ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IN VITRO REGENERATION, BIOLOGICAL ACTIVITIES AND PHENOLIC COMPOSITION OF *ASTRAGALUS GYMNOLOBUS* FISCHER, AN ENDEMIC PLANT

MASTER OF SCIENCE

ESRA UYAR

BOLU, SEPTEMBER 2015

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APPROVAL OF THE THESIS

IN VITRO **REGENERATION, BIOLOGICAL ACTIVITIES AND PHENOLIC COMPOSITION OF *ASTRAGALUS GYMNOLOBUS* FISCHER, AN ENDEMIC PLANT** submitted by **Esra UYAR** in partial fulfillment of there quirements for the degree of Master of Science in **Department of Biology, Abant Izzet Baysal University** by,

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Director,**Graduate School of Natural and Applied Sciences**

I dedicate this dissertation to all my family

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DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

ESRA UYAR

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ABSTRACT

IN VITRO REGENERATION, BIOLOGICAL ACTIVITIES AND PHENOLIC COMPOSITION OF _ASTRAGALUS GYMNOLOBUS_ FISCHER, AN ENDEMIC PLANT
MSC THESIS
ESRAUYAR
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
DEPARTMENT OF BİOLOGY
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Astragalus is a large genus covering about 3000 species belonging to Fabaceae family. *Astragalus* species have been used as antibacterial, antiviral, antifungal, antioxidant, anticancer, hepatoprotective, wound healing, anti-diabetic and diuretic in folk medicine. *Astragalus gymnolobus* Fischer (milkvetch weed) is an endemic dwarf cushion-forming shrub. An efficient plant regeneration system was developed for *A. gymnolobus*. Two different explants (leaf and petiole) were cultured on MS (Murashige and Skoog) medium with various plant growth regulator combinations. Leaf explants formed more shoots than petiole explants. The best shoot formation was obtained from leaf explants with 0.5 mg/l TDZ (thidiazuron). The best callus formation was obtained with 3.0 mg/l BA (benzyladenine) and 0.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) with leaf explant. Regenerated shoots were put on medium containing different concentrations of IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2,4-D or NAA (naphthalene acetic acid). However, rooting was not observed with regenerated shoots. Collected calluses were used for biological activity studies of *A. gymnolobus*.

Antibacterial and antioxidant activities, total phenol and flavonoid content, and HPLC (high performance liquid chromatograph) analysis of phenolic constituents of field-grown leaves and *in vitro*-grown callus parts of *A. gymnolobus* were evaluated. Antibacterial activity of water and methanol extracts of *A. gymnolobus* was determined with Kirby-Bauer disk diffusion assay. Generally, field-grown leaves showed better antibacterial activities than *in vitro*-grown callus. The free radical scavenging potency of methanol extracts of *A. gymnolobus* was determined with DPPH (2,2-diphenyl-1-picrylhydrazil) method. The best antioxidant activity was obtained with methanol extract of field-grown leaves at 25 µg/ml concentrations (91.47 %). Total phenolic content by using Folin-Ciocaltaeu method and the total flavonoid content by using aluminum chloride ($AlCl_3$) colorimetric method were determined. Methanol extract of field-grown leaves contained more phenols and flavonoid than *in vitro*-grown callus. Concentrations of phenolic compounds in field-grown leaves were found higher than the *in vitro*-grown callus with HPLC analysis.

KEYWORDS: *Astragalus gymnolobus*, *In vitro* culture, Micropropagation, Antibacterial, Antioxidant, Phenol, Flavonoid, HPLC.

ÖZET

ENDEMİK BİR BİTKİ OLAN *ASTRAGALUS GYMNOLOBUS* FİSCHER' IN *İN VİTRO* REJENERASYONU, BİYOLOJİK AKTİVİTELERİ VE FENOLİK BİLEŞİMİ
YÜKSEKLİSANS TEZİ
ESRAUYAR
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
BİYOLOJİ BÖLÜMÜ
TEZ DANIŞMANI: DOÇ. DR. ARZU UÇAR TÜRKER
BOLU, EYLÜL 2015

Astragalus, Fabaceae ailesine ait yaklaşık 3000 türü kapsayan geniş bir cinstir. *Astragalus* türleri halk arasında antibakteriyel, antiviral, antifungal, antioksidant, antikanser, karaciğeri koruyucu, yara iyileştirici, antidiyabetik ve idrar söktürücü olarak kullanılmaktadır. *Astragalus gymnolobus* Fischer (gevenotu) endemik, bodur, yastık formunda çalılardır. *A. gymnolobus* için güvenilir bir bitki rejenerasyonu protokolü geliştirilmiştir. İki farklı eksplant (yaprak ve yaprak sapı) çeşitli bitki büyüme düzenleyicisi kombinasyonlarının bulunduğu MS (Murashige and Skoog) ortamı üzerinde kültüre alınmıştır.Yaprak eksplantları, yaprak sapı eksplantlarından daha fazla sürgün oluşturmuştur. En iyi sürgün oluşumu 0.5 mg/l TDZ (thidiazuron) ile yaprak eksplantlarından elde edilmiştir. En iyi kallus oluşumu 3.0 mg/l BA (benziladenin) ve 0.5 mg/l 2,4-D (2,4-diklorofenoksiasetik asit) ile yaprak eksplantlarından elde edilmiştir. Rejenere olmuş sürgünler, farklı konsantrasyonlarda IAA (indol-3-asetik asit), IBA (indol-3-bütrik asit), 2.4-D veya NAA (naftalen asetik asit) içeren ortamlara konulmuştur. Ancak, rejenere olmuş sürgünler ile köklenme gözlemlenmemiştir. Toplanmış kalluslar *A. gymnolobus* bitkisinin biyolojik aktivite çalışmaları için kullanılmıştır.

A. gymnolobus bitkisinin *in-vitro* yetiştirilmiş kallus ve doğada yetişen yaprak parçaları ile antibakteriyel ve antioksidant aktivite, toplam fenol ve flavonoid içeriği ve fenolik bileşenlerin HPLC (yüksek performanslı sıvı kromatografisi) analizi değerlendirilmiştir. *A. gymnolobus* bitkisinin su ve methanol özütlerinin antibakteriyel aktivitesi disk difüzyon metodu (Kirby-Bauer) ile belirlenmiştir. Genellikle, doğada yetişen yapraklar, *in-vitro* yetiştirilen kalluslardan daha iyi antibakteriyel aktivite göstermiştir. *A. gymnolobus* bitkisinin metanol özütlerinin serbest radikal süpürme gücü DPPH (2,2-difenil-1-pikrilhidrazil) metodu ile belirlenmiştir. En iyi antioksidant aktivite 25 µg/ml konsantrasyonunda (91.47 %) doğada yetişen yaprakların metanol özütlerinde gözlemlenmiştir. Folin-Ciocaltaeu methodu kullanılarak toplam fenol içeriği ve alüminyum klorid ($AlCl_3$) kolorimetrik methodu kullanılarak toplam flavonoid içeriği belirlenmiştir. Doğada yetişen yaprakların metanol özütleri, *in-vitro* yetiştirilen kalluslardan daha fazla fenol ve flavonoid içermektedir. Doğada yetişen yaprakların fenolik bileşenlerinin konsantrasyonu HPLC analizi ile *in-vitro* yetiştirilen kalluslardan yüksek bulunmuştur.

ANAHTAR KELİMELER: *Astragalus gymnolobus*, *In vitro* kültür, Mikropropagasyon, Antibakteriyel, Antioksidant, Fenol, Flavonoid, HPLC.

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LIST OF ABBREVIATIONS AND SYMBOLS

2,4-D **:** 2,4-dichlorophenoxyacetic acid

AlCl3 **:** Aluminium chloride

ANOVA **:** Analysis of Variance

BA **:** Benzyladenine

DPPH **:** 2,2-diphenyl-1-picrylhydrazil

EtOH **:** Ethanol

MEtOH **:** Methanol

GA_3 **:** Gibberellic acid

GAE **:** Gallic acid equivalent

HPLC **:** High-Performance Liquid Chromatograph

IAA **:** Indole-3-acetic acid

IBA **:** Indole-3-butyric acid

KIN **:** Kinetin

MS **:** Murashige and Skoog

NAA **:** Naphthalene acetic acid

TDZ **:** Thidiazuron

TLC **:** Thin-Layer Chromatography Kinetin

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1. INTRODUCTION

Astragalus gymnolobus Fischer belonging to Fabaceae family is a perennial endemic plant that found throughout the Northern and Southern part of Turkey (Davis, 1970). *Astragalus* spp. is the largest genus of vascular plants, containing up to 3000 species (Lock and Simpson, 1991). In Turkey, the genus *Astragalus* spp. is represented by approximately 439 species, 204 endemic (Akan, 2000). Generally, *Astragalus* genus is found in steppe areas in Irano-Turanian phytogeographic region of Turkey (Ghahrema-Ninejad and Behçet, 2003).

