress is one of the potential abiotic stresses, occurs naturally in coastal salt marshes (especially estuaries) and desert areas as well as in long-term and highly irrigated agricultural areas, particularly in arid regions [18]. Salinity can be defined as the accumulation of excessive salt by plants in term of inorganic ions, including Mg^{2+}, Na^{+}, Ca^{2+}, K^{+}, $SO4^{2-}$, HCO_3^{-} and Cl^{-} [19, 20]. It exhibits significant negative impacts on almost all physiological phenomena, biochemical processes, and morphological traits, i.e., ion toxicity and a decrease in photosynthesis activity, increase in respiration rate, disruption in mineral conveyance, disruption of nitrate and carbon metabolisms, ionic imbalance in the cell membrane, and finally reduction of plant growth [21,22]. *In vitro* micropropagation of the plant with the salt-containing condition may cause secondary metabolite production as well as morphological changes [23]. However, there are limited studies on the secondary metabolite production of *Pistacia* species through *in vitro* tissue culture methods [6].

The khinjuk pistachio (*Pistacia khinjuk* Stocks) possesses strong acrid resinous smell and belongs to the family Anacardiaceae. They can grow naturally under severe arid and semi-arid conditions in the latitudes of 30-45°, and covers areas with the attitude of 700 - 2000 meters above sea level [24]. Different plant parts (resin, fruit, and leaves) of *Pistacia* species are used to treat several diseases for a long time due to their antimicrobial, anti-inflammatory, antiatherogenic, wound healing, blood pressure-lowering, and anticancer properties [25-28]. The effect of salinity on the quantity and quality of minerals and soluble sugars, proline accumulation, stomatal frequency and other morphological changes in leaves of different *Pistacia* species were reported; however, minimal information was found regarding the physiological response of khinjuk pistachio (*Pistacia khinjuk* Stocks) to saline stress [29-33].

Various *in vitro* propagation approaches have been reported in recent time to estimate the effects of salt stress on *Pistacia* and other species [34-36]. There is no study in the literature about the production of triterpenoids associated with the propagation of *Pistacia khinjuk* Stocks in *in vitro* salinity condition. In this context, this study aimed to investigate the effects of salt stress on *in vitro* shoot cultures of *P. khinjuk* Stocks considering some morpho-physiological responses on the germination. Besides, the production of some anticancer potential containing triterpenoids was also measured in this salt stress condition.

2. Experimental

2.1. Materials and Methods

2.1.1. Salt Treatments

For this study, salt solutions of 5 different concentrations (50, 100, 150, 200, and 250 mM) were prepared. These salt solutions were added into the MS medium [37] to investigate the responses of micropropagated plantlets regarding both triterpenoid content and morpho-physiological properties.

2.1.2. Obtaining Shoot Cultures and Elicitation Treatments

The seeds of *Pistacia khinjuk* Stocks were supplied by Gaziantep Pistachio Research Institute. The protocol developed by Tilkat et al. [38] was used for surface sterilization of seeds, culture initiation, and obtaining the stock cultures. The seeds were surface sterilized by immersing in a 20% (w/v) commercial bleach solution (NaOCl) for 30 min. Afterwards, the seed coats were removed, and the kernels were washed three times with sterile distilled water before inoculating onto the MS basal medium. MS basal medium supplemented with 100 mg/L, L-ascorbic acid, 3 % sucrose (w/v), and solidified with agar (0.7 %, w/v) was adjusted to pH 5.7 before autoclaving (120°C for 20 min). Cultures were maintained at 25 ± 2°C with a 16 h photoperiod (40 µmol $m^{-2}s^{-1}$) cycle. Approximately 1 cm long shoots from *in vitro* axenic stock medium of *P. khinjuk* Stocks were taken and cultured in MS medium containing different salt concentrations (0, 50, 100, 150, 200, 250 mM) along with a control group in a culture period of 28 days. After the end of this culture period, root, shoot, and leave of plantlets were dried separately and stored at 4°C for triterpenoid analysis.

