https://doi.org/10.31925/farmacia.2022.6.23 **ORIGINAL ARTICLE**

THE INVESTIGATION OF THE MEDICINAL POTENTIAL OF *ALCEA KURDICA* ALEF. IN NATURE AND TISSUE CULTURE

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Abstract

Alcea kurdica Alef. (AK) a hollyhock has been used in folk medicine to treat various diseases. Though its extensive utilization in traditional medicine, experimental studies regarding to its phytochemical composition is limited. The aim of this study was to determine the antioxidant capacity and phenolic composition of ethanol extract of AK plant collected from the natural environment in different vegetation periods (June-flowering, July-fruiting, August-seeding) and to control its antioxidant capacity and phenolic composition by *in vitro* shoot tip culture. AK plant at the flowering period had the highest antioxidant capacity, while the lowest activity was determined at the seeding period according to results. Phenolic acids dominated by gallic, chlorogenic and caffeic acids were determined as the major phenolics. According to *in vitro* results, the use of 1-naphthylacetic acid (NAA) and 6-benzylaminopurine (BA) alone caused significant decrease in the three phenolic acids content compared to the control. On the other hand, combination of NAA and BA caused significant increase in phenolic acids content compared to the control. Moreover, significant increase in ORAC was observed in AK extract obtained from *in vitro* Murashige & Skoog (MS) media culture supplemented with NAA and BA combination. 15 min of UV exposure negatively affected the antioxidant capacity and phenolic content.

Rezumat

Alcea kurdica Alef. (AK) a fost folosită în medicina populară pentru a trata diverse boli. Deși este utilizată pe scară largă, studiile experimentale privind compoziția sa fitochimică sunt limitate. Scopul acestui studiu a fost evaluarea caracterului antioxidant și compoziția în polifenoli a extractului etanolic din planta AK colectată din mediul natural în diferite perioade de vegetație (iunie-flori, iulie-fructe, august-semințe), precum și de a modula capacitatea antioxidantă și concentrația polifenolilor prin cultivarea *in vitro* a vârfurilor de lăstari. Planta AK în perioada de înflorire a avut cea mai mare capacitate antioxidantă, în timp ce cea mai redusă activitate a fost determinată în perioada de însămânțare. Acizii galic, clorogenic și cafeic au fost determinați ca polifenoli principali. Conform rezultatelor *in vitro*, expunerea la acid 1-naftilacetic (ANA) și la 6-benzilaminopurina (BA) a provocat o scădere semnificativă a conținutului celor trei acizi fenolici, în comparație cu martorul. Pe de altă parte, combinația dintre ANA și BA a determinat o creștere semnificativă a conținutului de acizi fenolici în comparație cu martorul. 15 min de expunere la UV au afectat negativ capacitatea antioxidantă și conținutul de fenoli.

Keywords: *Alcea kurdica*, antioxidant capacity, phenolic acids, medicinal plant

Introduction

Secondary metabolites are produced during the life cycle of plants, particularly when the plant is exposed to abnormal environmental conditions. The reasons that change the normal life cycle of the plant are abiotic (drought, salinity, temperature, altitude, UV light, agrochemicals, etc.) stress factors and biotic pests (insects and microorganisms). Plants produce secondary compounds to eliminate pest, survive against abiotic stresses, and optimise living conditions [1]. Antioxidants are of great importance in surviving and overcoming abnormal conditions that negatively affect metabolism and the life cycle of plants.

Antioxidants play an important role in preventing diseases caused by free radicals by scavenging reactive oxygen species that are produced due to internal or external factors in organisms and cause degenerative diseases such as cancer, atherosclerosis, Parkinson's, Alzheimer's diseases [2]. The increase in chronic diseases and the undesired side effects of synthetic drugs have led consumers to benefit from herbal ingredients. [3, 4]. Significant antioxidant effect of phenolic compounds, has been reported [5, 6].

The limitations of secondary metabolite production from wild sources are required *in vitro* plant regeneration for phytochemical production in nutraceutical, pharmaceutical, and cosmetical industries [7, 8]. *In vitro* plant regeneration is conducted in plant tissue

culture. Explants are incubated in sterile ready-made media in specific condition and a whole plant or desirable callus cell lines are produced [9]. Continuous and high quality micropropogation of medicinal plants in tissue culture prevents plant from extinction and plant habitat destruction, protects plants from the damage of agrochemicals threaten animal and environmental health and reduces field labour [10].

Alcea genus comprises of 40 taxa belonging to *Malvaceae* commonly known for colourful and splendid flowers and grown in Mediterranean countries including Turkey, Northern Iraq, and North-western Iran at an altitude zone of 1750 - 2500 meters [11]. In folk medicine, they have been used in the treatment of kidney and bladder stones and arthritis, stomach and throat pains, cough and asthma [11] as anti-inflammatory and antimicrobial [12], analgesic, diuretic [13], emmenagogue [14] and expectorant [15]. In addition, antioxidant, antimicrobial, antiviral and liver protective properties of *Alcea* species' extract in folk medicine and *in vivo* applications were reported [16].

Alcea kurdica Alef. is a perennial herbaceous plant of which antioxidant [17] and antimicrobial [18] activities were cited. The local name of the plant is "Devegülü" in Turkey. Medicinal applications of the plant in the treatment of tonsillitis, stomach and duodenal ulcer, pneumonia, urinary tract infections and alopecia were reported [19].

There are limited studies on investigating pharmaceutical potential of the AK plant in many diseases in folk medicine and *in vitro*. We firstly described a new sterilization and germination procedure for the plant seed collected from field of 1961 m altitude [20] and achieved a successful *in vitro* regeneration procedure [21]. Therefore, we aimed to investigate and compare phenolic content and antioxidant capacity between the AK plants collected from field at the different vegetation periods (June-flowering, July-fruiting, August-seeding) and *in vitro* regenerated and stressor applied shoot tip culture.

Materials and Methods

Chemicals and solutions

All chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and were of analytical or HPLC grade. Chemicals used in tissue culture were purchased from Duchefa Biochemie BV (2003 RV Haarlem, The Netherlands).

Plant material

Leaves of *Alcea kurdica* Alef. (AK) plant were collected from the highlands of Konalga village, at Van province, in the Eastern Anatolia Region of Turkey, (Global Positioning System coordinates 38º 033′ 1814″ N 41º 91′ 773 E; 1961 m). Collected samples were promptly (within 2 h) transferred to the laboratory. Plant samples were identified at the Van Pharmaceutical Herbarium (VPH), Faculty of Pharmacy, Van Yüzüncü Yil University (Van, Turkey) and voucher specimens were deposited at VPH (Herbarium codes: VPH-553; Collector code-DM350).

