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In vitro Culture of Endemic *Astragalus gymnolobus* Fischer and Comparison of its Antibacterial, Antioxidant, and Phenolic Profiles with Field Grown Plants

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ABSTRACT

Astragalus gymnolobus **Fischer (Leguminosae family) is an endemic plant to Turkey. Firstly, an *in vitro* regeneration system was achieved using leaf and petiole explants on Murashige and Skoog (MS) medium supplemented with different cytokinins [Thidiazuron (TDZ), Kinetin (KIN), Benzyladenine (BA)], auxins [Indole-3-Butyric Acid (IBA), Indole-3-Acetic Acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Naphthalene Acetic Acid (NAA)] and Gibberellic Acid (GA_3) at diverse concentrations. Best shoot formation was obtained with leaf explants and only TDZ alone or TDZ and IAA combinations were effective for shoot forming. The best shoot inducing response (17.60 shoots per explant at 23.81% shoot frequency) was recorded at 0.5 mg L^{-1} TDZ alone. Among the TDZ stand-alone treatments, 0.05 and 0.1 mg L^{-1} TDZ were also effective in terms of shoot induction frequency (31.82 and 30.43%, respectively). Root formation was obtained after 3 months (3.5 roots per shoot with 77.8% root frequency) only in auxin free MS medium (control) when regenerated shoots were cut off 0.5-1 cm from the base to exclude the callus part. This study also aimed to determine and compare the antibacterial and antioxidant properties and phenolic composition of *in vitro*-regenerated and field-grown *A. gymnolobus*. The results revealed that field-grown leaves generally showed higher biological activities and had higher amounts of phenolic compounds. Furthermore, *A. gymnolobus* leaves were noteworthy sources of rutin. This initial *in vitro* culture protocol for endemic *A. gymnolobus* is valuable for genetic resources conservation and can be used in stress application studies to increase the level of phenolic substances in *in vitro*-grown plants.**

Keywords HPLC, Micro-propagation, Murashige and Skoog (MS) medium, Phenol.

INTRODUCTION

Astragalus gymnolobus Fischer is an endemic, perennial plant belonging to the Leguminosae family and is found throughout the Northern and Southern part of Turkey (Davis, 1970). *Astragalus* species are some of the largest flowering plants, containing approximately 3000 species worldwide (Lock and Simpson, 1991). It is also the largest genus in Turkey with 439 species and about 47% of these species are endemic to Turkey (Akan and Civelek, 2001). Threat category of *A. gymnolobus* is determined as LC (Least Concern) (IUCN, 2015).

Members of *Astragalus* genus are used in traditional medicine to cure leukemia, healing of wound and injuries in Anatolia (Bedir *et al*., 2001). In traditional medicine, various *Astragalus* species have been used to treat nephritis, diabetes, leukemia and cancer. They have adaptogenic, vasodilator, immunostimulant, hepatoprotective, antiperspirant, antiviral, anti-diabetic, diuretic, and tonic properties (Tang and Eisenbrand,

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1992; Erisen *et al*., 2010; Bourezzane *et al*., 2018). *Astragalus* species are rich in polysaccharides, alkaloids, saponins and phenolics (Pistelli *et al*., 2003; Bourezzane *et al*., 2018). Antibacterial and antitumor potential of *A. gymnolobus* were investigated (Türker *et al*., 2009; Turker and Koyluoglu, 2012). Some members of the genus *Astragalus* are important for gum production and some of them are used as fodder (Baytop, 1999). They are also important for erosion control because of their tap root system (Erisen *et al*., 2010).

The most significant problems in cultivation of *Astragalus* species are low seed germination rates, low seed set number and slow seedling development due to the hard seed coat. Because of these drawbacks, their propagation by tissue culture techniques has been required (Yorgancilar and Erisen, 2011). *In vitro* culture is an effective method for *ex situ* conservation of plant diversity, especially in the conservation of the endemic or threatened plants. It is a potent agent for germplasm conservation and mass multiplication of many threatened plant species (Purohit *et al*., 2015; Turker *et al*., 2018).

While *Astragalus* species have a potential for commercialization and are used in many fields in medicine, to our knowledge, regeneration studies were not reported in *A. gymnolobus*. In this regard, we aimed to obtain a successful *in vitro* culture protocol of *A. gymnolobus* using plant tissue culture methods. This study also intended to reveal and compare the medicinal potential of *in vitro*-regenerated and field-grown *A. gymnolobus*, by investigating antibacterial activities, antioxidant properties and phenolic profiles of their leaves.

MATERIALS AND METHODS

Plant Materials and Seed Germination

Astragalus gymnolobus seeds were collected from Abant Lake, Bolu, Turkey, in September 2014. It was classified using “Flora of Turkey and The East Aegean Island” (Davis, 1970) and voucher specimens (AUT-2025) were deposited in Abant Izzet Baysal University Herbarium.

To germinate seeds aseptically, seeds were firstly sterilized in 70% ethanol for 10 minutes, then in 20% bleach (Domestos®-5% sodium hypochloride) for 10 minutes, and finally, washed five times with sterile water. Then, they were placed on basal MS medium (Murashige and Skoog, 1962) including 4.43 g L^{-1} Murashige and Skoog medium (Sigma Chemical Co., St. Louis, MO, USA), 30 g L^{-1} Sucrose (SC) and 8 g L^{-1} Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121°C and 105 kPa) in sterile petri plates and incubated in climate room conditions (22±2°C, 16-hours photoperiod, 200 μmol m^{-2} s^{-1} fluorescent light and 55-65% relative humidity). Germinated seeds were transferred into Magenta jars (GA-7 Vessel, Sigma Chemical Co.) containing MS medium for an additional three weeks.