2.2. Observation of Morphological Changes

After 28 days of seedling, three randomly selected plantlets from each treatment were selected for measuring the length of root and shoot. Symptoms developed in leaves were also observed in terms of morphological changes. Visible leaf damage was determined using a scale of 1-6 modified from Mohanty and Ong [39] as below:

1 = 0% no yellow leaves; 2 = 10-30% yellow leaves; 3 = 30-50% yellow leaves; 4 = 50-70% yellow leaves, 5 = most leaves were yellow; and 6 = all leaves were yellow.

2.3. Determination of Relative Water Content (RWC)

Relative water content (RWC) was determined according to previously described method in the literature [40]. Fresh 0.5 g of each leaf samples were placed in distilled water for 4 h. Then, the turgid weight of the disc of samples was measured. The dry weight of samples was also measured after drying the samples in an oven for 48 h at 65°C, and the following formula was used for calculating RWC of each sample:

$$RWC = [(\text{fresh weight} - \text{dry weight})/ (\text{turgid weight} - \text{dry weight})] \times 100.$$

2.4. Determination of Chlorophyll and Carotenoid Content

The primary pigment content was calculated based on the study of Arnon [41]. 100 mg of the fresh leaf was extracted by grinding in a mortar by using 15 ml of 80% acetone. The extract was filtered through white stripe filter paper; the absorbance values of these filtered extracts were measured against the distilled water for total chlorophyll content at 652 nm, for chlorophyll-a at 663 nm, for chlorophyll-b at 646 nm and carotenoid at 470 nm. Three replicates were used for each treatment, and the amount of pigment present in each sample was calculated according to the following equations [42] (A: Absorbance value reading):

Total chlorophyll = (A652x27.8) /mg specimen weight
Chlorophyll a (µg/mL) = (12.21 xA663 - 2.81 xA646) /mg specimen weight
Chlorophyll b (µg/mL) = (20.13 xA646 - 5.03 xA663) /mg specimen weight
Carotenoid (µg/mL) = (1000xA470 - 3.27[Cl- a] - 104[Cl- b]/227) /mg specimen weight

2.5. Triterpenoid Measurements by LC-MS/MS

Dried extracts from shoot cultures were analyzed by Shimadzu brand ultra-high-performance liquid chromatography (UHPLC). Dried plant materials were diluted with methanol (500 mg/kg), which was injected into the LC-MS/MS through a 0.22 µm filter.

2.6. LC-MS/MS Method Development and Validation

Quantitative analysis of the 6 triterpenoids used in this study was performed using the method developed within the TUBITAK project previously completed by Tilkat et al. [6]. According to the method, quantitative investigation of 6 triterpenoids in the studied samples was performed by using high-performance liquid chromatography-tandem mass spectrometry technique (LC-MS/MS). The system used was a Shimadzu brand LC–MS-8040 instrument. The column parameters were; 100 mm×2,1 mm, 2μm, The column temperature; 30 °C, Mobile phases; A: water, 10 mM ammonium formate and B: methanol, 10 mM ammonium formate. The gradient program; 85% B (35 min).

In this study, an LC-MS/MS method was optimized and validated for the quantification of 6 triterpenoids in the studied samples. The performance characteristics of the method were determined by using standard solutions, spiked and non-spiked samples. Within this context, the developed method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), limits of detection and quantification (LOD/LOQ) and relative standard uncertainty (U% at 95 % confidence level (k=2)). Parameters related to the LC-MS/MS method validation studies are given in Table 1.

2.6.1. Linearity

The linearity was assayed using external standard calibration curve with six concentration levels for each analyte, and each concentration level was assayed in triplicate. The developed method showed to be linear for all compounds, between the ranges of tested concentrations during the validation of the method with $R^2 \geq 0.989$. The equations for the calibration curves and the determination coefficients (R^2) are shown in Table 1.