Astragalus spp. contains annual or perennial species and some of them can resist to abiotic stress such as cold, salinity and drought. They can grow in a wide range of conditions. However, slow seedling development, poor seed germination capacity and low number of seed set are drawbacks affecting their wider adaptation capabilities requiring the application of tissue culture techniques for their propagation (Yorgancılar and Erisen, 2011). Due to the economic and medicinal importance of the *Astragalus* species, they have been investigated for their chemical compounds (Çalış et al., 1997; Bedir et al., 1998, 1999; Yahara et al., 2000).

Various species of *Astragalus* are mainly used for gum production (Akan, 2000). Some perennial *Astragalus* species are used for forage production (Towsend, 1970). Deep top root system can be used for erosion control (Erisen et al., 2010) establishing the pasture in the non-irrigated areas such as Karapınar (Acar et al., 2011).

Astragalus spp. is important and used in many different ways and areas in medicine due to their secondary metabolites (Tang, 1992; Karagöz et al.,2007).

In vitro micropropagation of *A. gymnolobus* can provide a rapid multiplication method for producing plantlets exhibiting desirable characteristics. These desirable characteristics may be defined as disease resistance, increased secondary product accumulation, superior genotype characteristics, or beneficial morphological characteristics; all of which result in superior planting stock. More uniform plantlets can be obtained using an *in vitro* protocol for production, thus ensuring a more consistent crop. In addition, an *in vitro* propagation method would eliminate seasonal

constraints with seedling by providing unlimited planting material on a consistent year-round basis (McCoy, 1998).

In vitro culture protocols provide multiple regenerants per explant that would be very useful for mass propagation of medicinal plants. So, there has been a significant increase in tissue culture of medicinal plants. Although medicinal plants can easily be found in the wild, they are generally subjected to some herbicides and attacked by some insects and pathogens. *In vitro* micropropagation of medicinal plants provides pesticide or disease-free plants and produces large numbers of vegetative planting stock. In addition, with an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year and uniform plant materials (less genetic diversity) can be produced that will be higher with seed germination (Turker et al., 2008).

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissues (Thorpe, 1994). There are three phases of organogenesis: dedifferentiation, induction, and differentiation (Christianson and Warnick, 1988). Dedifferentiation involves callus production and ends when cells become competent. During the induction phase, cells become determined and in the differentiation phase, cells form roots or shoots.

The first observation of controlled shoot formation *in vitro* was by White (1939) and root formation from callus was by Nobecourt(1939). Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced (Gurel and Turker, 2001).

Plants have been the best source of medicines and used as medicines for as long as human life has existed on earth (Balandrin et al., 1993). The World Health Organization forecasts that 80 percent of people in developing countries (65mpercent of Earth's population) still rely on folk medicine for their primary health care (Starbuck, 1999; Farnsworth et al., 1985). A total of 122 biologically active compounds have been identified and these compounds were derived from only 94 species of plants. A conservative estimate of the number of flowering plants occurring on the planet is 250.000. Of these, only about 6 % have been screened for biological activity, and a reported 15% have been evaluated phytochemically. There should be an abundance of drug sremaining to be discovered in these plants

(Fabricant and Farnsworth, 2001). Because of the host production of bioactive molecules, plants have been a rich source of medicines. Most of which probably evolved as chemical defenses against predation or infection (Cox and Balick, 1994). Bioassays are adaptable for the purpose of screening and testing plant extracts (McLaughlin et al., 1998).

Bioassays offer a special advantage in the standardization and quality control of heterogeneous botanical products. Such products can be "heterogeneous" due to the presence of mixture of bioactive components either from the same or from purposefully mixed botanical sources (McLaughlin et al., 1998). Screening studies for medicinal plants are important because folkloric usage of these plants gains some scientific justification.

The Kirby-Bauer Disc Diffusion Assay measures antimicrobial activity based on bacteriostatic/bacteriocidal properties. At the end of the 1950's, antimicrobial susceptibility testing was marked by lack of acceptable standardized procedures (Atlas, 1988). It was developed by Kirby and Bauer in the 1960's (Prescott et al., 1990). The Kirby-Bauer Disc Diffusion Assay (Barry and Thornsberry, 1985) has been accepted by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS). In this standardized assay, a culture is inoculated onto a Mueller-Hinton agar plate, followed by the addition of antibiotic impregnated discs to the agar surface. Inoculated plates are incubated at 37°C for 18-24 hours. At the end of the incubation period, a clear area (zone of inhibition) around the disc is measured and this area indicates the inhibition of microbial growth around the disc. These zones are compared to known values obtained with standard drugs (Prescott et al., 1990). Standardized zones for each antibiotic disc have been established to determine whether the microorganism is sensitive (S), intermediately sensitive (IS), or resistant (R) to the particular antibiotic. This method is not directly applicable to filamentous fungi, anaerobes, or slowly-growing bacteria (Atlas, 1988).

There is widely used method for the analysis of total phenolics. The Folin-Ciocalteau method has the advantage of a fairly equivalent response to different phenols. The Folin-Ciocalteau colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. Singleton and Rossi (1965) adapted this method to food phenolic analysis and has written two major reviews on its use (Singleton, 1974; Singleton et al., 1999). The products of the metal oxide

reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols (Waterhouse, 2002). The differential sensory effect of phenolics aside, a major advantage of the Folin-Ciocalteau procedure is that it has a fairly equivalent response to different phenolic substances. In general, the response of total phenol tests is comparable to antioxidant tests, with better correlations for antioxidant tests (Waterhouse, 2002). HPLC is becoming by far the most popular technique for the separation of phenolic compounds, both on preparative and analytical scales. Improvements in instrumentation, packing materials, and column technology are being introduced all the time, making the technique more and more attractive (Andersen and Markham, 2006).

1.1 Botany

Astragalus gymnolobus Fischer is a member of Fabaceae family and perennial endemic plant. It is a dwarf cushion forming shrub. The stem is erect, 5-30 cm long and bifurcate serious. Leaves are up to 15 cm and imparipinnate.Terminal leaflets are 5-15 mm long, triangular lanceolate, glabrous but ciliate both surfaces sparsely bifurcate serious.

The stipules are 5-10 mm long and triangular lanceolate and glabrous but ciliate. The peduncles are sparsely adpressed hairly and exceed leaves. The flowers is in a 6-18 flowered raceme, dense at first, elongating with age. Bracts are 4-10 mm long and linear lanceolate and bracteoles are 1-4 mm long and linear lanceolate.

The calyx is 8-11 mm long and tubular with very sparse, black and white adpressed bifurcate hairs. The corolla is white, cream or pale yellow color and 18-25 cm long. The legumes are 13-15 x 4-5 mm long and ovate oblong, glabrous, wrinkled and mucronate.

A. gymnolobus is found throughout the Northern and Southern part of Turkey and grows in Bolu, Kütahya, Afyon, Konya, Malatya, Niğde and Urfa (Davis, 1970).

Figure 1.1. Pictures of *Astragalus gymnolobus* Fischer (by Arzu Ucar Türker).

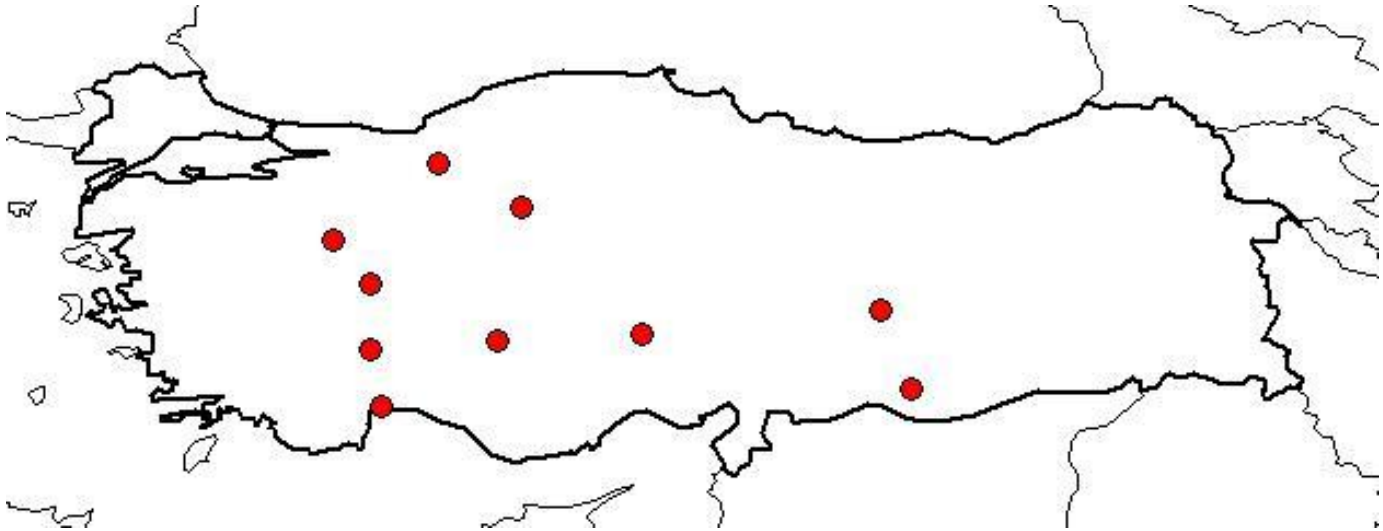

Figure 1.2. Map of the distribution of *Astragalus gymnolobus* in Turkey (http://www.tubives.com/index.php?sayfa=1&tax_id=2898).