Plant Regeneration and Rooting of Propagated Shoots

For shoot regeneration, petiole (4 mm; horizontally oriented) and leaf lamina explants (16 mm^2; adaxial side up) from one-month-old seedlings were placed in sterile petri plates containing MS medium supplemented with different combinations and concentrations of cytokinins [Thidiazuron (TDZ), Kinetin (KIN) and Benzyladenine (BA)] and auxins [Indole-3-Butyric Acid (IBA), Indole-3-Acetic Acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Naphthalene Acetic Acid (NAA)]: TDZ (0.01, 0.05, 0.1 and 0.5 mg L^{-1})+IAA (0.1, 0.25, 0.5 and 1 mg L^{-1}); TDZ (0.5 and 1 mg L^{-1})+2,4-D (0.5 and 1 mg L^{-1}); TDZ (0.5 and 1 mg L^{-1}) +2,4-D (0.5 and 1 mg L^{-1})+Gibberellic Acid (GA_3; 0.5 mg L^{-1}) ; TDZ (0.5 and 1 mg L^{-1})+IBA (0.5 and 1 mg L^{-1}); BA (0.5, 1, 3 and 5 mg L^{-1})+IAA (0.1, 0.25, 0.5 and 1 mg L^{-1}); BA (1, 2 and 4 mg L^{-1})+NAA (0.2, 0.5 and 1 mg L^{-1}); BA (0.5 and 1 mg L^{-1})+IBA (0.5 and 1 mg L^{-1}); BA (0.5, 1 and 3 mg L^{-1})+ 2,4-D (0, 0.1 and 0.5 mg L^{-1});

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KIN (0.5, 1 and 3 mg L^{-1})+IAA (0.1, 0.5 and 1 mg L^{-1}); KIN (0.5, 1 and 3 mg L^{-1})+2,4-D (0.1 and 0.5 mg L^{-1}); KIN (0.5, 1 and 3 mg L^{-1})+NAA (0.5 and 1 mg L^{-1}). All cultures were incubated in climate room. Two months later, regenerated shoots were transferred to Magenta containers on MS medium including 0.5 mg L^{-1} GA_3 for shoot elongation. On the 8th week of culture, shoot number per shooted explants and percentage of explants producing shoots were noted. Experiment was repeated three times for each treatment having 3 replications (each replication contained 5 explants and totally 15 explants were used for each treatment; totally 45 explants per treatment throughout the whole experiment).

After 10 weeks of culture, individual shoots were separated and their base callus was completely cut out. For root induction, shoots were placed in a medium with varying concentrations of MS, SC and four types of auxins: Control (4.43 g L^{-1} MS+30 g L^{-1} Sucrose (SC)+8 g L^{-1} agar), NAA (0.5, 1 and 3 mg L^{-1}), IBA (0.5, 1 and 3 mg L^{-1}), IAA (0.5, 1 and 3 mg L^{-1}), 2,4-D (0.1, 0.5 and 1 mg L^{-1}), ½ MS + NAA (0.5, 1 and 3 mg L^{-1}), ½ MS + IBA (0.5, 1 and 3 mg L^{-1}), ½ MS + IAA (0.5, 1 and 3 mg L^{-1}), ½ MS + 2,4-D (0.1, 0.5 and 1 mg L^{-1}), ½ MS + ½ SC + NAA (0.5, 1 and 3 mg L^{-1}), ½ MS +½ SC + IBA (0.5, 1 and 3 mg L^{-1}), ½ MS + ½ SC + IAA (0.5, 1 and 3 mg L^{-1}), ½ MS + ½ SC + 2,4-D (0.1, 0.5 and 1 mg L^{-1}), 0.5 mg L^{-1} GA_3 + NAA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} GA_3 + IBA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} GA_3 + IAA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} GA_3 +2,4-D (0.1, 0.5 and 1 mg L^{-1}), 0.5 mg L^{-1} ascorbic acid (AA) + NAA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} AA + IBA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} AA + IAA (0.5, 1 and 3 mg L^{-1}), 0.5 mg L^{-1} AA + 2,4-D (0.1, 0.5 and 1 mg L^{-1}), ½ MS+ ½ SC + activated charcoal (0.5, 1 and 2 mg L^{-1}), ½ MS+ ½ SC), Control + 1.5 g L^{-1} polyvinylpyrrolidone (PVP), ½ MS + ½ SC + 1.5 g L^{-1} PVP, 1.5 g L^{-1} PVP + NAA (0.5, 1 and 3 mg L^{-1}), 1.5 g L^{-1} PVP + IBA (0.5, 1 and 3 mg L^{-1}), 1.5 g L^{-1} PVP + IAA (0.5, 1 and 3 mg L^{-1}), 1.5 g L^{-1} PVP +2,4-D (0.1, 0.5 and 1 mg L^{-1}). Root number per shoots was recorded after 3 months of culture. Rooting experiment was repeated three times for each treatment having 10 replications (each replication contained 1 regenerated shoot, and totally 10 shoots were used for each treatment; thus, totally 30 regenerated shoots per treatment throughout the whole experiment).

After washing the roots of regenerated shoots with sterile water, they were placed in Magenta containers containing 25 gr of sterile vermiculite (Agrekal®) with 100 mL sterile distilled water for acclimatization for 3 weeks, followed by transfer to plastic pots containing potting soil (Mixflor®).

Experiments with a single factor - the one way ANOVA - in the completely randomized design were carried out and Duncan's multiple range tests using SPSS (Ver. 15, SPSS Inc, Chicago, IL, USA) were performed to evaluate the regeneration efficiency of leaf and petiole explants of *A. gymnolobus* against different Plant Growth Regulator (PGR) combinations. The significance level of the analysis was set to 0.05.