2.6.2. Accuracy (Recovery) and Precision (Repeatability)

Accuracy and precision studies of the method were performed by standard addition to a selected extract. For intra-day variability assessment, spiked samples were measured for six replicates within a single day, whereas spiked samples were examined in triplicate per day for three consecutive days to conduct inter-day assay. As a result of the studies conducted on the same day and on different days, recovery and % RSD values were calculated to determine the accuracy and precision (Table 1). The recovery was calculated with the following equation: recovery (%) = (amount found – original amount)/amount spiked ×100%.

2.6.3. Limits of Detection and Quantification (LOD/LOQ)

To determine the LOD and LOQ values for the phytochemicals used in the LC-MS/MS method, analyte mixture were spiked at 10 identical samples prepared from a selected extract at the lowest concentration signaled by the standards and injected to the instrument. LOD and LOQ values were calculated according to the following equations (Table 1):

LOD=Mean+3×Standard Deviation
LOQ= Mean+10×Standard Deviation

2.6.4. Relative Standard Uncertainty (U^{95})

Standard uncertainties of the analytes were determined by the accuracy (recovery) and precision (repeatability) studies according to EURACHEM Guide [43]

Table 1. Analytical parameters that belong to the LC-MS/MS method

No	Analytes	RT[a]	Parent ion (*m/z*)[b]	Daughter ion (*m/z*)[b]	Ion. mode	Equation	R^{2c}	RSD%[d]		Linearity Range (mg/L)	LOD/LOQ (µg/L)[e]	Recovery (%)		U[f]
								Inter day	Intra day			Inter day	Intra day	
1	Ursolic acid	18.9	457.4	411.1	Pos.	y=90374.5×-3952.85	0.998	0.004	0.007	0.25-4.5	0.131/0.147	100.5	99.9	0.013
2	Ursonic acid	19.5	453.2	453.3	Neg.	y=459342×-988.44	0.990	0.005	0.005	0.25-4.5	0.108/0.119	104.4	102.4	0.059
3	Moronic acid	23.5	453.2	453.3	Neg.	y=49695.9×-19247.8	0.997	2.699	0.009	1-18	0.420/0.455	102.2	101.8	0.020
4	Oleanonic acid	22.9	453.2	453.3	Neg.	y=541816×-566373	0.990	5.391	0.001	1-18	0.583/1.712	106.5	101.6	0.009
5	Oleanolic acid	1.0	208.9	59.1	Neg.	y=94087.1×-12986.1	0.999	2.711	0.007	1-18	0.108/0.122	102.0	101.7	0.015
6	Masticadienolic acid	25.47	455.3	453.3	Neg.	y=628603×-57141.4	0.999	0.018	0.005	0.5-9	0.213/0.231	100.8	101.1	0.010

[a]RT: Retention time, [b]Mother ion (*m/z)*: Molecular ions of the standard compounds (*m/z* ratio), [c]R^2: Coefficient of determination, [d]RSD: Relative standard deviation, [e]LOD/LOQ (µg/L): Limit of detection/quantification, [f] U (%): percent relative uncertainty at 95% confidence level (k = 2). Pos.: Positive, Neg.: Negative

2.7. Statistical Analysis

Using different parameters, measurements of all the plantlets were recorded after completing 28 days culture period of the salt treatment, and an average of 3-5 replicates were taken for per treatment. The significance was determined by the analysis of variance (ANOVA), and Duncan's new multiple range tests were used to calculate the differences among the treatment means. Data presented in percentages were subjected to chi-square ($\chi 2$) analysis.

3. Results and Discussion

In this study, micropropagation protocol developed by Tilkat et al. [38] was applied to initiate the shoot culture *from P. khinjuk* Stocks seeds, and successful results were obtained from this method. After applying different concentrations of salt (NaCl; 0, 50, 100, 150, 200, 250 mM) in shoot cultures, the triterpenoid content, and the morphological and physiological properties of plants were measured, which are given below.