1.2 Tissue Culture Studies of *Astragalus* spp.

There have been several plant regeneration studies in *Astragalus adsurgens* Pall. (Luo et al., 1999; Luo and Jia, 1997; 1998), *Astragalus cicer* L. (Uranbey et al, 2003; Başalma et al, 2007), *Astragalus melilotoides* Pall. (Hou and Jia, 2004a; 2004b), *Astragalus chrysochlorus* Boiss (Kara and Arı, 2008; Hasançebi et al, 2010), *Astragalus maximus* Willd (Kara and Arı, 2006), *Astragalus cariensis* Boiss (Erisen et al, 2009; Erisen et al, 2011), *Astragalus nezaketae* (Erisen et al, 2010) and *Astragalus schizopterus* (Yorgancılar and Erisen, 2011).

An efficient and reproducible procedure was established for the plant regeneration from hypocotyl explants and hypocotyl or stem-derived calli in *A. melilotoides* (Hou and Jia, 2004a). An other efficient and reproducible protocol was described for the regeneration of *A. melilotoides* Pall. protoplasts isolated from hypocotyl derived embryogenic callus (Hou and Jia, 2004b). A callus induction and plant regeneration protocol was developed from leaf and petiole explants of the endemic *A. nezaketae* (Erisen et al., 2010). Efficient plant regeneration through somatic embryogenesis was investigated in *A. adsurgens* (Luo et al., 1999). Efficient micropropagation and root culture protocols were developed for the endemic *A.chrysochlorus* (Hasançebi et al., 2010). Prolific shoot regeneration via organogenesis was investigated from leaf and leaf petiole explants of the endemic *A. cariensis* on Murashige and Skoog (MS) medium with NAA and BA within 8 week (Erisen et al., 2011). Regeneration protocol using TDZ with a high frequency *in vitro* root induction in *A. cicer* and high *in vitro* germination ratio (75%) for hard-seeds of *A. cicer* was also achieved (Başalma et al., 2007). A procedure has been developed for high frequency adventitious shoot regeneration from hypocotyls, cotyledon, stem and petiole explants of *A. cicer* (Uranbey et al., 2003). Yorgancılar and Erisen (2011) investigated the effect of TDZ on shoot regeneration of the endemic *A. schizopterus.* Effect of TDZ on callus formation and shoot regeneration from leaf and petiole explants of the endemic species *A. cariensis* Boiss was investigated (Erisen et al., 2009). *In vitro* plant regeneration was achieved from embryogenic cell suspension culture of *A. chrysochlorus* (Kara and Arı, 2008). A reproducible release of viable protoplasts was obtained from friable calli of *A.adsurgens* (Lou and Jia, 1998). An efficient procedure was developed for inducing callus and plant regeneration using

hypocotyl segments of *A. adsurgens* (Lou and Jia, 1997). Micropropagation of *A. maximus* Willd. through axillary bud culture was achieved (Kara and Arı, 2006).

1.3 Medicinal Usage of *Astragalus spp.*

Astragalus species are important and are used in many different way and areas in medicine due to their secondary metabolites (Tang, 1992; Karagöz et al., 2007). They are very old and well known curative plants with immunostimulant, hepatoprotective, antiperspirant, diuretic, and tonic properties (Tang and Eisenbrand, 1992). In Anatolia, an aqueous extract of *Astragalus* roots were traditionally used for treatment of leukemia and wound-healing (Bedir et al., 2001). *Astragalus* roots from various species have been used in the traditional Chinese medicine, as immunostimulant, hepatoprotector, antiperspirant, diuretic and for the treatment of nephritis, diabetes, leukemia, and cancer (Tang, 1992). This species is traditionally used for wound healing and also a crude ethanol extract of the roots has shown antioxidant, enhancement effects on the phagocytic activity of lymphocytes and cytotoxic activity (Karagöz et al., 2007).

1.4 Biological Activity and Constitutents of *Astragalus* spp.

Moderate antibacterial activities of some members of *Astragalus* spp. (*A. siculus, A. gummifer, A. membranaceus, A. malanophrurius* and *A. verrucosus*) were recorded against Gram-positive and Gram-negative bacteria (Pistelli et al., 2002; Bisignano et al.,1994). *Astragalus siculus* showed antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus faecalis, Proteus mirabilis, Citrobacter freundii, Pseudomonas aeruginosa* and *Klebsiella oxytoca* (Bisignano et al.,1994). *Astragalus membranaceus*, a commonly used Chinese medicinal plant, has been shown to be capable of restoring the impaired T cell functions in cancer patients (Cho and Leung, 2006). Lim et al (2011) showed that acetone extract of *Astragalus sinicus* seed might be a valuable antioxidant of natural source applicable in the medicine, cosmetics and the food industry.

Turker and Koyluoğlu (2011) showed that alcoholic extracts of *Astragalus gymnolobus* exhibited little inhibition against only *Streptococcus pyogenes* and no antitumor activity was observed with *A. gymnolobus*.

Astragalus brachypterus showed strong inhibition against only *S. pyogenes* (Turker and Yıldırım, 2013). Auyeng et al. (2009) showed that *Astragalus*

membranaceus has been used to ameliorate the side effects of antineoplastic drugs due to its immunomodulating nature and total *Astragalus* saponins (AST) possess anticarcinogenic and proapoptotic properties in human colon cancer cells and tumor xenograft.

Cytotoxic activity of *Astragalus chrysochlorus* crude extracts (hexane, chloroform, ethylacetate, 80 % ethanol and water extracts prepared from roots and stems) were tested for cytotoxic activity on Vero (V) cells using the MTT assay. The hexane-root and water-stem extracts of *A. chrysochlorus* were not cytotoxic at 500 µg/ml. Both the ethanol-stem and water-root extracts exhibited weak cytotoxic activity. The hexane-stem, chloroform-root and stem, ethylacetate root and stem or ethanol-root extracts showed stronger cytotoxic activity than the others. However, the chloroform-root extract exhibited the most effective cytotoxic activity at 500 µg/ml (70.3 %) (Karagöz et al., 2007).

Yan et al. (2005) showed that *Astragalus mongholicus* contains lectin that is known to bind to cancer cell membranes or receptors thereby and causing cytotoxicity, apoptosis and inhibition of tumor growth (de Mejıa and Prisecaru, 2005). Yan et al. (2010) showed that administration of *Astragalus mongholicus* polysaccharides could significantly increase serum and liver antioxidant enzyme activitiesin mice and decrease peroxidative lipid levels and *A.mongholicus* polysaccharides may offer good protection against oxidative stress.

Astragalus membranaceus has been widely used as food material as well as medicinal ingredients for therapeutic effects and therefore, these suggest that the extracts of *A. membranaceus* can be used as a data to develop more efficient material in functional food (Kim et al., 2009).

Turker et al. (2009) reported that *Aeromonas hydrophila* was the only inhibited bacteria from all alcoholic and aqueous extracts *of A. gymnolobus.*

Methanol extracts of antioxidant activities of four wild type *Astragalus* species namely, *A. microcephalus, A. lagurus, A. macrocephalus* and *A. galegiformis* have been investigated and extract obtained from the roots of *A. macrocephalus* exhibited the strongest antioxidant properties among the studied *Astragalus* species (Sokmen et al., 2009).

Adıgüzel et al. (2009) investigated that antimicrobial activities of the hexane and methanol extracts of the above ground parts and roots of some *Astragalus* species (*Astragalus ponticus*, *A. aduncus* Willd, *A. microcephalus*

Willd, *A. pinetorum* Boiss, *A. atrocarpus* Champ. & Matthews, *A. lagurus* Willd, *A. macrocephalus* Willd, *A. erinaceus* Fisch. & Mey. ex Fischer, *A. galegiformes* L, *A. trichostigma* Bunge, *A. bicolor* Lam, *A. psoraloides* Lam, *A. argyroides* Becker ex Stapf) grown in the vicinity of Erzurum, Turkey. Antibacterial and cytotoxic activities of extracts from the leaves of wild *Astragalus gombiformis* Pomel was investigated (Teyeb et al., 2010). Pistelli et al. (2002) investigated the antimicrobial and antifungal properties of several crude extracts and pure saponins, astraverrucins I-VI, from the aerial parts of *Astragalus verrucosus* and antimycotic activity was observed on more polar extracts, but only one saponin showed an appreciable activity.