Leaf Extract Preparation

Field-grown leaves of *A. gymnolobus* were collected from Abant Lake, Bolu/Turkey in May 2014. *In vitro*-grown leaves obtained from 0.5 mg L^{-1} TDZ were obtained from *in vitro*-regenerated *A. gymnolobus*. The collected plant sources were powdered after drying for preparation of extracts. Ten grams of plant materials were extracted with 100 mL methanol at 40ºC by using water bath for 24 hours and then filtered. After extraction, methanol was concentrated using rotary evaporator to obtain the crude extract. Extract yields were determined with the following formula:

Yield (%)= [Weight of extract (g)/Powdered plant material (g)]×100

Antibacterial Assay

The antibacterial effect of each plant extract against 10 human pathogenic bacterial strains

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was evaluated by using disc diffusion method (Kirby-Bauer Method) (Andrews, 2009). Gram-positive bacteria [*Streptococcus pyogenes* (ATTC 19615), *Staphylococcus aureus* (ATTC 25923), and *S. epidermidis* (ATCC 12228) and Gram-negative bacteria [*Escherichia coli* (ATCC 25922), *Enterobacter cloacea* (ATCC 23355), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 13315) and *Pseudomonas aeruginosa* (ATCC 27853)] (Becton Dickinson Laboratories, France) were tested.

Test organisms were subcultured at 37°C in Tryptic Soy agar and then pure bacterial colonies were inoculated in 0.9% sterile saline water. The turbidity of each bacteria broth culture was then adjusted to 0.5 McFarland standards with sterile saline water. Adjusted broth cultures were separately inoculated on the entire surface of Mueller Hinton Agar plates. Extracts were dissolved in Dimethyl Sulfoxide (DMSO) for antibacterial test. Sterile 6 mm paper discs (Glass Microfibre filters, Whatman®) were impregnated with 13 µL plant extracts (100 mg mL^{-1}) or DMSO as negative control. Loaded discs and standard antibiotic discs [ampicillin (10 µg), erythromycin (15 µg) and tetracycline (30 µg)] were placed on the inoculated plates, incubated for one day at 37°C, and then inhibition zone diameter (mm) around the disc was recorded. Three independent experiments were performed.

Antioxidant Assay

Antioxidant potentials of *A. gymnolobus* methanol extracts were determined using 2,2-DiPhenyl-1-PicrylHydrazil (DPPH Sigma-Aldrich Chemie, Steinheim, Germany) radical photometric assay according to Blois (1958) method. DPPH radical was dissolved in methanol to get approximately 1.4 absorbance unit (0.13 mM DPPH solution) at 517 nm. Different concentrations of extracts and quercetin as an antioxidant standard were prepared in methanol and mixed with DPPH solution to determine antioxidant potential of extracts: 0.1 mL of extract, quercetin or methanol (control) was added into 1.4 ml DPPH solution and samples were incubated in the dark at 25°C. Then, absorbance was measured against blank (methanol) with Hitachi U-1900, UV-VIS Spectrophotometer 200V. The experiment was repeated at least four times. The following equation was used to calculate the capability of *A. gymnolobus* samples to scavenge the DPPH· radical:

DPPH Scavenging effect (% inhibition)= $[(A_0{-}A_1/A_0)\times100]$

Where, A_0 is the absorbance of the control reaction and A_1 is the absorbance of *A. gymnolobus* extracts.

Estimation of Total Phenolic Content

To determine total phenolic content, Folin–Ciocalteu method was used (Slinkard and Singleton, 1977). Gallic acid stock solution, as a reference phenol, was prepared and diluted with distilled water at different concentrations. Twenty µL of gallic acid solutions of various concentrations and plant extract or distilled water as a blank were mixed well with 1.58 mL water and 100 µL of Folin-Ciocalteu regent (Sigma®). Two min later, 20% Na_2CO_3 (300 µL) was added to each solution, and was shaken vigorously. Each solution was incubated at 25 °C for 2 h and the absorbance of each solution was measured at 765 nm against the blank using the spectrophotometer. These data were used to determine total phenolic content of the extracts using standard curve of gallic acid and was expressed as mg Gallic Acid Equivalents (GAE) 100 g^{-1} dried mass. All analyses were made in triplicate.

Estimation of Total Flavonoid Content

To determine total flavonoid content, aluminum colorimetric assay with some modifications was used (Chang *et al.*, 2002). Rutin as a reference flavonoid was used and diluted with methanol at different

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concentrations. Briefly, 500 μL of extract, rutin or methanol as a blank was added to vial containing 2 ml distilled water. At zero time, 150 μl $NaNO_2$ (5%) was added to each vial. After 5 minutes, 150 μL $AlCl_3$ (10%) was added and 6 min later, 1,000 μL NaOH (1M) was added to each mixture. Immediately, the reaction tube was made up to 5 mL adding distilled water and shaken thoroughly. Each solution was incubated in dark at 25°C for 10 minutes and its absorbance was measured at 510 nm against the blank using the spectrophotometer. The standard rutin curve was prepared by plotting absorbance value versus known concentrations of standard, then, total flavonoid contents were estimated by using the equation obtained from the standard calibration curve.

Analysis of Methanol Extracts by High Performance Liquid Chromatography

Methanolic extracts were analyzed for some standard phenols using a HPLC-DAD system (VWR-Hitachi LaChrom Elite®). Caffeic acid, coumarin, apigenin, rutin hydrate, myricetin, quercetin and luteolin-7-O-β-D glucoside were used as phenol standards (Sigma®). Various concentrations (10, 20, 40, 60, 80, 100 and 200 mg L^{-1}) of standards were prepared for obtaining standard curve. The operating conditions were arranged as described previously in our laboratory (Yildirim *et al.*, 2017). HPLC grade (Merck) solvents were used and eluent was composed of 0.1% orthophosphoric Acid (OA) in water (solvent A) and 0.1% OA in methanol (solvent B). A gradient elution was used with 60% of A and 40% of B at 0 minute and adjusted to 50%, 40%, 40%, 60% A at 10, 15, 25 and 25.1th min, respectively. Twenty µL of each extract was injected into the HPLC and separations were done at 25°C oven and one mL min^{-1} flow rate. The chromatograms were recorded at 255 nm for luteolin, rutin and quercetin, 277 nm for coumarin, 325 nm for caffeic acid, 340 nm for apigenin and 370 nm for myricetin standard.