3.1.1. Morphological Observations

Morphological changes (root and shoot length and visible leaf damages) were evaluated after a culture period (28 days) of the NaCl treatment. According to Table 1 and Figure 1, the growth of the seedlings was decreased with the increase of salt concentration. Thick, large-sized, dark green-colored leaves were observed at control condition, which turned into light green color, especially at 50 and 100 mM NaCl concentrations, and most of the leaves turned smaller and yellow color at 200-250 mM NaCl concentrations. Chlorosis is one of the most common symptoms of stress due to the decrease of chloroplastic pigments. In this study, this process (chlorosis) increased gradually with the increase of salt concentration. Visible leaf damage was also increased with the increase of salt concentration level. Furthermore, salinity also reduced root, shoot, and leaf lengths and relative water content values compared to the control.

3.1.2. Physiological Observations

The physiological changes in terms of the RWC values and the pigment contents are given in Table 2, and Table 3, respectively. With the increase in salinity (0 to 250 mM), salinity stress in this study gradually harms most of the physiological characteristics (Table 2). Pigment content is considered as one of the remarkable stress symptoms in salt-treated tissues. Compared with the control, the maximum inhibiting effect was recorded at high salt concentrations (100-250 mM) for a culture period of 28 days. The relative total chlorophyll (Cl-a, the Cl-b), and the carotenoid contents of *in vitro P. khinjuk* Stocks shoots with different salt stress concentrations are presented in Table 3.

Table 2. The effect of increased NaCl concentrations on the root-shoot lengths, the visible leaf damage and the RWC of the *P. khinjuk* Stocks seedlings*

Salt con. (mM)	Root lengths (cm)	Shoot lengths (cm)	Visible leaf damage	RWC
0	18.80±1.14^{a}	4.50±0.29^{a}	1.00±0.00^{a}	53.11±0.13^{a}
50	18.75±1.80^{a}	3.00±0.14^{b}	1.20±0.10^{b}	50.0 ±1.08^{b}
100	14.45±0.62^{b}	2.50±0.16^{c}	2.40±0.11^{c}	45.89±0.05^{c}
150	5.40±0.55^{c}	2.30±0.11^{c}	3.20±0.64^{d}	41.48±0.07^{d}
200	4.45±0.43^{c}	1.80±0.06^{d}	4.30±0.65^{e}	38.84±0.09^{e}
250	1.19±0.11^{d}	1.15±0.07^{e}	5.30±5.59^{f}	34.9 ±0.09^{f}

*Data represents an average of 3 replicates per treatment after 28 days of culture. Means in a row followed by the same lowercase letters are not significantly different at $P \leq 0.05$ levels of significance according to Duncan's multiple range tests.

The control group showed the highest amounts of total chlorophyll (36.47), chlorophyll-a (25.27), chlorophyll-b (11.20) and carotenoid (5.72) among all groups. The lowest chlorophyll content was measured at 250 mM concentration. Total chlorophyll, chlorophyll-a, chlorophyll-b, and carotenoid values were very close to each other or similar from 100 to 250 mM NaCl concentration levels (Table 3).

Table 3. The effect of increased NaCl concentrations on the pigment contents of the *P. khinjuk* Stocks*

Salt con. (mM)	Total Cl	Cl-a	Cl-b	Carotenoid
0	36.47±0.75^{a}	25.27±1.20^{a}	11.20±0.45^{a}	5.72±0.71^{a}
50	11.11±0.56^{b}	8.41±0.59^{b}	2.70±0.02^{b}	2.59±0.16^{b}
100	5.41± 0.98^{c}	3.68±0.19^{c}	1.70 ±0.09^{c}	1.52±0.06^{c}
150	5.23± 0.59^{c}	4.31±0.10^{c}	0.92±0.04^{d}	1.75±0.07^{c}
200	4.50± 0.81^{d}	4.00±0.05^{c}	0.50±0.02^{e}	1.68±0.05^{c}
250	3.46± 0.67^{e}	2.94±0.04^{d}	0.52±0.01^{e}	0.84±0.01^{d}

*Data represents an average of 3 replicates per treatment after 28 days of culture. Means in a row followed by the same lowercase letters are not significantly different at $P \leq 0.05$ levels of significance according to Duncan's multiple range tests.