Leaves of the plant were collected from their natural environment in different vegetation times (June-flowering, July-fruiting, August-seeding). They were cleaned from the dust and other contaminants avoiding the loss of bioactive components and were put to dry at room temperature (22 ± 2°C) for 14 - 21 days in a dark place as applied in folk medicine. The dried materials were pulverised into a fine powder using a grinding mill (Isolab laboratory mill 602, Interlab, İstanbul, Turkey) and stored at -20°C until analysed. The seeds of the plant were collected from nature in 2017 and used in tissue culture studies.

Sterilization

All glass and plastic equipment and Murashige & Skoog (MS) media were sterilized by autoclave. The pH of the cultures was adjusted to 5.8 before plant agar is added. After sterilization, MS medium was poured into petri dish under sterile conditions in a biosafety cabinet after addition of filter sterilized plant growth regulators (PGRs). Tissue culture studies were conducted in a laminar flow cabinet and incubated in controlled plant tissue culture room (25 ± 1°C with 50 - 60% moisture, under a photoperiod of 16/8 h (light/dark)). Samples were bi-weekly sub-cultured.

Surface sterilization of seeds was performed with 10% of sodium hypochlorite for 10 minutes and rinsed thrice with sterile distilled water [20]. Stocks of plant growth regulators (PGRs) were prepared as 2 mg/L of 1-naphthylacetic acid (NAA) and 2 mg/L 6-benzylaminopurine (BA). They were filter sterilized and stored in a freezer at -20°C until use.

Explant source

After seed surface sterilization, the seed coat was nicked with a scalpel and put into MS medium containing 4.4 g/L of MS, 30 g/L of sucrose and 8 g/L plant agar to germinate seedlings (20 - 21). They were incubated in the tissue culture room for 28 days. Shoot tip of 28 days old seedlings was used as explant source in plant tissue culture studies.

In vitro manipulation of antioxidant capacity and phenolic content

The effects of sucrose concentration, PGRs and UV-C exposure time on the antioxidant capacity and phenolic content of the plant were investigated.

Different sucrose concentrations were tested Control (MS + 30 g/L sucrose), Suc-15 (MS + 15 g/L sucrose) and Suc-45 (MS + 45 g/L sucrose). Shoot tips explants were removed from seedlings under aseptic conditions, and put into Control, Suc-15 and Suc-45 media to investigate how different sucrose concentration affect antioxidant capacity and phenolic content of *in vitro* plant. Samples were incubated for 28 days, harvested

from media and cleaned from medium contaminants. They were stored at -20°C until processing.

Shoot tips were incubated in MS medium supplemented with different concentrations and combinations of PGRs (NAA and BA). PGRs were tested as NAA-1 (1 mg/L NAA), BA-1 (1 mg/L BA) and NAA-0.5 + BA-0.5 (0.5 mg/L NAA + 0.5 mg/L BA). Samples were incubated for 28 days, harvested, cleaned from medium contaminants, and stored at -20°C until processing.

Shoot tips incubated in MS medium were exposed to Osram/Puritec HNS 30W G13 UV-C (254 nm) source with a distance of 20 cm at different exposure time as UV-Control (without UV exposure), UV-15 (15 min. UV-C exposure) and UV-30 (30 min. UV-C exposure). Samples were harvested on the 4th day after application, cleaned from medium residuals and stored at -20°C until processing.

Preparation of ethanol extracts

Leaf extracts were prepared according to previous studies [22] with minor modifications. Briefly, powdered plant leaves collected form nature at different vegetation time were well mixed with ethanol (80% ethanol + HCl 1% + ultrapure water 19%) at a ratio 1:20 (w:v) and shaken at 4°C for 2 hours. Homogenized mixture was centrifuged at 10000 rpm for 20 min. Supernatant was filtrated from 0.45 µm hydrophylic filter. Ethanol was evaporated with a rotavapor system. Extracts were lyophilized and dissolved in extraction solvent as 1 mg/mL. They were filtrated with a 0.45 µm hydrophylic filter to get ready for analysis after 15 min sonication at 37°C.

Tissue culture samples were firstly lyophilized and subsequently powdered. They were extracted with ethanol (80% ethanol + HCl 1% + ultrapure water 19%) at a ratio 1:20 (w:v) according to previous studies [22]. Samples were sonicated for 15 min. after continuously vortex. Subsequently, the solutions were centrifuged at 10000 rpm for 10 min. Supernatant was filtrated from 0.45 µm hydrophylic filter. Ethanol was evaporated with a rotavapor system. Extracts were lyophilized, dissolved in extraction solvent as 1 mg/mL and filtrated with a 0.45 µm hydrophylic filter to get ready for analysis after 15 min sonication at 37°C.

Determination of antioxidant capacity

Folin-Ciocâlteu reducing (FCR) commonly known as total phenolic content assay was performed according to previous study [22]. The absorbance was measured at 600 nm. The results were calculated as mg gallic acid (GA) E/g DW based on a standard curve of GA.

Ferric reducing antioxidant power (FRAP) capacity of the AK extracts was determined according to the previous report [22]. FRAP capacity of AK extracts measured at 595 nm were calculated as mmol of iron (Fe^{2+})/g DW based on an iron sulphate standard curve against a blank control.

Oxygen radical absorbance capacity (ORAC) assay was conducted to the previous report [23]. The ORAC capacity of the AK samples was expressed as mmol of Trolox equivalent per gram of dry weight (mmol TE/g DW) based on a Trolox standard curve.

Identification and quantification of phenolic compounds

Phenolic compounds were identified and quantified using high performance liquid chromatography-diode array detector (HPLC-DAD) and liquid chromatography-photo-diode array-mass spectrometry (LC-DAD–MS/MS) on a Quantum triple period quadrupole mass spectrometer equipped with a quaternary solvent delivery system. Phenolic compound levels were quantified as authentic standard E/g DW based on a calibration curve.

Statistical analysis

The experiments were conducted at least in triplicate. Data were presented as mean ± standard deviation. Statistical significance was accepted as $p < 0.05$. Statistical analysis of variance was performed according to One-Way ANOVA (Dunnet) comparison test.