Alrumman et al. (2012) reported that leaf extracts of *Astragalus atropilosulus* subsp. *abyssinicus* could be source of compounds, which can be used to combat pathogenic microorganisms. Extracts of aerial parts and roots of wild *Astragalus gombiformis* Pomel were tested for their antibacterial, antioxidant and contents of phenolic compounds. Among the tested extracts, three extracts (methanol, chloroform, and ethyl acetate) from aerial parts and two extracts (water, methanol) from roots exhibited diameters of inhibition zone equal or above 12 mm (at 150 µ/disk) and minimum inhibitor concentrations ranging between 233 and 1250 µ/ml. Spectrophotometric and HPLC analyses showed that contents of both total polyphenols and flavonoids, as well as antioxidant activity were higher in the methanolic extract of aerial parts as compared to roots (Teyeb et al., 2012).

The methanol extracts of antioxidant capacity of the flowering aerial parts of *Astragalus squarrosus* was determined by DPPH radical scavenging and ferric thiocyanate methods and the phenolic and flavonoid content was also measured. *A. squarrosus* showed weak free radical scavenging activity with the DPPH method which might be due to its low phenolic constituents (23.3 mg/g) and flavonoid content (26.0 mg/g) (Asgarpanah et al.,n2011). Aqueous extracts of *Astragalus membranaceus* (AMEE) were prepared and *in vitro* antioxidant and antitumour activities of aqueous extracts of *A. membranaceus* were evaluated and the results indicated that the aqueous extracts of *A. membranaceus* have a very potent antioxidant and antitumour activity (Peng et al., 2011).

Antibacterial bioassay guided fractionation of acetone extracts of *Astragalus brachystachys* was investigated in isolation of sclareol and two related

labdane-type diterpenoids, 14R-epoxysclareol and 6β-hydroxysclareol (Jassbi et al., 2002).

From the aerial parts of *Astragalus verrucosus* Moris, a novel cycloartane-type triterpeneglycoside, named astraverrucin VII, was isolated along with cycloaraloside D (peregrinosideII) and cycloaraloside C (astrailienin A) and fifteen known flavonoids, were isolated and identified. All structural elucidation was performed by spectral means (Pistelli et al., 2003). Nine flavonoids were identified in aerial parts of *Astragalus corniculatus* by liquid chromatography coupled with ionspray mass spectrometry in the tandem mode (LC/MS/MS) with negative ion detection (Krasteva and Nikolov, 2008). From the roots of *Astragalus melanophrurius* eight known saponins were isolated. Based on spectral data, the structures were established as astrasieversianins II and X, astragalosides I, II, IV and VI, and cyclocanthosides E and G (Çalış et al., 1997). *Astragalus membranaceus* (Fisch.) Bunge was investigated for the content of isoflavones and some other polyphenolic compounds in roots and aerial parts by means of TLC and HPLC. The total amount of isoflavones in leaves, was 0.55 mg/g dry weight, and of the flavonols up to 3.54 mg/g. In the roots isoflavonoid content was extremely variable, but reached 3.04 mg/g, whereas flavonols content was 0.49 mg/g (Matkowski et al., 2003).

2. AIM AND SCOPE OF THE STUDY

Astragalus spp. is important and used in many different ways and areas in medicine due to their secondary metabolites. The main objectives of this study were a) to obtain an *in vitro* culture protocol for *A. gymnolobus* using plant tissue culture methods; b) to screen and compare the biological activities of field-grown leaves and *in vitro*-grown callus of *A. gymnolobus* using specific bioassays (antibacterial and antioxidant); c) to analyze the phenolic content of field-grown leaves and *in vitro*-grown callus of *A.gymnolobus* by means of HPLC. *In vitro* culture protocols provide multiple regenerants per explant that would be very useful for mass propagation of medicinal plants. So, there has been a significant increase in tissue culture of medicinal plants. *A. gymnolobus* is a valuable medicinal herb, but there are no reports on an *in vitro* culture protocol of this species. The present study describes, to our knowledge for the first time, an efficient *in vitro* plant regeneration protocol for *A.gymnolobus* via adventitious shoot development from leaf and petiole explants cultured on medium containing different concentrations and combinations of various plant growth regulators.

Astragalus species have been used as antibacterial, antiviral, antifungal, antioxidant, anticancer, hepatoprotective, wound healing, anti-diabetic and diuretic in folk medicine. The objective of this study was a) to evaluate the antibacterial and antioxidant activities of *in vitro*-grown callus and field-grown leaves of *A. gymnolobus*; b) to determine the phenolic constituents of *in vitro*-grown callus and field-grown leaves of *A. gymnolobus* by means of HPLC analysis.

3. MATERIALS AND METHODS

3.1. Plant Material and Extraction

3.1.1. *In vitro* culture of *Astragalus gymnolobus*

Seeds of *Astragalus gymnolobus* were collected from Abant Lake, Bolu/Turkey in September of 2012. Identification of the species was made by using "Flora of Turkey and The East Aegean Island" (Davis, 1970) and voucher specimens (AUT-2001) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Seeds were washed with an anti-bacterial soap, rinsed with distilled water and surface sterilized by shaking in 70% ethanol for 10 minutes followed by rinsing well with sterilized distilled water and then dipped into 20% Domestos® (5% sodium hypochloride) for 10 minutes. They were finally washed with sterile distilled water three times. After surface sterilization of the seeds, seeds were placed in sterile, disposable petri dishes containing Murashige and Skoog (MS) medium (4.43 g/l MS, Sigma Chemical Co., St. Louis, MO, USA; Murashige and Skoog1962) with 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121°C and 105 kPa). After a one week incubation in this medium, seedlings were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing the same medium for an additional three weeks. For shoot regeneration, leaf and petiole were excised from four week old sterile seedlings and placed in sterile disposable petri plates containing 4.43 g/l MS with different combinations and concentrations of plant growth regulators; thidiazuron (TDZ; 0.01, 0.05,0.1 and 0.5 mg/l) + indole-3-acetic acid (IAA; 0.1, 0.25, 0.5 and 1 mg/l); TDZ (0.5 and 1 mg/l) + 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 and 1 mg/l); TDZ (0.5 and 1 mg/l) + 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 and 1 mg/l) + gibberellic acid (GA3; 0.5) ;TDZ (0.5 and 1 mg/l) + indole-3-butyric acid (IBA; 0.5 and 1 mg/l); benzyladenine (BA; 0.5, 1, 3 and 5 mg/l) + IAA (0.1, 0.25, 0.5 and 1 mg/l); BA (1.0, 2 and 4 mg/l) + naphthalene acetic acid (NAA; 0.2, 0.5 and 1mg/l); BA (0.5 and 1 mg/l) + IBA (0.5 and 1 mg/l); BA (0.5, 1 and 3 mg/l) + 2,4-D (0, 0,1 and 0,5 mg/l); Kinetin (KIN; 0.5, 1 and 3mg/l) + IAA (0.1, 0.5 and 1 mg/l); KIN (0.5, 1 and 3 mg/l) + 2,4-D (0.1 and 0.5mg/l); KIN (0.5, 1 and 3 mg/l) + NAA (0.5 and 1 mg/l). GA3 was used after

filter sterilization. All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 μmolm^{-2}s^{-1}).

After two months, regenerated explants were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MS for shoot elongation for an additional two weeks. Shoots were then separated individually and placed in rooting medium containing different concentrations of sucrose, MS and different auxins; NAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), IAA (0.5, 1 and 3 mg/l), 2,4-D (0.1, 0.5 and 1 mg/l), ½ MS + NAA (0.5, 1 and 3 mg/l), ½ MS + IBA (0.5, 1 and 3 mg/l), ½ MS + IAA (0.5, 1 and 3 mg/l), ½MS + 2,4-D (0.1, 0.5 and 1 mg/l), ½ MS + ½ sucrose + NAA (0.5, 1 and 3 mg/l), ½ MS +½ sucrose + IBA (0.5, 1 and 3 mg/l), ½ MS + ½ sucrose + IAA (0.5, 1 and 3 mg/l), ½ MS + ½ sucrose + 2,4-D (0.1, 0.5 and 1 mg/l), 0.5 GA3+ NAA (0.5, 1 and 3 mg/l), 0.5 GA3 + IBA (0.5, 1 and 3 mg/l), 0.5 GA3 + IAA (0.5, 1 and 3 mg/l), 0.5 GA3 + 2,4-D (0.1, 0.5 and 1 mg/l), ascorbic acid + NAA (0.5, 1 and 3 mg/l), ascorbic acid + IBA (0.5, 1 and 3 mg/l), ascorbic acid + IAA (0.5, 1 and 3 mg/l), ascorbic acid + 2,4-D (0.1, 0.5 and 1 mg/l), ½ MS + ½ sucrose + activated charcoal (0.5, 1 and 2 mg/l).