RESULTS AND DISCUSSION

In Vitro Propagation of *A. gymnolobus*

Surface sterilized *A. gymnolobus* seeds were germinated within 2-3 weeks and additional 3 weeks were required for seedling development. Leaves and petioles dissected from *in vitro* seedlings were cultured on MS medium containing BA, TDZ or KIN, either alone or in combination with IAA, IBA, NAA or 2,4-D.

Of the tested PGRs, shoot regeneration was observed only in the presence of TDZ, alone or in combination with IAA, in both types of explants (Table 1, Figures 1-A and -B). However, shoot development was not observed using combinations of BA or KIN with different auxins in either type of explants. Besides, the addition of GA_3 into the combination of PGRs was not successful for shoot regeneration in both explants (data not shown).

When TDZ was used alone with leaf explants, the most efficient concentration on shoot formation was 0.5 mg/L TDZ (Table 1; Figure 1). However, lower TDZ concentrations were more successful in regard to shoot frequency (Table 1). Moreover, shoot formation was not observed when TDZ was used alone at 0.01 mg L^{-1} for leaf explants. Generally, TDZ stand-alone treatments (0.05, 0.1 and 0.5 mg L^{-1}) were more effective in mean number of shoots and the addition of any amount of IAA reduced the shoot numbers. However, there was essentially no statistically significant difference between any of the TDZ+IAA combinations (except for 0.05 mg L^{-1} TDZ+0.1 or 0.5 mg L^{-1} IAA) compared to 0.5 mg L^{-1} TDZ alone. When 0.05 mg L^{-1} TDZ were combined with 0.1 mg L^{-1} IAA, a reduction was observed in terms of the mean number of shoots per leaf explant (6.11 shoots), but an increase appeared in terms of shoot frequency (37.50%) compared with 0.05 mg L^{-1} TDZ alone treatment. Although

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Table 1. Shoot regeneration from leaf and petiole explants cultured on MS medium containing different combinations of TDZ with IAA.

Treatments		Explants			
		Leaf		Petiole	
		Mean no. of shoots per shooted explant [a]	% Explants forming shoots	Mean no. of shoots per shooted explant [a]	% Explants forming shoots
Control (No PGR)		-	-	-	-
TDZ ($mg L^{-1}$)	IAA ($mg L^{-1}$)				
0.01	-	-	-	-	-
0.01	0.1	-	-	-	-
0.01	0.25	-	-	-	-
0.01	0.5	10.75 ±1.89^{ab}	19.05	-	-
0.05	-	12.57 ±2.60^{ab}	31.82	-	-
0.05	0.1	6.11 ±1.32^{b}	37.50	0.67 ±0.67^{b}	5.88
0.05	0.25	-	-	-	-
0.05	0.5	6.50 ±1.43^{b}	26.08	-	-
0.1	-	12.43 ±1.23^{ab}	30.43	-	-
0.1	0.1	-	-	-	-
0.1	0.25	10.50 ±2.40^{ab}	18.18	-	-
0.1	0.5	12.80 ±4.82^{ab}	20.83	-	-
0.5	-	17.60 ±3.17^{a}	23.81	-	-
0.5	0.1	12.75 ±2.56^{ab}	20.00	5.00 ± 1.73^{a}	20.00
0.5	0.25	-	-	0.33 ± 0.33^{b}	6.25
0.5	0.5	10.00 ±1.00^{ab}	13.64	-	-

[a] Means within columns followed by the same letters are not significantly different (P> 0.05).

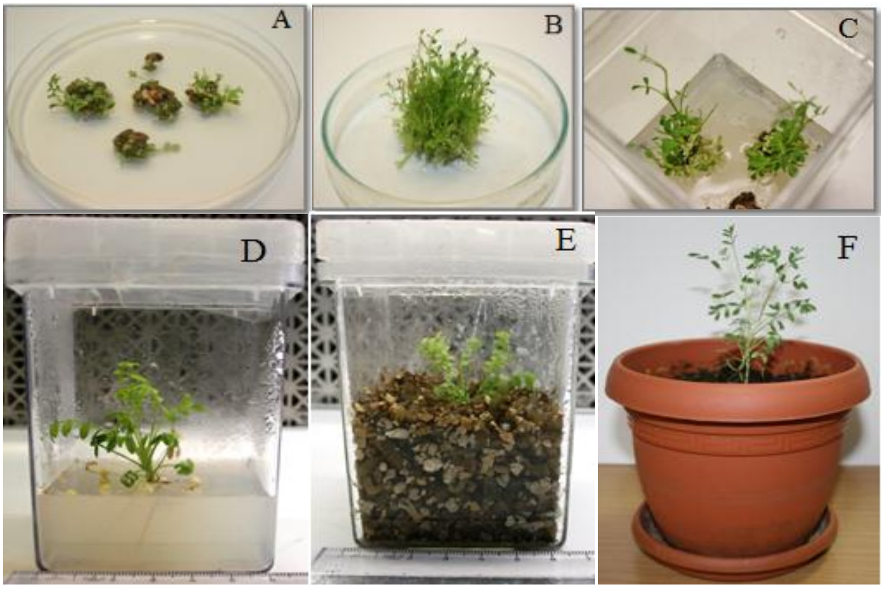

Figure 1. *In vitro* micropropagation of *A. gymnolobus*: Shoot regeneration from leaf explants containing 0.5 mg L^{-1} TDZ (A, B); shoot elongation on MS+0.5 mg L^{-1} GA_3 (C); root development on MS medium (D); rooted plants in vermiculite for acclimatization (E), and regenerated plant in plastic pot containing sterile soil under growth room conditions (F).