Results and Discussion

Antioxidant capacity of lyophilized AK extract

FCR, FRAP and ORAC assays were conducted to determine antioxidant capacity of lyophilized plant extracts in different vegetation periods (flowering, fruiting and seeding) (Figure 1). Extract of the plant at the flowering period had the highest FCR and ORAC capacities and they were found to be significantly higher than that of fruiting and seeding period ($p < 0.0001$) (Figure 1). Additionally, FRAP capacity in the flowering period was significantly higher than that of seeding period.

Phenolic composition of lyophilized AK extract

The phenolic composition of lyophilized leaf extract obtained from AK plant collected from nature in different vegetation period was investigated by HPLC-DAD-MS/MS. Chromatographic analyses revealed the presence of phenolic acids dominated by gallic, chlorogenic, and caffeic acids in the extracts (Table I). Gallic acid was observed at 6.5 minutes and showed the highest absorption at 280 nm. 125 *m/z* fragment was obtained in negative mode as fragmentation ion. In SRM mode, 125 *m/z* fragments were obtained at 169 *m/z* in negative mode. Chlorogenic acid was observed at 8.7 minutes and showed the highest absorption at 326 nm. 191 m/z fragment was obtained in negative mode as fragmentation ion. In SRM mode, 191 *m/z* fragments were obtained at 353 *m/z* in negative mode. Caffeic acid was observed at 9.45 minutes and showed the highest absorption at 326 nm. 135 *m/z* fragment was obtained in negative mode as fragmentation ion. In SRM mode, 135 m/z fragments were obtained at 179 *m/z* in negative mode.

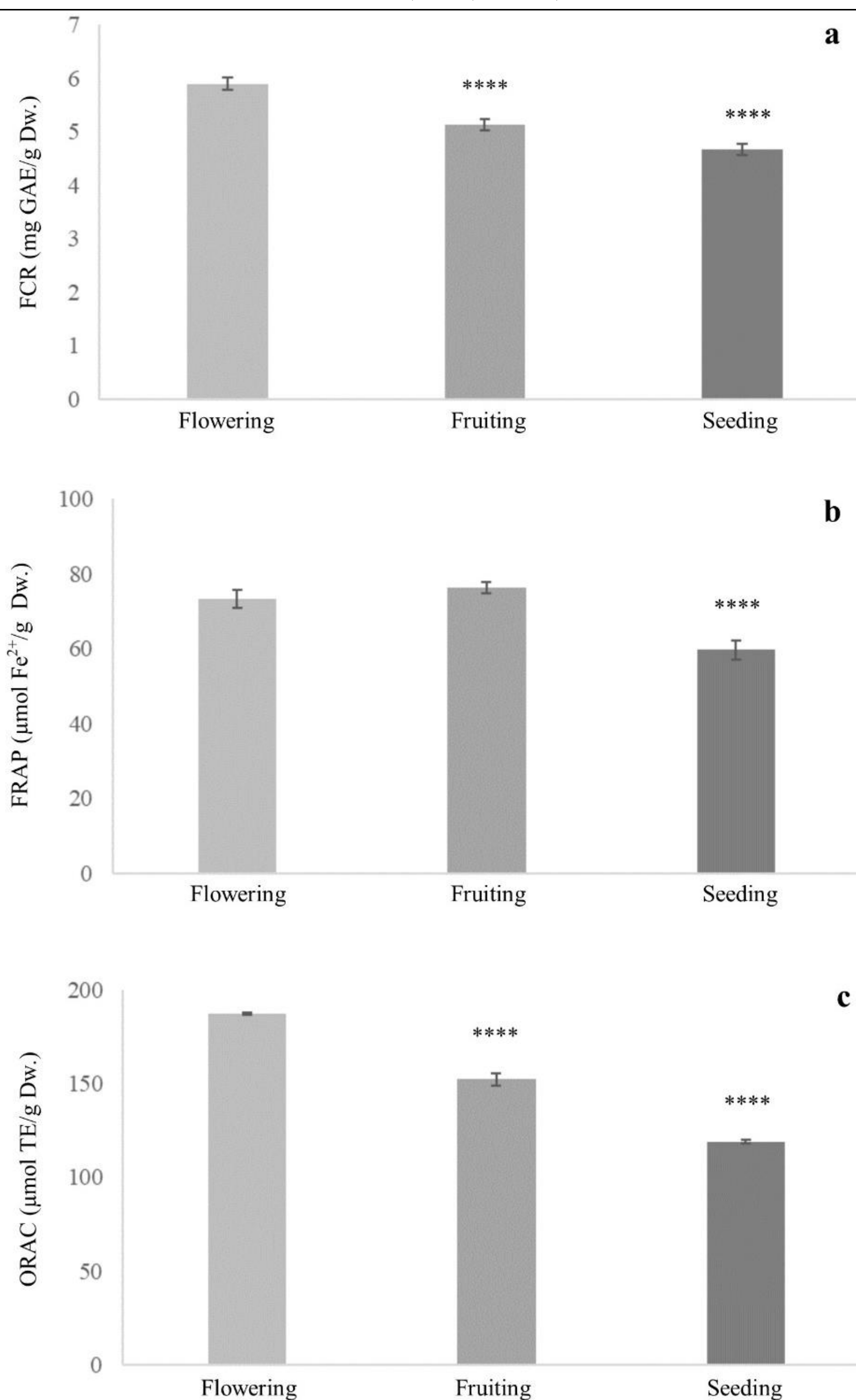

Figure 1.

Antioxidant capacity of lyophilized extracts of AK plant collected from nature. a: FCR, b: FRAP and c: ORAC

* $p < 0.05$, ** $p < 0.01$, *** $p<0.001$, **** $p < 0.0001$ according to Flowering period

Table I

Phenolic composition of lyophilized extracts of AK plant collected from nature

Sample	MS/MS	Gallic acid (mg/g DW)	Chlorogenic acid (mg/g DW)	Caffeic acid (mg/g DW)
	$[M+1]^+/[M+1]^-$	-/169	-/353	-/179
	Fragments (m/z) (+/-)	-/125	-/191	-/135
Flowering		0.361 ± 0.032	0.488 ± 0.006	0.844 ± 0.041
Fruiting		0.315 ± 0.007	0.436 ± 0.017*	0.752 ± 0.042*
Seeding		0.264 ± 0.029*	0.376 ± 0.010**	0.631 ± 0.024**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to Flowering period.