Leaf explants were also transferred to MS medium containing BA (0.5, 1 and 3 mg/l) + 2,4-D (0.1 and 0.5 mg/l) and TDZ (0.5 and 1 mg/l) + 2,4-D (0.5 and 1 mg/l) for callus formation.

Finally, all data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc., Chicago, IL, USA).

3.1.2 Biological activity and constituents of *Astragalus gymnolobus*

Two different sources of plant (field-grown leaves and *in vitro*-grown callus) were used for extractions. Leaves of *A. gymnolobus* (field-grown plants) were collected from Abant Lake, Bolu/Turkey in May of 2014. Identification of the species was made by using "Flora of Turkey and the East Aegean Islands" (Davis, 1972) and voucher specimens (AUT-2001) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Calluses were obtained from *in vitro* cultured *A. gymnolobus* leaves. Collected leaves and calluses were dried in oven at 45 °C and then ground into a powder. Plant materials were extracted with methanol (MeOH) and water. 20 grams from each plant sample were extracted with 300 ml

water and methanol at 45 °C for 12 hours and then filtered. For water extraction, frozen filtrate was lyophilized by using freeze-dryer at -65 °C and for methanol extractions; they were evaporated under vacuum using rotary evaporator at 60 °C. Each residue was then dissolved in sterile distilled water to produce a final concentration of 100 mg/ml (Table 3.1).

3.1.2.1 Antibacterial Assay

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott et al., 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC® 25922), *Pseudomonas aeruginosa* (ATCC® 27853), *Salmonella typhimurium* (ATCC® 14028), *Serratia marcescens* (ATCC® 8100), *Proteus vulgaris* (ATCC® 13315), *Enterobacter cloacae* (ATCC® 23355) and *Klebsiella pneumoniae* (ATTC®13883) which are Gram-Negative bacteria and *Streptococcus pyogenes* (ATTC® 19615), *Staphylococcus aureus* (ATTC® 25923), *Staphylococcus epidermidis* (ATCC® 12228) which are Gram-Positive bacteria (Table 3.2). BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated for 3 hr at 37°C. After 3 hr, one bacteriological loop from each broth was streaked on Tryptic Soy Agar (TSA) plates and incubated for 2 days at 37°C. After 2 days, a single colony was removed and streaked on a new TSA plate for pure culture and incubated 37°C for 2 additional days. The turbidity of each broth culture was adjusted with saline to obtain turbidity visually comparable to that of a 0.5 McFarland standard. A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface of a (60 X 15 mm) Mueller-Hinton (M-H) agar plate. Agar plates were streaked three times, each time turning the plate at a 60° angle and finally rubbing the swab through the edge of the plate. All extracts were sterilized by filtering through a 0.22 µm filter (Pal-Gelman Laboratory) and sterile filter paper discs (Glass Microfibre filters, Whatman®; 6 mm in diameter) were impregnated with 50 µl of extract. There were five replicates in each plate and two plates for each extract tested for each bacterium. Positive controls consisted of five different antimicrobial susceptibility test discs (Bioanalyse®): Erythromycin (15 µg) (E-15), Ampicillin (10 µg) (AM-10), Carbenicillin (100µg) (CB-100), Tetracycline (30 µg) (TE-30) and Chloramphenicol (30 µg) (C-30). Four

antibiotic discs were used for each plate and run in duplicate. Negative control consisted of water. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hrs of incubation, inhibition zone diameter (mm) was measured. All experiments were repeated two times. All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc., Chicago, IL, USA).

Table 3.1. Designation of extracts in bioassays and their extraction procedures.

Test Materials	Designations	Materials in Treatment	Extraction Procedure	Yield (%)
Water extracts	**AW**	Field-grown leaves	20 g field-grown leaves were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	25
	IW	In vitro- grown callus	20 g in vitro -grown leaf callus were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	33
Methanol extracts	**AM**	Field-grown leaves	20 g field-grown leaves were extracted with methanol at 45 ºC for 12 hr. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	32
	IM	In vitro- grown callus	20 g in vitro -grown leaf callus were extracted with methanol at 45 ºC for 12 hr. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	10

Table 3.2. Bacteria used in antibacterial assay and their representative diseases (Levinson and Jawertz, 2002).

Pathogen	Diseases	Habitat	Treatment
Escherichia Coli	Urinary tract infection (UTI), sepsis, neonatal menengitis, "traveler's diarrhea'	Human colon, colonize the vagina and urethra.	Ampicilin or sulfonamides for UTI, Cephalosporin for menengitis and sepsis, trimethoprimsulfamethoxazole for diarrhea
Proteus Vulgaris	UTI and sepsis	Human colon and environment (soil and water)	Trimethoprimsulfamethoxazole or ampicillin, cephalosporin for serious infections.
Enterobacter Cloacae	Hospital-acquired pneumonia, UTI, and sepsis.	Enteric tract	Higly antibiotic-resistant.
Salmonella Typhimurium	Enterocolitis, diarrhea	Enteric tract	Ampicillin, ciprofloxacin or ceftriaxone
Serratia Marcescens	Nosocomial infections and hospital-acquired pneumonia, UTI, and sepsis	Enteric tract also soil and water	Gentamicine and cephalosporin
Klebsiella Pneumoniae	Pneumonia, UTI, and sepsis.	Human upper respiratory and enteric tracts.	Cephalosporin or with aminoglycosides
Pseudomonas Aeruginosa	Wound infections, UTI, pneumonia and sepsis.	Environmental water sources, hospital respirators and humidifiers and also skin, upper respiratory tract and colon	Antipseudomonal penicilin and aminoglycoside eg, gentamicin or amikacin
Streptococcus Pyogenes	Suppurative (pusproducing) diseases,cellulitis, immunologic diseases such as rheumatic fever and acute glomerulonephritis	Human throat and skin	Penicilin G
Staphylococcus Aureus	Abscesses of many organs,endocarditis, gastroenteritis, toxic shock syndrome, sepsis, hospital acquired pneumonia	Human nose and also human skin.	Penicilin G, Nafcillin
Staphylococcus Epidermidis	Endocarditis, neonatal sepsis, prosthetic hip infection	Human skin and mucous membranes.	Vancomycin plus either rifampin or an aminoglycoside.

3.1.2.2 Antioxidant Assay

3.1.2.2.1 Free Radical Scavenging Activity-DPPH (2,2-diphenyl-1-picrylhydrazil) Method

DPPH is a free radical and when it was dissolved in methanol, it has a blue-violet color. The solution loses color which depends upon the number of electrons taken up. Hence, the loss of color indicates radical scavenging activity of test material (Angayarkanni et al., 2010). DPPH free radical scavenging activities of the extracts were determined according to the method described by Brand-Williams et al. (1995) with some modifications such as 80% methanol instead of methanol and molarity of DPPH concentration ($1.5x10^{-5}$ M instead of $6x10^{-5}$M) (Cai etal., 2003). The free radical-scavenging activities of the samples were measured in terms of hydrogen donating or radical-scavenging ability to the stable radical DPPH, as a reagent. The samples of extracts were weighted as 0.003 g (AM and IM) and they were dissolved in 3 ml of 80 % methanol. Finally, this stock solution was prepared as 1000 µl/ml concentration. The extract solutions were diluted with 80% methanol (25, 50, 100, 200 µl/ml) from the stock extract solutions. 1.5 ml of the diluted samples was mixed vigorously with 0.5 ml of $1.5x10^{-5}$ M DPPH in 80% methanol. After 30 min at room temperature in the dark, absorbance was measured against blank (methanol of 80%) at 517 nm with Hitachi U-1900, UV-VIS Spectrophotometer 200V. The solution which includes only 1.5 ml of the 80% methanol and 0.5 ml of the $1.5x10^{-5}$ M DPPH in 80% methanol were positive control (Milardovic et al., 2006; Brand-Williams et al., 1995). All analyses were made in triplicate. The capability of *A. gymnolobus* samples to scavenge the DPPH· radical was calculated using the following equation: DPPH· Scavenging Effect (%) (% inhibition) = [(A0−A1/A0) x 100] (Gülçin et al., 2003) where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample of *A. gymnolobus* extracts.