0.25 mg L^{-1} IAA in combination with 0.01, 0.05 or 0.5 mg L^{-1} TDZ was not effective in shoot formation, 0.1 mg L^{-1} TDZ in combination with 0.25 mg L^{-1} IAA caused shoot formation (10.50 shoots). Differences in shoot formation efficiency among TDZ and IAA combinations may be the reason for a synergism between TDZ and both endogenous and exogenous auxins (Huetteman and Preece 1993).

Petiole explants gave the best shoot formation with media containing 0.5 mg L^{-1} TDZ+0.1 mg L^{-1} IAA (20% of explants formed 5 shoots on average). Generally, petiole explants were not effective for shoot regeneration (Table 1). Leaf lamina having higher amount of vascular tissue and thus higher levels of endogenous hormones and metabolites might be liable for the increase in shoot regeneration capacity.

Callus formation was observed with auxin and cytokinin combinations. Greater amount of callus was obtained with BA (0.5, 1 and 3 mg L^{-1})+2,4-D (0.1 and 0.5 mg L^{-1}) and TDZ (0.5 and 1 mg L^{-1})+2,4-D (0.5 and 1 mg L^{-1}) combinations than other tested PGR combinations. When TDZ was used alone on the leaf explants, callus formation was not observed. Leaf explants produced greater amount of callus than petiole explants. 2.4-D was the most effective auxin on callus development. If 2,4-D concentration was increased from 0.1 to 0.5 mg L^{-1}, leaf explants produced greater amount of callus. Best callus formation was obtained with MS medium containing 3.0 mg L^{-1} BA and 0.5 mg L^{-1} 2,4-D (data not shown).

Shoot elongation of regenerated shoots occurred in MS medium supplemented with 0.5 mg L^{-1} GA_3 for 2 weeks (Figure 1-C). In order to promote root induction in *A. gymnolobus*, single shoots were obtained by cutting, to remove callus from regenerated shoots, and placed on rooting medium. Regenerated shoots rooted only in basal MS medium (control) with 3.5 root number per shoot and 77.8% rooting ratio (Figure 1-D). Roots were observed after at least 3 months. Media supplemented with different auxin types and concentrations in combination with full and half-strength MS and SC, GA_3, PVP, activated charcoal or ascorbic acid was not effective for rooting. Vermiculite was used for acclimatization for 3 weeks (Figure 1-E) and then all rooted shoots were finally transferred to plastic pots containing sterile soil and kept in growth room conditions at 22°C under a 16-hour photoperiod (Figure 1-F). The rooted plants had 50% survival rate through the hardening off process.

So far, there has been no regeneration study with *A. gymnolobus*, but some *Astragalus* species were reported for their regeneration capacities. Dilaver *et al*. (2017) studied *in vitro* regeneration of *A. vulnerariae* for its response to KIN-NAA and BAP-NAA media using 5 different explant types (hypocotyl, epicotyl, cotyledon, shoot node, and leaf explant). Their results indicated that combinations of KIN-NAA induced shoot regeneration in all types of explants, whereas BA-NAA medium was less effective for shoot regeneration. The highest regeneration was noted from hypocotyl explants with KIN-NAA (4.47 shoots per explants). With combinations of BAP-NAA, the highest regeneration rate (3 shoots per explant) was reported for epicotyl explants (Dilaver *et al*., 2017). Erisen *et al*. (2010) reported that high concentrations of BA with NAA were the most effective for shoot formation of *A. nezaketae*. Similarly, Luo and Jia (1998) noted that MS medium containing BA and NAA strongly stimulated shoot formation from hypocotyl explants of *A. adsurgens*. Using different BA-NAA combinations to study shoot regeneration in *A. cicer*, Uranbey *et al*. (2003) tested petiole, hypocotyl, stem and cotyledon explants and showed that hypocotyl explants had better regeneration capability than other used explants. High regeneration capacity from *A. cicer* hypocotyl explants was also observed in another study using MS media that contained TDZ, in which hypocotyls produced more shoots than cotyledon explants (Basalma *et al*., 2008). In a different *Astragalus* species, Erisen *et al*. (2011) evaluated the effect of TDZ alone or

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in combination with NAA for shoot development from leaf and petiole explants. According to their results, leaf explant of *A. cariensis* was the best for shoot development and the combination of TDZ with low NAA concentration was more inductive than the use of TDZ alone. Esmaeili *et al*. (2016) investigated hypocotyl, cotyledon and apical meristem of *A. adscendens* for *in vitro* regeneration, using BA, KIN and Zeatin, alone or in combinations with NAA. They reported that neither hypocotyl nor cotyledon explants were induced to shoot formation, whereas apical meristem gave shoot regenerants at high concentration of BA. Similarly, in *A. schizopterus* axillary meristems, best shoot proliferation had been reported with the usage of BA alone (Yorgancilar and Erisen, 2011). Micropropagation of *A. maximus* from axillary buds using trans-Zeatin Riboside (ZR) was reported (Turgut-Kara and Arı, 2006). In *A. chrysochlorus*, shoot formation was also achieved using ZR (Hasancebi *et al*., 2011). In our study, leaf and petiole explants gave shoot regeneration only with TDZ alone or in combination with IAA. On the other hand, KIN-NAA, BAP-NAA or TDZ-NAA media were not effective for shoot regeneration in *A. gymnolobus* leaf and petiole explants (Table 1).