Caffeic acid was found to be the major phenolic compound in the ethanol extracts of leaves collected from natural environment. The highest caffeic acid content was in the flowering period that was significantly higher than that of fruiting ($p < 0.05$) and seeding periods ($p < 0.01$) (Table I). Chlorogenic acid was detected as a second major phenolic acid in the extracts. The highest chlorogenic acid content was determined in the flowering period being importantly higher than that of fruiting ($p < 0.05$) and seeding period ($p < 0.01$). Another phenolic acid detected in the ethanol extracts of plant leaves from nature was gallic acid. Gallic acid contents of the plant extracts at the flowering period were found to be significantly higher than that of seeding period ($p < 0.05$) (Table I).

In the present study, the highest concentration of phenolic content (caffeic, chlorogenic and gallic acids) and antioxidant activity were determined in the flowering periods of plant collected from the field. The changes in the phenolic composition and antioxidant capacity of the plants in different vegetation periods were in consistence with the studies, reported that bioactive compounds may show great differences in different vegetative periods depending on the metabolic and physiological changes in different plants [24, 25] and with studies reported that morphological changes in the physiological ontogenesis process cause changes in the synthesis of secondary metabolites [26].

Higher *in vitro* and *in vivo* antioxidant effects of caffeic, chlorogenic and gallic acids detected as major phenolic compounds of the plant were reported [27, 28]. Results obtained from the present study were in agreement with the studies [29, 30], in which caffeic, chlorogenic, and gallic acids were reported to be the dominant key compounds of *Alcea* taxa.

The plant, grown at an altitude of more than 2000 meters in the Eastern Anatolian Mountains, the coldest region of Turkey, is exposed to lower temperatures and shorter daylight during flowering and seeding periods compared to fruiting period. During the flowering period, the plant has just emerged from the cold winter conditions, the young leaves can synthesize limited primary metabolites for its own growth in short daylight. In the fruiting period, the expansion of the leaf surface, the temperature, and the effect of seasonal sun rays may increase the conversion of photosynthesis products from phospho-phenol pyruvate to phenolic acids (caffeic, gallic and chlorogenic acid) *via* shikimic acid pathway. The increase in phenolic acids in fruiting period caused an increase in the antioxidant capacity as well. The decrease in photosynthesis during the seeding period may cause a decrease in the production of secondary metabolites (phenolics) and thus a decrease in antioxidant capacity. The linear relationship between total phenolic content and antioxidant capacity has also been reported [31].

Phenolic compounds and antioxidant properties may show seasonal variations with the effect of climatic factors such as temperature, solar radiation, precipitation and hydro thermic coefficient between different growing seasons [32]. Seasonal changes can cause stress in the plant, increasing or decreasing phenolic synthesis and thus antioxidant capacity. While the bioactive molecules are at the highest level in the fruiting period, the chloroplast DNA begins to break down with the onset of senescence in the seeding development period, causing chlorophyll degradation, which suddenly reduces the photosynthetic capacity of the leaf resulted in nucleic acid, lipid and protein degradation [33]. Phenolics synthesized in chloroplasts and stored in vacuoles under the influence of light are found in greater concentration in spring leaves than that of in autumn [34]. During the transition to the seeding period (August), the decrease in photo-synthesis may lead to a decrease in the production of secondary metabolites and thus a decrease in antioxidant capacity.

In vitro manipulation of antioxidant capacity and phenolic content

Sucrose concentration and different PGRs concentration and combinations significantly affected antioxidant capacity of shoot tip culture of the plant. FCR content was importantly decreased in Suc-15 application compared to the control. Additionally, BA-1 and NAA-1 application caused statistically significant decrease in FCR compared to the control (Figure 2). FRAP capacity of *in vitro* shoot tip culture subjected to Suc15, Suc-45, BA-1 and NAA-1 was significantly reduced compared to the control. Additionally, application of high concentration of sucrose (45 mg/L) resulted with statistically significant decrease in ORAC capacity compared to the Control. Moreover, similar results were provided from NAA-1 application. However, BA-0.5 + NAA-0.5 application caused statistically significant increase in ORAC capacity compared to the control MS (Figure 2).

Caffeic acid was determined as the major phenolic acid compound in the plant extracts obtained from *in vitro* shoot tip culture. While the highest caffeic acid content was found in the BA-0.5 + NAA-0.5 application, the lowest caffeic acid was in the NAA-1 application (Table II). Application of PGRs combination resulted in 1.9 folds increase in caffeic acid compared to the control. The highest sucrose application (Suc-45) caused statistically significant increase in caffeic acid concentration compared to the control. On the other hand, caffeic acid concentration was found to be statistically significant lower in the application of BA-1 and NAA-1 than that of the control.

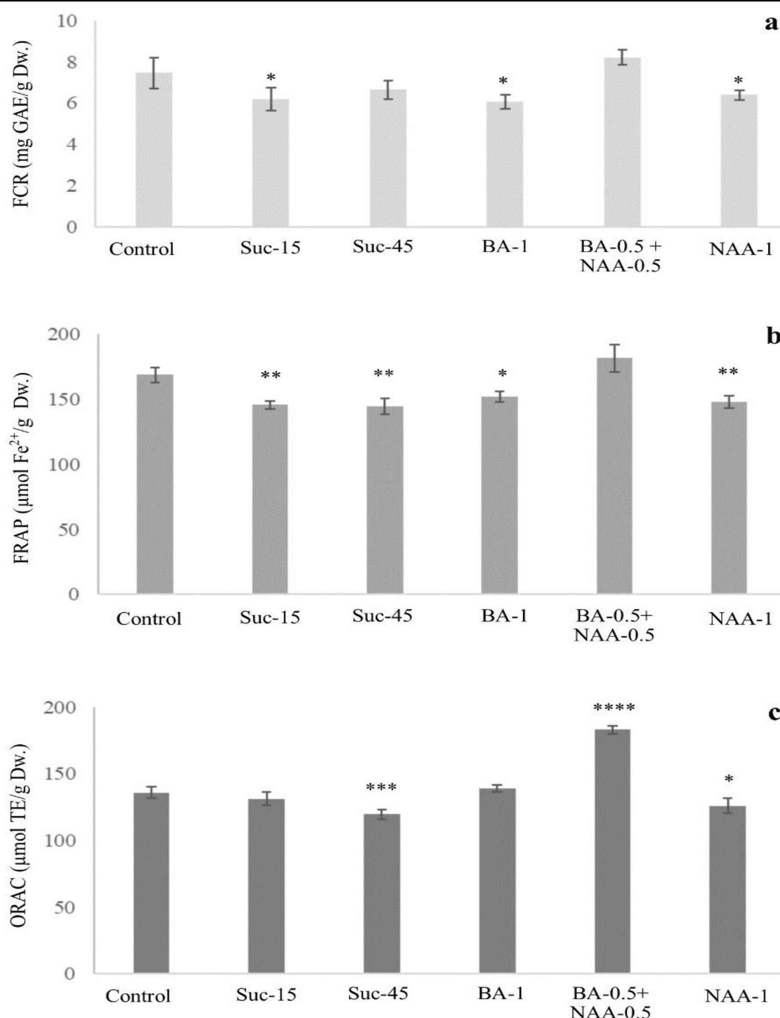

Figure 2.