3.1.2.2.2 Total Phenolic Assay (Folin-Ciocalteau Method for Total Phenolic Content)

Preparation of extract solution (stock solution): 2 ml of distilled water was added to 0.01 g plant extracts of *A. gymnolobus* (AM and IM). Prepared stock

solution was then diluted to 1mg/ml. Gallic acid stock solution: In a 100 ml volumetric flask, 0.5 g of dry gallic acid was dissolved in 10 ml of pure methanol and was diluted to required volume with water. This stock solution was used as phenol control. The accepted standard is gallic acid (Figure 3.1). It is a particularly good standard because it is relatively inexpensive in pure form and is stable in its dry form (Waterhouse, 2002). Sodium Carbonate (Na_2CO_3) Solution: 20 g of anhydrous sodium carbonate was dissolved in 100 ml of water in a 100 ml volumetric flask. To prepare a calibration curve: The phenol concentrations of 0, 25, 50, 100, 150 and 200mg/l gallic acid was prepared. 20 µl from each calibration solution, sample, or blank was placed into separate cuvettes. 1.58 ml water and 100 µl Folin-Ciocalteu reagent (Sigma®) was added to each, and then mixed well. After 2 minutes, 300 µl Na_2CO_3 solution was added and was shaken very well. The solutions were incubated at 200 C for 2 hours and measured the absorbance of each solutions at 765 nm against the blank (the "0 ml" solution) using the spectrophotometer. The absorbance vs. concentration was plotted. After the created calibration curve with gallic acid (Figure 3.1), phenol levels in the samples were determined. The results were reported at Gallic Acid Equivalent, GAE. The phenol content was expressed as GAE calibrated (Jeong et al., 2010). The total phenol content of samples of *A. gymnolobus* was expressed as mg gallic acid equivalents (GAE)/100g dried mass. All analyses were made in triplicate.

3.1.2.2.3 Total Flavonoid Assay-Aluminum Chloride ($AlCl_3$) Colorimetric Assay for Total Flavonoids

The amount of total flavonoid was measured by aluminum chloride ($AlCl_3$) colorimetric assay. Catechol was used as a reference flavonoid (Figure 3.2). Preparation of catechol solution: 0.0125 g catechol was dissolved in 25 ml of 80% ethanol (EtOH) and this stock solution was adjusted to concentration as 500 mg/ml. In order to obtain calibration curve of catechol, 20, 40, 60, 80 and 100 mg/ml concentrations were prepared (Figure 3.2). Preparation of extract solution: 0.005 g of each *A. gymnolobus* extracts was dissolved in 2 ml of 80% ethanol (2500 mg/ml). Later, this stock solution was diluted to 1250 mg/ml and 500 mg/ml concentrations. 500 µl of extract solution or standard solution of catechol was added to a 10 ml test tube containing 2 ml distilled water. At zero time, 150 µl 5% $NaNO_2$ was added to

the test tubes. After 5 min, 150 µl of 10 % $AlCl_3$ was added. At 6 min, 1000 µl of 1M NaOH was added to the mixture. Immediately, the reaction tube was diluted to volume 5 ml with the addition of 1200 µl distilled water and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm versus a blank. Samples were analyzed in three replications (Marinova et al., 2005; Chang et al., 2002).

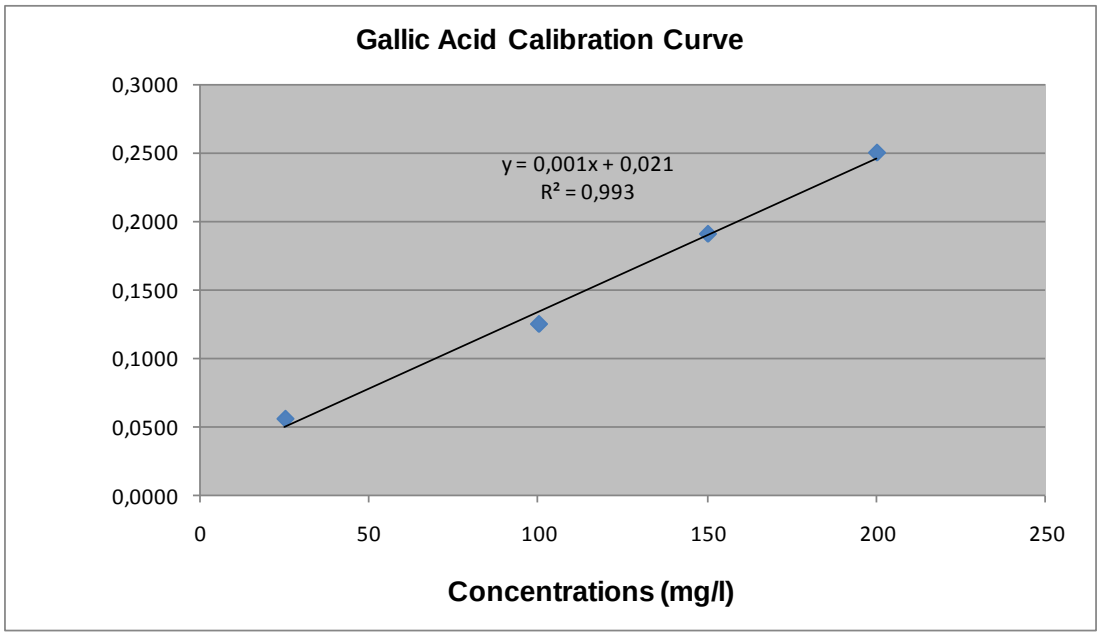

Figure 3.1. Gallic acid calibration curve.

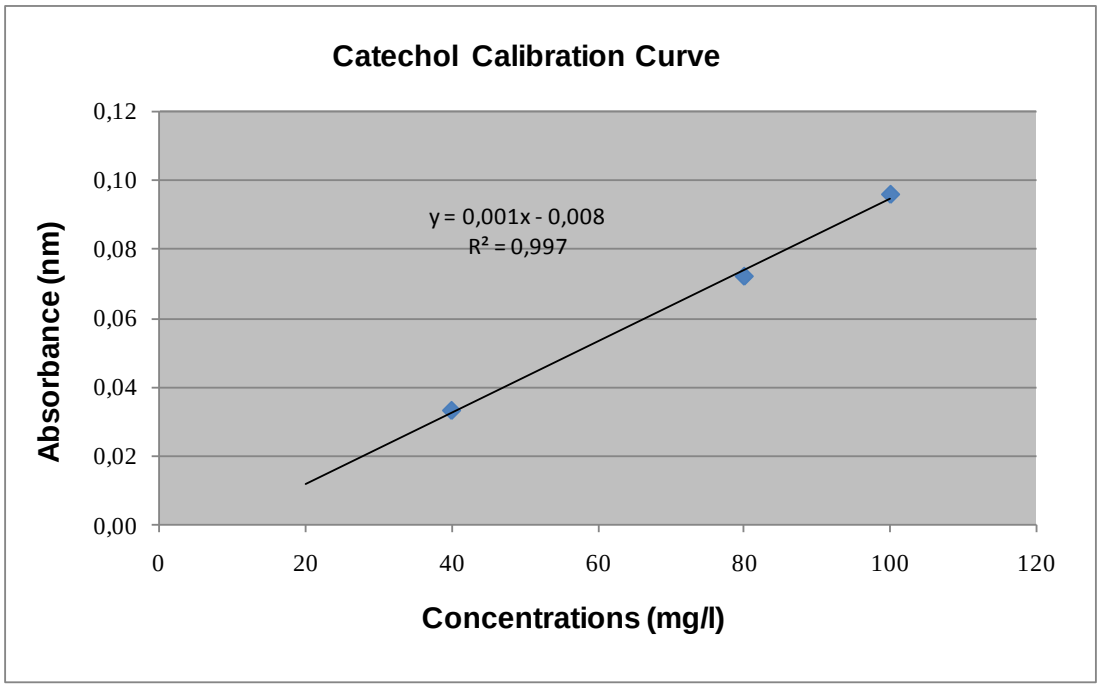

Figure 3.2. Catechol calibration curve.

3.1.2.3 HPLC-DAD Analysis of Phenolic Compounds

Methanolic extracts were analyzed using a HPLC system (VWR-Hitachi LaChrom Elite®) equipped with a Hitachi L-2455 Diode-Array Detector (DAD), Hitachi L-2130 Pump, Hitachi L-2200 Autosampler. Chromatographic seperation was achieved using Hitachi column oven L-2300 and Venusil XBP C18 column (Bonna-Agela Technologies, particle size 5 µm, 4.6 x 250 mm). Flow rate was 1ml/min with 25 ºC oven and injection volume was 20 µl. All solvents were HPLC grade (Merck) and mobile phased was composed of solvent (A) acetonitrile (ACN) and solvent (B) 0.1% acetic acid. A gradient elution was used as shown in Table 3.3. Mobile phases and ultrapure water (SG Labostar) were filtered through a 0.45 µm hydrophilic polypropylene membrane filter (47 mm) (Pall Corporation) prior to HPLC injection. Spectra data were recorded from to 200 to 400 nm during the entire run. The chromatograms were obtained at 280 nm.