3.1.3. Terpenoid Content

In the control group, the triterpenoids were absent in the roots and leaves; meanwhile, all triterpenoids (from Ursonic acid to Ursolic acid) were estimated in shoot extracts (Table 4). The triterpenoid synthesis was activated in leaves and roots at 50 mM and 150 mM salt levels, respectively. Besides, increased amount of all triterpenoid types was found in the root, shoot, and leaf at the salt elicitation of 150 mM level; on the other hand, 200 mM salt elicitation, however, was marked as the beginning of the adverse salt concentrations since it significantly reduced the triterpenoids contents in shoots and leaves. Moreover, the amount of triterpenoid in almost all parts was decreased at 250 mM level (Table 4).

Table 4. The effect of NaCl elicitation on the triterpenoid content of the *P. khinjuk* Stocks root, shoot and leaves*

Explant Type	Salt con. (mM)	Ursonic Acid (µg/g)	Moronic Acid (µg/g)	Oleanonic Acid (µg/g)	Masticadienolic Acid (µg/g)	Oleanolic Acid (µg/g)	Ursolic Acid (µg/g)
Root	**Control**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	50 mM	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	100 mM	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	150 mM	0.668±0.04^{b}	0.003±0.01^{b}	0.016±0.01^{b}	0.028±0.01^{b}	0.023±0.01^{b}	0.028±0.01^{b}
	200 mM	2.512±0.15^{a}	0.007±0.01^{a}	0.068±0.01^{a}	0.110±0.01^{a}	0.135±0.01^{a}	0.114±0.01^{a}
	250 mM	0.439±0.03^{c}	0.001±0.01^{c}	0.008±0.01^{c}	0.016±0.01^{c}	0.021±0.01^{c}	0.020±0.01^{c}
Shoot	**Control**	0.984±0.06^{a}	0.082±0.02^{a}	0.033±0.01^{b}	0.037±0.01^{b}	0.084±0.01^{a}	0.052±0.01^{b}
	50 mM	0.807±0.05^{b}	0.024±0.01^{d}	0.023±0.01^{c}	0.031±0.01^{c}	0.030±0.01^{d}	0.051±0.01^{b}
	100 mM	1,067±0.06^{a}	0.016±0.01^{d}	0.015±0.01^{e}	0.043±0.01^{a}	0.024±0.01^{e}	0.024±0.01^{d}
	150 mM	1.114±0.07^{a}	0.016±0.01^{d}	0.020±0.01^{d}	0.043±0.01^{a}	0.047±0.01^{b}	0.036±0.01^{c}
	200 mM	1.103±0.07^{a}	0.002±0.01^{e}	0.003±0.01^{f}	N.D.	0.033±0.01^{c}	0.003±0.01^{e}
	250 mM	1.063±0.06^{a}	0.021±0.01^{c}	0.095±0.01^{a}	N.D	0.017±0.01^{f}	0.094±0.01^{a}
Leaf	**Control**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	50 mM	6.205±0.37^{a}	0.026±0.01^{b}	0.064±0.01^{b}	0.288±0.01^{a}	0.246±0.01^{b}	0.204±0.01^{a}
	100 mM	0.442±0.03^{d}	0.013±0.01^{d}	0.018±0.01^{d}	0.020±0.01^{d}	0.051±0.01^{d}	0.011±0.01^{d}
	150 mM	4.947±0.30^{b}	0.135±0.01^{a}	0.129±0.01^{a}	0.206±0.01^{b}	0.388±0.01^{a}	0.153±0.01^{b}
	200 mM	0.783±0.05^{c}	0.024±0.01^{c}	0.046±0.01^{c}	0.031±0.01^{c}	0.067±0.01^{c}	0.032±0.01^{c}
	250 mM	0.104±0.01^{e}	0.002±0.01^{e}	0.003±0.01^{e}	0.005±0.01^{e}	0.006±0.01^{e}	0.003±0.01^{e}