The effects of sucrose and PGRs on antioxidant capacity of lyophilized extracts obtained from AK shoot tip culture. a: FCR, b: FRAP and c: ORAC

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to Control

Chlorogenic acid content in the treatment of BA-1 and NAA-1 was found to be significantly lower than that of the control whereas, it was found statistically significant higher in Suc-15, Suc-30 and BA-0.5 + NAA-0.5 applications than the control (Table II). The highest chlorogenic acid content was determined in the BA-0.5 + NAA-0.5 application. Gallic acid results were similar to chlorogenic acid results. The highest gallic acid was determined in BA0.5 + NAA-0.5 application. The results revealed that the combination of BA and NAA caused statistically significant increase in all the three phenolic acid concentration of *in vitro* regenerated plant.

Table II

The effects of sucrose and PGRs on phenolic composition of lyophilized extracts of AK shoot tip culture

Sample	MS/MS	Gallic acid (mg/g DW)	Chlorogenic acid (mg/g DW)	Caffeic acid (mg/g DW)
	$[M+1]^+/[M+1]^-$	-/169	-/353	-/179
	Fragments (m/z) (+/-)	-/125	-/191	-/135
Control		0.318 ± 0.003	0.456 ± 0.005	0.904 ± 0.010
Suc-15		0.337 ± 0.007*	0.474 ± 0.005*	0.944 ± 0.037
Suc-45		0.516 ± 0.008****	0.514 ± 0.011***	1.036 ± 0.015****
BA-1		0.212 ± 0.006****	0.338 ± 0.012****	0.578 ± 0.011****
BA-0.5+NAA-0.5		0.628 ± 0.007****	0.711 ± 0.010****	1.725 ± 0.016 ****
NAA-1		0.277 ± 0.006**	0.414 ± 0.014**	0.648 ± 0.023****

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to Control.

Phenolics are generally synthesized under adverse environmental conditions when the plant is under biotic and abiotic stress [35]. There are a number of physical and chemical factors (e.g. light, pH, drought, water, UV, pathogen infection from injured surfaces) that affect the synthesis of target secondary metabolites in medicinal plant in tissue culture [34-37]. Biotic and abiotic stress cause an increase in the concentration of some enzymes, such as PAL (phenylalanine ammonium lyase) and peroxidase (POD), that activate the phenolic acid metabolic pathway [38].

Plant cell and organ cultures are often grown hetero-trophically using simple sugars as a carbon source. The sucrose level has been shown to affect the growth of cells/organs and the productivity and accumulation of secondary metabolites in tissue culture. [39]. In addition to being a carbon and energy source, sucrose can also cause osmotic stress in plants [40]. High sucrose concentration results in cell plasmolysis causing osmotic pressure in the cell cytoplasm, [41]. Also, previous studies have reported that sucrose affects the expression of genes encoding enzymes involved in carbon and nitrogen metabolism, stress responses, photosynthesis and phenolic synthesis in the shikimic acid metabolic pathway [42, 43]. High sucrose concentration causes the activation of genes that enable the production of phenolics by stressing the cell. In the present study, the highest phenolic content was determined in the plant incubated in Suc-45 compared to that of the control. The findings were in agreement with those of *Phoenix dactylifera* [41], *H. adenotrichum* [44], and *H. sabdariffa* [45]. Compatible with our study, an induction of total phenolic compounds has been reported in previous studies after the addition of plant growth regulators [46, 47]. Plants need different concentration and combination of PGRs (Plant Growth Regulator) for secondary metabolite increase, depending on its genotype. It was reported that higher gallic acid and antioxidant capacity was found in the MS containing BA in *Habenaria edgeworth* callus than that of the plant grown in the natural environment [48], and that the highest phenolic content in *Strelitzia reginae* tissue culture in medium containing 0.5 mg/l NAA and 6 mg/l BA [49]. However, in the present study conducted with AK plant, phenolic acid concentration and antioxidant capacity of the samples treated with 0.5 mg/L NAA + 0.5 mg/L BA were found to be higher than the Control. It was determined that the ORAC value of BA-0.5 + NAA-0.5 application was found to be significantly higher than the control. Additionally, significant increase in the concentration of all three phenolic acids was determined in BA-0.5 + NAA-0.5 application. It was concluded that 0.5 mg/L NAA + 0.5 mg/L BA is the appropriate dose of PGR that can be applied to increase phenolic acid in AK plant. On the other hand, BA and NAA applied alone caused decrease in the antioxidant activity compared to the control. Additionally, administration of BA-1 and NAA-1 alone reduced the amount of all three phenolic acids compared to the control group, thus suppressing the phenolic synthesis.

Some stress factors such as UV, drought, excessive water, pathogen infection and carbohydrate (sugar) supplements affect phenolics production in plants [34-50]. Plants need different concentration and combination of PGR depending on their genotype for secondary metabolite increase [9]. It was reported that the highest phenolic acid and antioxidant capacity was detected in the medium supplemented with 1 mg/L NAA in *Rosmarinus officinalis* callus [51]. Higher gallic acid and antioxidant capacity was seen in *Habenaria edgeworthii* callus grown in the medium containing BA than the plant grown in natural environment [48]. The highest phenolic content was reported in tissue culture of *Strelitzia reginae* regenerated in the medium containing 0.5 mg/L NAA and 6 mg/L BA [49]. In the present study we reported for the first time, that the phenolic acid amounts and antioxidant capacities of *in vitro* culture exposed to BA-0.5 + NAA-0.5 were found to be higher than both the control and those that were collected from field.