3.1.2.3.1 Sample Preparation for HPLC Analysis

MeOH extracts (10 mg) were dissolved in 1 ml MeOH (10000 mg/l). Gallic acid monohydrate, caffeic acid, rutin hydrate, luteolin-7-O-β-D glucoside, kaempferol, myricetin, quercetin, coumarin, rosmarinic acid and apigenin were used as reference standarts (Sigma®). All standards were prepared at 1 mg/ml in MeOH and mixed together to make five different concentrations (1, 5, 10, 20, 40, 60, 80 and 100 mg/l) to generate the standard curve. HPLC analyzes of extracts were performed with or without 10 phenolic standards. All extracts and standards were filtered through a 0.2 µm GHP Acrodisc (25 mm) (Pall Corporation) into 2 ml HPLC vials. Procedures were repeated 3 times for each sample tested.

Table 3.3. HPLC solvent system, (A) Acetonitrile (ACN) and (B) 0.1% Acetic acid.

	Solvent (%)	
Time(min)	A	B
0.0	10	90
5.0	20	80
10.0	40	60
15.0	60	40
20.0	80	20
20.01	10	90
25.0	10	90

4. RESULTS AND DISCUSSIONS

4.1 *In vitro* culture of *Astragalus gymnolobus*

Although *Astragalus* species have valuable medicinal properties, there is no study about *in vitro* propagation of *Astragalus gymnolobus*. We therefore aimed to develop an *in vitro* culture protocol for high frequency regeneration of *A. gymnolobus* by adventitious organogenesis.

Two different explants (leaf and petiole) were used for experiment and were excised from 4 or 5-weeks-old sterile seedlings and cultured on MS medium containing BA in combination with IAA, IBA, NAA and 2,4-D; TDZ in combination with IAA, IBA, 2-4-D and 2-4-D+ 0.5 GA_3; KIN in combination with IAA, NAA and 2,4-D. Leaf explants formed more shoots than petiole explants (Table 4.1; Figure 4.1 and 4.2). TDZ was used in combination with IAA, IBA, 2,4-D and 2,4-D + 0.5 GA_3 (Table 4.1 and 4.2). Greater shoot regeneration was observed in all leaf explants with TDZ in combination with IAA (Table 4.1; Figure 4.1 and 4.2). BA or KIN in combination with different auxins were not effective for shoot regeneration with all two explants (Table 4.3). Shoot development was not observed with the addition of 0.5 mg/l GA_3 into 0.5 TDZ and 1 mg/l TDZ+ 0.5, 1 mg/l 2,4-D (Table 4.2).

When TDZ were used alone at different concentrations (0.01, 0.05, 0.1and 0.5 mg/l) on the leaf explants, the best shoot formation was obtained with 0.5 mg/l TDZ (17.60 ± 8.17 shoots per shooted explant; 23.81 % explants formed shoots). But, 0.05 mg/l TDZ (12.57 ± 2.60 shoots per shooted explant; 31.82 % explants formed shoots) and 0.1 mg/l TDZ (12.43 ± 1.23 shoots per shooted explant; 30.43 % explants formed shoots) were more successful in terms of shoot frequency (Table 4.1; Figure 4.3 and 4.4). When TDZ were used alone at 0.01 mg/l for leaf explants, shoot development was not observed. When 0.05 mg/l TDZ were combined with 0.1 mg/l IAA, reduction was observed in terms of the mean number of shoots per shooted leaf explant (6.11 ± 1.32 shoots per shooted explant, 37.50 % explants formed shoots). Addition of 0.25 mg/l IAA into 0.05 mg/l TDZ caused no shoot formation. When 0.5 mg/l IAA were combined with 0.05 mg/l TDZ, significant

increased were observed in terms of the mean number of shoots per shooted leaf explant from zero to 6.50 ± 1.43 shoots per shooted explant (Table 4.1).

Shoot development was not observed with the addition of 0.1 mg/l IAA into 0.1 mg/l TDZ with leaf explants. If IAA concentration was increased from 0.1 to 0.25 mg/l substantially, shoot number increased from 0 to 10.50 ± 2.40 for leaf explant. If IAA concentration was increased from 0.25 to 0.5 mg/l substantially, shoot number increased for leaf explant from 10.50 ± 2.40 to 12.80 ± 4.82. When TDZ was used alone at 0.5 mg/l on the leaf explants, the shoot formation was increased (17.60 ± 8.17 shoots per shooted explant; 23.81% explants formed shoots). If 0.5 mg/l TDZ were combined with 0.1 mg/l IAA, decline was observed in terms of the mean number of shoots per shooted leaf explant from 17.60 ± 8.17 shoots to 12.75 ± 2.56 shoots. If IAA concentration was increased from 0.1 to 0.25 mg/l substantially, shoot number significantly decreased and shoot development was not observed. When IAA concentration was increased from 0.25 to 0.5 mg/l substantially, shoot number significantly increased from 0 to 10.00 ± 1.00 (Table 4.1).

Petiole explants gave the best shoot proliferation with media containing 0.5 mg/l TDZ + 0.1 mg/l IAA (5.00 ± 1.73 shoots per shooted explant; 20.00 % explants formed shoots) (Table 4.1; Figure 4.2). Generally petiole explant was not effective for shoot regeneration (Table 4.1, 4.2, 4.3 and 4.4).

Callus was observed for leaf and petiole explants before shoot formation (indirect organogenesis). In control experiments (no plant growth regulators added to media) none of the explants formed shoots.

The promoting effect of TDZ on *in vitro* development has been lately reported for many species (Ahn et al., 2007; Jones et al., 2007; Wang and Bao 2007; Yucesan et al., 2007). TDZ was more successful for leaf and petiole explant than BA or KIN for shoot formation in our study. Generally, with leaf explants in our study, higher concentrations of TDZ were effective for shoot regeneration (0.05, 0.1 and 0.5 mg/l) and the best shoot formation was obtained with 0.5 mg/l TDZ.

Yorgancılar and Erisen (2011) reported the effect of thidiazuron (TDZ) on shoot regeneration of the endemic *Astragalus schizopterus.* Explants (leaf and petiole) were cultured on the MS medium containing various plant growth regulators to induce shoot bud induction. The highest number of shoots was obtained on MS medium containing 1 mg/l TDZ followed by MS medium containing 1 mg/l BA. It was observed that shoots didn't have healthy development in all media containing

TDZ (Yorgancılar and Erişen, 2011). Erisen et al. (2009) reported that the highest number of shoots from leaf and petiole explants of the endemic *Astragalus cariensis* species was obtained from leaf explants cultured on MS medium with 0.5 mg/l NAA and 4 mg/l BA.

Regenerated shoots were separated individually and put on rooting medium containing IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0,01, 0,5 and 1 mg/l) and NAA (0.05, 1 and 3 mg/l). But, root formation was not observed with these treatments (Table 4.5). Further treatments were tried for root formation [½ MS + IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0,01, 0,5 and 1 mg/l) or NAA (0.05, 1 and 3 mg/l); ½ MS + ½ sucrose + IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0,01, 0,5 and 1 mg/l) or NAA (0.05, 1 and 3 mg/l); 0.5 mg/l ascorbic acid + IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), NAA (0.05, 1 and 3 mg/l) and 2,4-D (0,01, 0,5 and 1 mg/l); activated charcoal (0.5, 1 and 2 mg/l) + IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0,01, 0,5 and 1 mg/l) or NAA (0.05, 1 and 3 mg/l); 0.5 GA_3 + IAA (0.5, 1 and 3 mg/l), IBA(0.5, 1 and 3 mg/l), 2,4-D (0,01, 0,5 and 1 mg/l) or NAA (0.05, 1 and 3 mg/l)]. Unfortunately, root development was not achieved after these much trials (Table 4.5).

Calluses were collected from leaf explants for biological activity studies. More callus formation was obtained with TDZ (0.5 and 1 mg/l) + 2,4-D (0.5 and 1 mg/l) and BA (0.5, 1 and 3 mg/l) + 2,4-D (0.1 and 0.5 mg/l) combinations than other tested PGR combinations (Figure 4.5, 4.6 and 4.7). When TDZ was used alone at different concentrations (0.5 and 1 mg/l) on the leaf explants, callus formation was not observed. Effect of 2,4-D on callus formation is the greatest and if 2,4-D concentration was increased from 0.1 to 0.5 mg/l, leaf explants produced greater amount of callus. Best callus formation was obtained with MS medium containing 3.0 mg/l BA and 0.5 mg/l 2,4-D (Figure 4.5 and 4.6).