* Analyzes performed on the LC-MS/MS are given as the results of 3 replicates. Results are given as µg/g extract. Each explant in a column has been evaluated among themselves. Means in a row followed by the same lowercase letters are not significantly different at $P \leq 0.05$ levels of significance according to Duncan's multiple range tests, **N.D. = Not Determined.

In terms of salt concentrations applied at different levels, the ursonic acid was not observed in the leaves of the control group, while in the 50 mM salt elicitation was synthesized at the rate of 6.205 ppm and 150 mM salt elicitation at a rate of 4.947 ppm. Again in terms of salt concentrations applied at different levels, ursonic acid was not observed in the leaves of the control group; however, it was found that at the level of 6.205 ppm and 4.947 ppm in 50 mM and 150 mM salt concentrations, respectively.

The production of secondary metabolites using biotechnological methods, including elicitor and precursor treatments, has many advantages over classical methods. To date, although there are many studies about the secondary metabolite content and cytotoxic activity in plants growing in nature [44,45], several studies have shown that many commercial and medicinal plants can accumulate a higher amount of secondary metabolites through shoot cultures. For example, a high amount of bacoside from *Bacopa monnieri*; camptothecin from *Nothapodytes nimmoniana*; digitoxin from *Digitalis purpurea* L.; stevioside from *Stevia rebaudiana*; xanthone from *Gentiana dinaricawere*; anthraquinones from *Oldenlandia umbellata* L. rosmarinic acid from *Ocimum basilicum* L. were able to be synthesized from their shoot culture than naturally occurring plants [46, 47, 48, 49, 50, 51, 52]. Moreover, it is reported that the effect of elicitor applications for metabolite production depends on the conditions of the culture, applied time-period, and the concentration of the elicitor [12].

In this study, *in vitro Pistacia khinjuk* Stocks shoot culture with different levels of salt concentrations was used, and the amount and type of triterpenoids in leaves, shoots, and roots were investigated. Different levels of elicitation applied to *P. khinjuk* shoot culture produced several triterpenoids at different levels. For instance, 50 mM and 25 mM NaCl elicitation in leaves increased the amount of ursonic acid 2.16 and 3.71 times compared to the control group, respectively. Moreover, the length of shoots and the synthesis of total chlorophyll, Chl-a, Chl-b, and carotenoid pigments decreased at 50 mM salt level. Likewise, *in vitro* root culture of *P. khinjuk* Stocks with different salt concentrations showed that the amounts of various secondary metabolites were increased whereas root length started to decrease from 150 mM salt level. All these outcomes indicated that at stress conditions, plants focus on the synthesis of secondary metabolites rather than primary metabolite production. It is well known that stress is one of the most critical environmental impacts that limits the growth and development of plants, changes the physiological and biochemical properties of plants, and increases the secondary metabolites contents, which play the crucial roles in the protection and defense systems of plants [23].

Although no studies on triterpenoid production from *Pistacia* species with salt stress have been found in the literature yet, there is a report on the application of 0.05 M potassium chloride salt on *Catharanthus roseus* which indicated the increased production of ajmalicin four times compared to the control group [53]. In another study, *Stevia rebaudiana* plant was exposed to 6 different salt concentrations (0, 30, 60, 90, 120 and 150 mM) for 62 days in which the highest amount of steviosid and rebaudiosid-A diterpenes was obtained from 30 mM salt concentration [9].