After phenolics are synthesized in leaves, they are transported to other tissues and organs [52]. Since no leaf formation is observed in callus cultures, it is thought that shoot tips carrying fresh leaves are more active in phenolic production than callus. The amount of phenolic matter was found to reach the highest level in the third and fourth weeks (between 20 and 30 days) after culturing the leaves and shoots of the cotton plant, which is in the same family with AK plant [52]. Similar results have also been reported in sugarcane cultures [53], and in potato cell cultures [54].

The effects of different exposure time of UV-C application on FCR, FRAP and ORAC capacities of the plant were evaluated. High exposure time (UV-30) caused significant decrease in FCR ($p < 0.001$), FRAP ($p < 0.001$) and ORAC ($p < 0.01$) capacities compared to the UV-Control. UV-15 application resulted in statistically significant decrease in FRAP and ORAC activities compared to the UV-Control ($p < 0.05$) (Figure 3). UV exposure caused significantly decrease in gallic acid and caffeic acid contents when compared to UV-Control, while chlorogenic acid content was statistically increased at UV-15 application (Table III). Statistically significant decreases in the all three phenolic acid content were detected at UV-30 compared to the UV-Control (Table III).

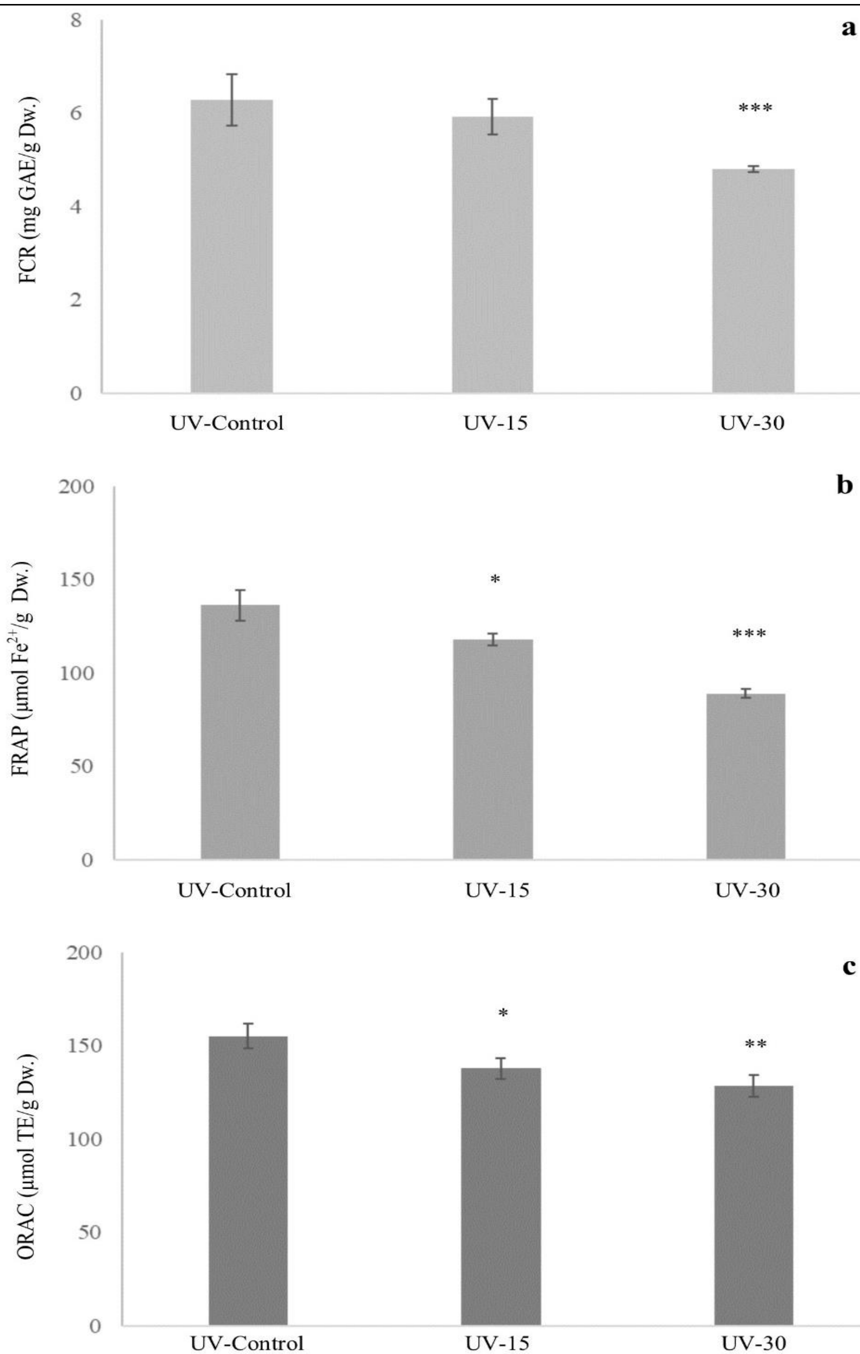

Figure 3.

The effects of UV-C exposure time on antioxidant capacity of lyophilized extracts obtained from AK shoot tip culture. a: FCR, b: FRAP and c: ORAC

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to UV-Control

Table III

The effects of UV-C exposure time on phenolic composition of lyophilized extracts of AK shoot tip culture

Sample	MS/MS	Gallic acid (mg/g DW)	Chlorogenic acid (mg/g DW)	Caffeic acid (mg/g DW)
	$[M+1]^+/[M+1]^-$	-/169	-/353	-/179
	Fragments (m/z) (+/-)	-/125	-/191	-/135
UV-Control		0.242 ± 0.008	0.298 ± 0.007	0.708 ± 0.007
UV-15		0.183 ± 0.005****	0.417 ± 0.014****	0.663 ± 0.009**
UV-30		0.139 ± 0.006****	0.216 ± 0.005****	0.456 ± 0.010****

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to UV-Control.

The increase in the amount of chlorogenic acid as a result of UV application is in consistent with previous studies [35, 55, 56]. The results similar to ours indicating that the amounts of secondary metabolites in *Genista tinctoria* callus cultures increased or decreased depending on the wavelength of UV radiation and the application time [57]. Likewise, in another study [58] it was reported that although hypericin in *Hypericum triquetrifolium* increased with 15 minutes of UV application, it decreased after 60 minutes of application. UV application might suppress the synthesis pathway of phenolics other than chlorogenic acid, but activate the gene encoding chlorogenic acid due to the UV stress.