Root formation in *A. gymnolobus* was not observed with tested auxins. There was a darkening at the bottom of regenerated shoots. Tested auxins were tried again in combination with GA_3, ½ MS, ½ SC, activated charcoal, PVP and ascorbic acid and these combinations were also inefficient for root formation. Later, all basal parts of regenerated shoots were cut off 0.5-1 cm from the bottom and single shoots were transferred to rooting medium including IAA, IBA, 2.4-D or NAA at different concentrations. Rooting was observed only in MS medium (control) after 3 months, and there was no browning of the shoots. Callus forming at the bottom of the shoots may have prevented the root formation. TDZ alone did not produce callus before shoot formation, but regenerated shoots obtained from TDZ alone treatments did not form roots. Similar to our results, root induction was only obtained on plant growth regulator-free medium in *A. adsurgens* (Luo and Jia, 1998), *A. maximus* (Turgut-Kara and Arı, 2006) and *A. chrysochlorus* (Hasancebi *et al*., 2011). On the other hand, regenerated shoots of *A. schizopterus* were successfully rooted in the presence of NAA or IBA (Yorgancilar and Erisen, 2011). Basalma *et al*. (2008) also reported that best rooting in *A. cicer* was achieved on 1/2 MS medium containing NAA. In *A. adscendens*, highest root induction was also obtained from MS medium with NAA (Esmaeili *et al*., 2016). However, rooting was achieved by IBA treatment in *A. vulnerariae* (Dilaver *et al*., 2017), *A. nezaketae* (Erisen *et al*., 2010) and *A. cariensis* (Erisen *et al*., 2011).

Antibacterial Activity

Methanol extracts of field-grown (24.03% yield) and *in vitro*-grown (26.75% yield) leaves were evaluated for the antibacterial potential of *A. gymnolobus* against 10 bacteria. Only field-grown leaves exhibited antibacterial potential and only Gram positive bacteria (*S. pyogenes*, *S. aureus*, and *S. epidermidis*) were sensitive to this extract (Table 2). The highest inhibitory activity was obtained against *S. pyogenes*. Antibacterial activity of the extract against *S. aureus*, *S. epidermidis* and *S. pyogenes* may explain why *Astragalus* species have been used in folk medicine to treat nephritis, immunologic diseases (caused by *S. pyogenes*), sepsis, and urinary tract infections (caused by *S. aureus* and *S. epidermidis*). Similar to our results, Turker and Koyluoglu (2012) reported that methanol and ethanol extracts of aerial parts of *A. gymnolobus* including flowers showed little inhibition against only *S. pyogenes*. Furthermore, Turker and Yildirim (2013) demonstrated that ethanol, methanol and aqueous extracts of aerial parts of *A. brachypterus* including flowers exhibited

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Table 2. Antibacterial activities of methanol extracts of field-grown and *in vitro*-grown *A. gymnolobus* leaves.

Treatments	Mean diameter of inhibition zones (mm±SE) [a]		
	S. aureus	*S. epidermidis*	*S. pyogenes*
Field-grown leaves	9.8±0.4 [c]	10.2±0.4[c]	11.0±0.3[d]
In vitro-grown leaves	-	-	-
Ampicillin	35.6±1.1[a]	35.0±1.4[b]	45.0±0.3[a]
Erythromycin	30.6±0.3[b]	45.0±0.3[a]	38.9±0.3[b]
Tetracycline	35.5±0.3[a]	-	35.8 ± 0.3[c]
DMSO	-	-	-

[a] Data are presented as a mean diameter of inhibition zones±Standard Error (SE). Means within columns followed by the same letter are not significantly different at P> 0.05.

strong antibacterial inhibition against only *S. pyogenes*.

Some *Astragalus* species were investigated for their antibacterial activity against Gram positive and Gram negative bacteria (Bisignano *et al*., 1994; Pistelli *et al*., 2002). Teyeb *et al*. (2012) reported the antibacterial activity of four extracts (methanol, dichloromethane, petroleum ether and alkaloid extract) from aerial parts of *A. gombiformis* and the methanol extract was the most active against *S. typhimurium* and *P. aeruginosa*. Jaradat *et al*. (2017) examined the antibacterial activity of four *Astragalus* species against *S. aureus*, *E. coli* and *P. aeruginosa*. Their results indicated that *A. boeticus* exhibited higher bioactivity against the growth of the studied bacteria compared to that of other *Astragalus* species. Adigüzel *et al*. (2009) studied antibacterial activity of some *Astragalus* species against 24 different microorganisms; however, they could not get any activity against the tested bacteria. Similarly, Albayrak and Kaya (2017) demonstrated that studied *Astragalus* species had no antibacterial potential except against *P. aeruginosa*. Kanaan *et al*. (2017) showed the highest bacteriostatic effect of whole plant ethanolic extract of *A. angulosus* against *P. aeruginosa*, *E. coli*, and *S. epidermidis*.

Antioxidant Activity

The antioxidant activity of methanol extracts of *A. gymnolobus* leaves was determined by free radical scavenging activity (DPPH), total phenol, and flavonoid content (Table 3). IC_{50} values (concentration of extracts that inhibits the DPPH radical to 50%) of *A. gymnolobus* extracts were assessed and field-grown *A. gymnolobus* leaves exhibited better radical scavenging activity than *in vitro*-grown leaves having IC_{50} value of 960.1 μg mL^{-1} (Table 3). Both extracts demonstrated a concentration-dependent free radical scavenging activity (%) by scavenging DPPH radical.

Total phenolic content of field- and *in vitro*-grown *A. gymnolobus* methanolic extracts was estimated by using Folin Ciocalteu reagent and

Table 3. IC_{50} values, total phenol and flavonoid content of field-grown and *in vitro*-grown *A. gymnolobus* leaf methanol extracts.[a]

Extracts	Antioxidant activity IC_{50} (μg mL^{-1})	Total Phenolics mg GAE g^{-1} dry extract	Total Flavonoids mg RE g^{-1} dry extract
Field-grown leaves	960.1±6.9	96.7±0.0	184.5±0.0
In vitro-grown leaves	> 5000	23.7±0.0	79.6±0.0

[a] Data are presented as a mean number±standard error (SE). IC_{50}: The half maximal inhibitory concentration. GAE: Gallic acid equivalent, RE: Rutin equivalent.