For woody plants, the early developing stage, i.e., the seedling is usually the most salt-sensitive phase; hence, young seedlings could be a valuable material to detect initial salt responses [29]. Besides, visible alteration and damage in the leaf are other critical indicators for screening salt tolerance. Several studies indicated that leaf relative water content (RWC), total chlorophyll contents, and the carotenoid contents of leaves decreased during salt stress. It was observed that NaCl decreases the total chlorophyll content of both *Pistacia atlantica* Defs., and *P. vera* L. seedlings [32]. Furthermore, the decrease of chlorophyll content in leaves due to salt stress was observed from the effects of SO_4^{-2}, Cl^- and boron on pistachio rootstocks [54]; and the effects of NaCl and $CaCl_2$ salt combinations on *P. khinjuk* Stocks and *P. mutica* L. [55]. The reduction of chlorophyll content may be the result of the adverse effects of salt on membrane stability [56]. Besides, it was reported that the total biomass of the shoots of *P. vera* L. seedlings reduced on the 30th and 60th days due to salt stress [57]. Besides, in a study on *P. vera* L. Badami-Zarand variety showed that the root-shoot length, the number of leaves, dry weight of shoots, and the rate of photosynthesis were decreased under salt stress [58].

The present study showed that increased NaCl concentrations increased terpenoid production at a certain level. Additionally, this given salt concentration exhibited adverse effects on the morphological and physiological properties of *P. khinjuk* Stocks, which disrupted the growth and development of plants. Production of valuable terpenoids using *in vitro* micropropagated *P. khinjuk* Stocks is very significant since this secondary metabolite group processes many biological functions, especially anticancer activity [28]. For instance, antiproliferative and apoptosis capacity of oleanolic acid in diverse tumor cells such

as human pancreatic, colorectal, prostate, and bladder cells has been observed by several *in vitro* studies [59]. The cytotoxic effects of oleanolic acid and ursolic acid against the proliferation, growth and invasion of a variety of liver cancer cell lines were confirmed by several studies [60]. Oleanolic acid was found to have the potential to decrease the viability of different liver cancer cell lines i.e., HepG2, Hep3B, Huh7 and HA22T cells [59, 61]. In a similar study, ursolic acid was found to have the inhibition capacity in the proliferation of R-HepG2 and HepG2 cell lines [62].

Furthermore, chios mastic gum and oil isolated from *Pistacia lentiscus* L. were identified to exhibit beneficial effects on a wide range of human disorders [63]. The inhibitory effects of mastic gum on the proliferation and cell cycle progression were observed in human prostate cancer PC-3 cells [64], promyelocytic leukemia HL-60 cells and myeloblastic leukemia ML-1 and KG-1 cells [65] etc. Triterpenoid analogues i.e., masticadienolic acid, oleanolic acid, oleanonic acid, ursolic acid, ursonic acid, dammaradienone, tirucallol, moronic acid etc. from mastic gum appeared to be mainly responsible for its anticancer potential [63].

Nevertheless, this study will be useful for elucidating the tolerance mechanisms of the *Pistacia* genus against salt stress, particularly for surviving high salt containing agricultural land in arid and in semi-arid climate conditions.

4. Conclusions

This study was conducted to investigate the effects of NaCl applications on growth and triterpenoid content of *P. khinjuk* Stocks. The result showed that the morpho-physiological properties of *in vitro* seedling were decreased as salt concentrations were increased. However, salt application synchronously increasing the amount and types of triterpenoids due to the induction of cellular defense mechanisms against salt stress. Therefore, the protocol established in this study could provide the guideline for carrying out detailed research on elicitation for producing a high amount of secondary metabolites from various plants, especially cytotoxic property containing triterpenoids for pharmaceutical benefits.

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Supporting Information

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ORCID
Emine Ayaz-Tilkat: 0000-0001-5111-425X

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