Conclusions

In the present study, phenolic composition and antioxidant activity of both wild plants collected from natural environment and *in vitro* regenerated culture of AK plant were evaluated. Phenolic acids comprised of gallic, chlorogenic, and caffeic acids were dominant compounds of AK. The effects of vegetation time showed that flowering period had higher antioxidant capacity and phenolic acids. Sucrose applications significantly affected phenolic acid content.

Additionally, combination of BA and NAA caused significantly increase in phenolic acid content of *in vitro* culture. In general, UV exposure negatively affected antioxidant capacity and phenolic acid content except for chlorogenic acid in the UV-15 application. The present study suggests the enhancement protocol of antioxidant capacity and phenolic acids of AK plant regenerated *in vitro*. Investigation of the possible ameliorative effects of AK plant against different *in vivo* models could be considered as further studies.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

References

1. Güven A, Knorr D, Isoflavonoid production by soy plant callus suspension culture. *J Food Eng.*, 2011; 103(3): 237-243.
2. Pisoschi AM, Negulescu GP, Methods for total antioxidant activity determination: a review. *Biochem Anal Biochem.*, 2012; 1(1): 106: 1-10.
3. Boğa, M, Ertaş, A, Haşimi, N, Demirci, S, Yılmaz, MA, Temel H, Kolak U, Phenolic profile, fatty acid and essential oil composition analysis and antioxidant, antialzheimer and antibacterial activities of *Verbascum flavidum* extracts. *Chiang Mai J Sci.*, 2016; 43(5): 1090-1101.
4. Ng ZX, Samsuri SN, Yong PH, The antioxidant index and chemometric analysis of tannin, flavonoid, and total phenolic extracted from medicinal plant foods with the solvents of different polarities. *J Food Process Pres.*, 2020; 44: e14680: 1-11.
5. Orhan DD, Orhan N, Demir O, Konuklugil B, Phenolic content, antioxidant and *in vitro* antidiabetic effects of thirteen marine organisms from Mediterranean Sea. *Farmacia*, 2021; 69(1): 68-74.
6. Dudonné S, Vitrac X, Coutiere P, Woillez M, Mérillon JM, Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J Agric Food Chem.*, 2009; 57(5): 1768-1774.
7. Vanisree M, Lee C, Lo S, Nalawade SM, Lin CY, Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot Bull Acad Sinica*, 2004; 45(1): 1-22.
8. Güven A, Gürsul I, Secondary metabolite synthesis in plant tissue cultures. *Gıda*, 2014; 39(5): 299-306 (in Turkish).
9. Mansuroğlu S, Gürel E, Micropropogation, In: Plant biotechnology: Tissue culture and applications, Babaoğlu M, Gürel E, Özcan S Eds: Selçuk University Press, Konya, Turkey, 2002; 1: 374-375.
10. Murch SJ, KrishnaRaj S, Saxena PK, Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. *Plant Cell Rep.*, 2000; 19(7): 698-704.
11. Uzunhisarcıklı ME, Vural M, The taxonomic revision of *Alcea* and *Althaea* (*Malvaceaee*) in Turkey. *Turkish J Bot.*, 2012; 36: 603-636.
12. Iauk L, Bue AML, Milazzo I, Rapisarda A, Antibacterial activity of medicinal plant extracts against periodontopathic bacteria. *Phytother Res.*, 2003; 17(6): 599-604.
13. Elçi B, Erik S, Ethnobotanical properties of Güdül Ankara and Near Environs. *Hacet Univ J Fac Pharm.*, 2006; 25: 57-64.
14. Dudek M, Matlawska I, Szkudlarek M, Phenolic acids in the flowers of *A. rosea* Var. *nigra*. *Acta Pol Pharm*, 2006; 63(3): 207-211.
15. Shome U, Mehrotra S, Sharma HP, Comparative pharmacognosy of two *Althaea* spp. and "gulkhairo" samples. *Pharm Biol.*, 1992; 30(1): 47-55.
16. Azab A, *Alcea*: Traditional medicine, current research and future opportunities. *Eur Chem Bull.*, 2017; 5(12): 505-514.
17. Bouayed J, Piri K, Rammal H, Dicko A, Desor F, Younos C, Soulimani R, Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. *Food Chem.*, 2007; 104(1): 364-368.
18. Qader SW, Awad HM, Evaluation of Antioxidant, Antimicrobial and Cytotoxicity of *Alcea kurdica* Alef. *Jordan J Biol Sci.*, 2014; 147(1572): 1-5.
19. Mati E, de Boer H, Ethnobotany and trade of medicinal plants in the Qaysari Market, Kurdish Autonomous Region, Iraq. *J Ethnopharm.*, 2011; 133(2): 490-510.