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calculated from the calibration curve (R^2= 0.9996). Methanol extract from field-grown *A. gymnolobus* contained about four-fold higher amount of phenol than that of *in vitro*-grown plants (Table 3). The flavonoid content of *A. gymnolobus* methanol extracts from field-grown plants was about 2-fold higher than that from *in vitro*-grown plants (Table 3).

Adigüzel *et al*. (2009) reported the antioxidant activity of the methanol extracts obtained from the aerial part of several *Astragalus* species and showed mild free radical scavenging activity with 50% inhibition between 68.8 and 400.4 µg mL^{-1} concentrations. Haşimi *et al*. (2017) reported moderate antioxidant activity with 50% inhibition ranging between 54.61 and higher than 200 µg mL^{-1} from three endemic *Astragalus* species. Similarly, Albayrak and Kaya (2017) reported that four different *Astragalus* species exerted slight antioxidant activity in DPPH assay. In the investigation of Bourezzane *et al*. (2018), n-butanol extract of *A. monspessulanus* possessed a moderate radical scavenging effect (IC_{50}= 63.60 µg mL^{-1}). On the contrary, Langari and Salehi (2015) studied methanol and dichloromethane extracts of *A. glaucacanthus* and found significant antioxidant activity with 0.196 µg mL^{-1} IC_{50} for methanol and 0.536 µg mL^{-1} IC_{50} for dichloromethane extracts.

HPLC Analysis of Phenolic Compounds

Methanol extracts from field- and *in vitro*-grown *A. gymnolobus* leaves were investigated for the presence of coumarin, apigenin, caffeic acid, rutin hydrate, quercetin, luteolin-7-O-β-D glucoside and myricetin (Table 4). Chromatogram of these phenol standards is presented in Figure 2. Rutin hydrate was detected in much higher amounts in field-grown compared to *in vitro*-

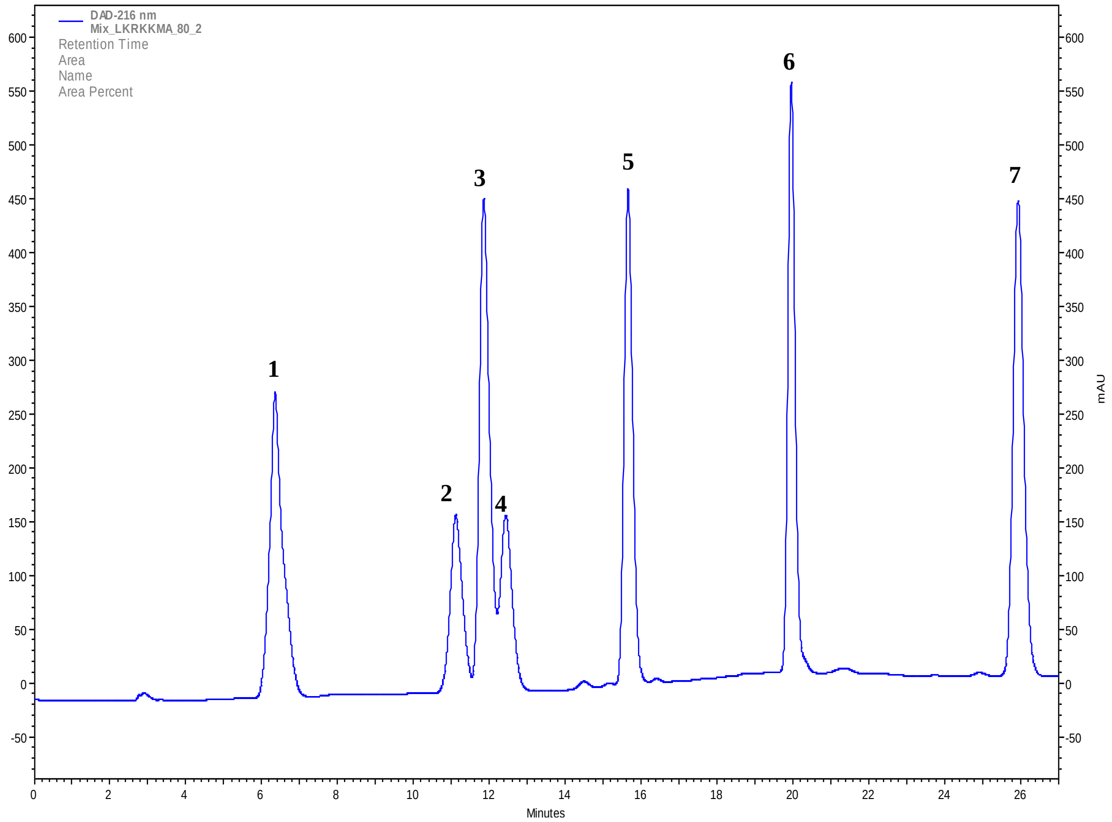

Figure 2. Chromatogram of the phenolic standards: (1) Caffeic acid, (2) Luteolin-7-O-β-D glucoside, (3) Coumarin, (4) Rutin hydrate, (5) Myricetin, (6) Quercetin, (7) Apigenin.

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grown leaves (50.48 and 2.59 µg mg^{-1} dry extract, respectively). Coumarin and apigenin were not present in *A. gymnolobus* methanol extracts. The rest of the phenols were found in both methanolic extracts, ranging from 0.06 to 0.99 mg g^{-1} dry extract for field-grown plants and from 0.01 to 0.27 mg g^{-1} dry extract for *in vitro*-grown plants (Table 4).