20. Battal A, Görmez G, Türker M, Tıbbi Öneme Sahip Hatmiler İçin Etkili Bir Çimlendirme Yöntemi: Tohum Kabuğuna Çentik Atılması. *Bitlis Eren Üni.Fen Bil. Drg.*, 2019; 8(3): 843-851.
21. Görmez G, Battal A, Türker M, The investigation of *in vitro* callus formation, regeneration and micropropagation of Devegulu hollyhock, a medicinal plant. *Cauc J Sci.*, 2019; 6(2): 111-123.
22. Dalar A, Türker M, Konczak I, Antioxidant capacity and phenolic constituents of *Malva neglecta* Wallr. and *Plantago lanceolata* L. from Eastern Anatolia Region of Turkey. *J Herb Med.*, 2012; 2(2): 42-51.
23. Dalar A, Uzun Y, Turker M, Mukemre M, Konczak I, Health attributes of ethnic vegetables consumed in the Eastern Anatolia Region of Turkey: antioxidant and enzyme-inhibitory properties. *J Ethn Foods*, 2016; 3(2): 142-149.
24. Nair BR, Biochemical changes associated with fruit development in *Abelmoschus esculentus* cv. arka anamika. *Int J Agric Technol.*, 2013; 9(2): 373-382.
25. Şavşatlı Y, Özcan A, Çatal Mİ, Yurteri E, Seyis F, The effect of pruning age and diurnal variability on the antioxidant activity of tea (*Camellia sinensis* (L.) O. Kuntz) leaves in organic tea farming. *Turkish J Agric Food Sci Technol.*, 2018; 6(2): 163-168.
26. Schwob I, Bessiere JM, Masotti V, Viano J, Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochem Sys Ecol.*, 2004; 32: 735-745.
27. Gonthier MP, Verny MA, Besson C, Rémésy C, Scalbert A, Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *J Nutr.*, 2003; 133: 1853-1859.
28. Sato Y, Itagaki S, Kurokawa T, Ogura J, Kobayashi M, Hirano T, Sugawara M, Iseki K, *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. *Int J Pharm.*, 2011; 403: 136-138.
29. Valiei M, Shafaghat A, Salimi F, Chemical composition and antimicrobial activity of the flower and root hexane extracts of *Althaea officinalis* in Northwest Iran. *J Med Plant Res.*, 2011; 5: 6972-6976.
30. Al-Snafi AE, The pharmaceutical importance of *Althaea officinalis* and *Althaea rosea*: A review. *Int J of PharmTech Res.*, 2013; 5: 1387-1385.
31. Velioğlu YS, Mazza G, Gao L, Oomah BD, Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J Agric Food Chem.*, 1998; 46: 4113-4117.
32. Xu C, Zhang Y, Zhu L, Huang Y, Lu J, Influence of growing season on phenolic compounds and antioxidant properties of grape berries from vines grown in subtropical climate. *J Agric Food Chem.*, 2011; 59: 1078-1086.
33. Sağlam NG, Leaf senescence: A view of its physiological and molecular regulation. *Marmara J Pure App Sci.*, 2005; 27: 83-92.
34. Kefeli VI, Kalevitch MV, Borsari B, Phenolic cycle in plants and environment. *J Cell Mol Biol.*, 2003; 2: 13-18.
35. Fini A, Brunetti C, Di Ferdinando M, Ferrini F, Tattini M, Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. *Plant Signal Behav.*, 2011; 6: 709-711.
36. Collin HA, Secondary product formation in plant tissue cultures. *Plant Growth Regul.*, 2001; 34: 119-134.
37. Wang J, Li J, Li J, Liu S, Huang L, Gao W, Production of active compounds in medicinal plants: from plant tissue culture to biosynthesis. *Chinese Herb Med.*, 2017; 9: 115-125.
38. Ruiz JM, Rivero RM, Lopez-Cantarero I, Romero L, Role of Ca^{2+} in the metabolism of phenolic compounds in tobacco leaves (*Nicotiana tabacum* L.). *Plant Growth Regul.*, 2003; 41: 173-177.
39. Cui XH, Murthy HN, Wu CH, Paek KY, Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. *PCTOC*, 2010; 103: 7-14.
40. Liu CZ, Cheng XY, Enhancement of phenylethanoid glycosides biosynthesis in cell cultures of *Cistanche deserticola* by osmotic stress. *Plant Cell Rep.*, 2008; 27: 357-362.
41. Zouine J, El Hadrami I, Somatic embryogenesis in *Phoenix dactylifera* L. effect of exogenous supply of sucrose on proteins, sugars, phenolics and peroxidases activities during the embryogenic cell suspension culture. *Biotechnol.*, 2004; 3: 114-118.
42. Rolland F, Moore B, Sheen J, Sugar sensing and signaling in plants. *Plant Cell.*, 2002; 14: S185-S205.
43. Khatri D, Chhetri SBB, Reducing Sugar, Total Phenolic Content, and Antioxidant Potential of Nepalese Plants. *BioMed Res Int.*, 2020; 2020: 7296859: 1-7.
44. Yamaner Ö, Propagation of *Hypericum adenotrichum* Spach using tissue culture techniques and investigation of secondary metabolite variations under *in vitro* conditions. Doctoral dissertation, Adnan Menderes University, Aydın, Turkey 2011; 75-77.
45. Raoul SS, Gilbert C, Hamidou S, Protocol for callus and somatic embryogenesis initiation for *Hibiscus sabdariffa* L. (*Malvaceaee*): Influence of explant type, sugar and plant growth regulator. *Aust J Crop Sci.*, 2010; 4: 98-106.
46. Karalija E, Paric A, The effect of BA and IBA on the secondary metabolite production by shoot culture of *Thymus vulgaris* L. *Biol Nyssana.*, 2011; 2(1): 29-35.
47. Liang Z, Ma Y, Xu T, Effects of abscisic acid, gibberellin, ethylene and their interactions on production of phenolic acids in *Salvia miltiorrhiza* Bunge hairy roots. *Plos One*, 2013; 8: e72806: 1-9.
48. Giri L, Dhyani P, Rawat S, *In vitro* production of phenolic compounds and antioxidant activity in callus suspension cultures of *Habenaria edgeworthii*: A rare Himalayan medicinal orchid. *IndCrops Prod.*, 2012; 39: 1-6.
49. North JJ, Ndakidemi PA, Laubscher CP, Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of *Strelitzia reginae*. *Int J Phys Sci.*, 2012; 7: 638-646.
50. Lux-Endrich A, Treutter D, Feucht OW, Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. *PCTOC*, 2000; 60: 15-21.
51. Celiktas OY, Kocabas EH, Bedir E, Antimicrobial activities of methanol extracts and essential oils of

Rosmarinus officinalis, depending on location and seasonal variations. *Food Chem.*, 2007; 100: 553-559.
52. Özyigit II, Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. *Afr J Biotechnol.*, 2008; 7: 1145-1150.
53. Lorenzo JC, Blanco MD, Pelaez O, Sugarcane microporopogation and phenolic excretion. *PCTOC*, 2001; 65: 1-8.
54. İslam IK, Okuno S, Yoshimoto M, Yamakawa O, Composition of phenolics and anthocyanins in a sweet potato cell suspension culture. *Biochem Eng J.*, 2003; 14: 155-161.
55. Pakseresht G, Kahrizi D, Mansouri M, Ghorbani T, Kazemi N, Study of callus ınduction and cell culture to secondary metabolite production in *Hyssopus officinalis* L. *J Rep Pharm Sci.*, 2016; 5: 104-111.
56. Jiao J, Gai QY, Yao L, Ultraviolet radiation for flavonoid augmentation in *Isatis tinctoria* L. hairy root cultures mediated by oxidative stress and biosynthetic gene expression. *Ind Crops Prod.*, 2018; 118: 347-354.
57. Tůmová L, Tůma J, The effect of UV light on isoflavonoid production in *Genista tinctoria* culture *in vitro*. *Acta Physiol Plant.*, 2011; 33: 635-640.
58. Namlı S, Toker Z, Işıkalan Ç, Özen HC, Effect of UV-C on production of hypericin in *Hypericum triquetrifolium* Turra grown under *in-vitro* conditions. *Fresenius Envir Bull.*, 2009; 18: 123-128.