Haşimi *et al*. (2017) used LC-MS/MS to detect twenty-four phenolic compounds and three non-phenolic organic acids in three *Astragalus* species. Their results showed that rutin (1.03-13.35 µg mg^{-1} extract), hesperidin (1.60-9.70 µg mg^{-1} extract) and hyperoside (0.23-1.99 µg mg^{-1} extract) were the most abundant flavonoids in *Astragalus* species. Similarly, rutin was found to be present in the highest amounts in our analyzed extracts. Some flavonoids from aerial parts of *A. verrucosus* Moris were isolated and identified as rutin, quercetin 3-*O*-robinobioside and apigenin (Pistelli *et al*., 2003). In another study, Bourezzane *et al*. (2018) isolated rutin and quercetin 3-*O*-(2,6-α-L-dirhamnopyranosyl-β-D-glucopyranoside) as flavonoids, two saponins, two sterols, and one triterpenoid from *A. monspessulanus* n-butanol extract.

Exposure of plants to different stress circumstances in the field environment causes the raising of phenolic constituents for adaptation (Türker and Yıldırım, 2018; Turker *et al*., 2018). It is obvious that *in vitro*-grown plants encounter no stress conditions and no need of much phenol production for survival, in our study.

CONCLUSIONS

In this study, *in vitro* culture protocol for endemic *A. gymnolobus* plant was developed for the first time. A comparison was performed between field-grown and *in vitro*-grown leaves, by determining antibacterial and antioxidant activities, total phenolic and flavonoid content, and also analyzing phenolic composition in *A. gymnolobus* by HPLC. Our data clearly showed that field-grown *A. gymnolobus* had higher bioactivity and phenolic content than *in vitro*-grown plants. It was also found that *A. gymnolobus* has moderate antioxidant and antibacterial activities. Besides, it contains remarkable amount of rutin. Future studies should focus on increasing the quantity of phenolics like rutin in *in vitro*-grown leaves by implementing different types of stress.

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Table 4. Contents of phenolic compounds in field-grown and *in vitro*-grown *A. gymnolobus* methanol extracts.

Standard compounds			Plant extracts (mg g^{-1} dry extract)[a]	
Name	Peak number	RT (Min)	Field-grown leaves	*In vitro*-grown leaves
Caffeic acid	1	6.27	0.21 ± 0.00	0.17 ± 0.00
Luteolin	2	11.17	0.99 ± 0.00	0.27 ± 0.00
Coumarin	3	11.87	-	-
Rutin	4	12.89	50.48 ± 0.00	2.59 ± 0.00
Myricetin	5	16.85	0.43 ± 0.00	0.02 ± 0.00
Quercetin	6	19.49	0.06 ± 0.00	0.01 ± 0.00
Apigenin	7	25.91	-	-

[a] Data are presented as means±standard error (SE).

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کشت درون شیشه ای گیاه بومی *Astragalus gymnolobus* Fischer و مقایسه مشخصات آنتی باکتریایی، آنتی اکسیدانی، و فنولیک آن با گیاه کشت شده در مزرعه

ا. ب. ایلدیریم، ا. اویار، و ا. ی. توکر

چکیده

گیاه *Astragalus gymnolobus* Fischer (از خانواده Leguminosae) از گیاهان بومی ترکیه است . در این پژوهش، نخست سامانه ای برای کشت این گیاه در شیشه با استفاده ازریز نمونه های برگ و دمبرگ روی بستر Murashige and Skoog (MS) همراه با غلظتهای متفاوت مواد تکمیلی شامل مواد زیر به دست آمد: سیتوکینین ها [thidiazuron (TDZ), kinetin (KIN), benzyladenine (BA)]، اکسین ها (auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) و نفتالین اسید استیک(NAA) و اسید جیبرالیک (GA_3). بهترین تشکیل شاخساره با ریز نمونه های برگ به دست آمد و TDZ به تنهایی یا ترکیب TDZ و IAA در تشکیل شاخساره نقش موثری داشتند. بهترین واکنش القای شاخساره یا ساقه (به تعداد ۱۷/۶ ساقه در هر ریزنمونه با بسآمد ۲۳/۸۱٪) در تیمار ۰/۵ میلی گرم در لیتر TDZ به تنهایی ثبت شد. در تیمارهایی که TDZ به تنهایی مصرف شد، تیمار ۰/۵ و ۰/۱ میلی گرم در لیتر نیز از لحاظ بسآمد القای ساقه(به ترتیب به میزان ۳۱/۸۲ ٪ و ۳۰/۴۳٪) موثر بودند. ریشه بعد از ۳ ماه فقط در بستر MS عاری از اکسین (شاهد) تشکیل شد(۳/۵ ریشه در هر شاخساره یا ساقه با بسآمد ریشه برابر ۷۷۵/۸) و در آن زمان ریشه های باز زایی شده (regenerated roots) در فاصله ۱–۰/۵ سانتی متری از پایه بریده شد تا قسمت پینه(callus) جدا شود .هدف دیگر این پژوهش این بود که خواص آنتی باکتریایی و آنتی اکسیدانی و ترکیبات فنولی کشت درون شیشه ای وکشت مزرعه ای *A. gymnolobus* تعیین و مقایسه شود. نتایج آشکار ساخت که برگ های کشت مزرعه ای به طور کلی دارای فعالیت زیستی بیشتر و مقادیر بیشترترکیبات فنولی بودند. افزون بر این، برگ های *A. gymnolobus* منبع قابل توجهی از ماده Rutin بودند. این دستورالعمل اولیه برای کشت درون شیشه ای گیاه بومی *A. gymnolobus* با هدف حفاظت منابع ژنتیکی ارزشمند است و می تواند در مطالعات کار برد تنش ها برای افزایش سطح تولید مواد فنولیک در کشت درون شیشه ای مورد استفاده قرار گیرد.

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