Erisen et al. (2011) studied for the regeneration of *Astragalus cariensis* Boiss and reported that the highest number of shoots was obtained from leaf explants on MS medium containing 0.4 mg/l TDZ and 0.2 mg/l NAA. Regenerated shoots were rooted in MS medium containing 0.5 mg/l IBA. But, root formation was not observed in our study.

Başalma et al. (2007) showed that the highest frequency of shoot regeneration (53.3%) of *Astragalus cicer* L.was achieved from hypocotyl segments through an initial callus growth stage on MS medium containing 0.25 mg/l TDZ. Shoots were

cultured on the different strength (1/1, 3/4, 1/2 and 1/4) of basal Murashige and Skoog medium containing different concentrations of NAA and high rooting (100%) and survival (100%) were achieved using half strength MS medium supplemented with 0.25 and 0.50 mg/l NAA.

Erisen et al. (2010) developed callus induction and plant regeneration protocol from leaf and petiole explants of endemic *Astragalus nezaketae* on media containing 0.5 mg/l NAA and 4 mg/l BA and the regenerated shoots transferred to rooting medium (MS with 0.5 mg/l IBA) were successfully rooted (100%).

Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Schwarz and Beaty, 1996). Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success. In our study, when leaf and petiole explants were compared, leaf explants were much more productive for shoot formation than petiole explants with all plant growth hormone combinations and concentrations.

Table 4.1. Shoot regeneration from leaf and petiole explants cultured on MS medium containing different concentrations and combinations of TDZ with IAA. Mean values with the same letters within vertical columns are not significantly different (P>0.05).

		EXPLANTS			
Treatments		**Leaf**		**Petiole**	
		Mean # of shoots per shooted explant	**% explants forming shoots**	**Mean # of shoots per shooted explant**	**% explants forming shoots**
Contol (No PGR)		**0**	**0**	**0**	**0**
TDZ (mg/l)	**IAA (mg/l)**				
0.01	0	0	0	0	0
0.01	0.1	0	0	0	0
0.01	0.25	0	0	0	0
0.01	0.5	10.75 ± 1.89^{ab}	19.05	0	0
0.05	0	12.57 ± 2.60^{ab}	31.82	0	0
0.05	0.1	6.11 ± 1.32^{b}	37.50	0.67 ± 0.67^{b}	5.88
0.05	0.25	0	0	0	0
0.05	0.5	6.50 ± 1.43^{b}	26.08	0	0
0.1	0	12.43 ± 1.23^{ab}	30.43	0	0
0.1	0.1	0	0	0	0
0.1	0.25	10.50 ± 2.40^{ab}	18.18	0	0
0.1	0.5	12.80 ± 4.82^{ab}	20.83	0	0
0.5	0	17.60 ± 8.17^{a}	23.81	0	0
0.5	0.1	12.75 ± 2.56^{ab}	20.00	5.00 ± 1.73^{a}	20.00
0.5	0.25	0	0	$0,33 \pm 0,33^{b}$	6.25
0.5	0.5	10.00 ± 1.00^{ab}	13.64	0	0

Table 4.2. Effects of TDZ in combination with IBA, 2,4-D and 2,4-D+0.5 mg/l GA_3 for shoot regeneration from leaf and petiole explants.

		EXPLANTS			
Treatments		**Leaf**		**Petiole**	
		Mean # of shoots per explant	**% explants forming shoots**	**Mean # of shoots per explant**	**% explants forming shoots**
Contol (No PGR)		-	-	-	-
TDZ (mg/l)	**IBA (mg/l)**				
0.5	0.5	-	-	-	-
0.5	1.0	-	-	-	-
1.0	0.5	-	-	-	-
1.0	1.0	-	-	-	-
TDZ (mg/l)	**2,4-D (mg/l)**				
0.5	0.5	-	-	-	-
0.5	1.0	-	-	-	-
1.0	0.5	-	-	-	-
1.0	1.0	-	-	-	-
TDZ (mg/l)	**2,4-D+0.5 GA3 (mg/l)**				
0.5	0.5	-	-	-	-
0.5	1.0	-	-	-	-
1.0	0.5	-	-	-	-
1.0	1.0	-	-	-	-

Table 4.3. Effects of BA in combination with IAA, IBA, NAA and 2,4-D for shoot regeneration from leaf and petiole explants.

Treatments		EXPLANTS			
		Leaf		Petiole	
		Mean # of shoots per explant	% explants forming shoots	Mean # of shoots per explant	% explants forming shoots
Contol (No PGR)		-	-	-	-
BA (mg/l)	IAA (mg/l)				
0	0	-	-	-	-
0	0.1	-	-	-	-
0	0.25	-	-	-	-
0	0.5	-	-	-	-
0.5	0	-	-	-	-
0.5	0.1	-	-	-	-
0.5	0.25	-	-	-	-
0.5	0.5	-	-	-	-
0.5	1	-	-	-	-
1	0.5	-	-	-	-
1	0.1	-	-	-	-
1	0.25	-	-	-	-
1	0.5	-	-	-	-
1	1	-	-	-	-
3	0	-	-	-	-
3	0.1	-	-	-	-
3	0.25	-	-	-	-
3	0.5	-	-	-	-
3	1	-	-	-	-
BA (mg/l)	IBA (mg/l)				
0.5	0.5	-	-	-	-
0.5	1.0	-	-	-	-
1.0	0.5	-	-	-	-
1.0	1.0	-	-	-	-
BA (mg/l)	NAA (mg/l)				
1	0.2	-	-	-	-
1	0.5	-	-	-	-
1	1	-	-	-	-
2	0.2	-	-	-	-
2	0.5	-	-	-	-
2	1	-	-	-	-
4	0.2	-	-	-	-
4	0.5	-	-	-	-
4	1	-	-	-	-
BA (mg/l)	2,4-D (mg/l)				
0.5	0	-	-	-	-
0.5	0.1	-	-	-	-
0.5	0.5	-	-	-	-
1.0	0	-	-	-	-
1.0	0.1	-	-	-	-
1.0	0.5	-	-	-	-
3.0	0	-	-	-	-
3.0	0.1	-	-	-	-
3.0	0.5	-	-	-	-

Table 4.4. Effects of Kinetin in combination with IAA, NAA and 2,4-D for shoot regeneration from leaf and petiole explants.

Treatments		EXPLANTS			
		Leaf		Petiole	
		Mean # of shoots per explant	% explants forming shoots	Mean # of shoots per explant	% explants forming shoots
Contol (No PGR)		-	-	-	-
KINETIN (mg/l)	IAA (mg/l)				
0.5	0	-	-	-	-
0.5	0.1	-	-	-	-
0.5	0.5	-	-	-	-
0.5	0	-	-	-	-
1.0	0.1	-	-	-	-
1.0	0.5	-	-	-	-
1.0	0	-	-	-	-
1.0	0.1	-	-	-	-
3.0	0.5	-	-	-	-
3.0	0	-	-	-	-
3.0	0.1	-	-	-	-
3.0	0.5	-	-	-	-
KINETIN (mg/l)	NAA (mg/l)				
0.5	0	-	-	-	-
0.5	0.5	-	-	-	-
0.5	1.0	-	-	-	-
1.0	0	-	-	-	-
1.0	0.5	-	-	-	-
1.0	1.0	-	-	-	-
3.0	0	-	-	-	-
3.0	0.5	-	-	-	-
3.0	1.0	-	-	-	-
KINETIN (mg/l)	2,4-D (mg/l)				
0.5	0	-	-	-	-
0.5	0.1	-	-	-	-
0.5	0.5	-	-	-	-
1	0	-	-	-	-
1	0.1	-	-	-	-
1	0.5	-	-	-	-
3	0	-	-	-	-
3	0.1	-	-	-	-
3	0.5	-	-	-	-

Table 4.5. Effects of tested auxins on root formation from regenerated shoots.

	EXPLANTS	
Treatments	**Mean # of roots per explant**	**% explants forming roots**
Control	-	-
IAA (mg/l)		
0.5	-	-
1	-	-
3	-	-
IBA (mg/l)		
0.5	-	-
1	-	-
3	-	-
NAA (mg/l)		
0.5	-	-
1	-	-
3	-	-
2,4-D (mg/l)		
0.1	-	-
0.5	-	-
1	-	-
$1/_2$ MS + IAA, IBA, NAA		
0.5	-	-
1	-	-
3	-	-
$1/_2$ MS + 2,4-D (mg/l)		
0.1	-	-
0.5	-	-
1	-	-
$1/_2$ MS + $1/_2$ sucrose + IAA,IBA,NAA (mg/l)		
0.5	-	-
1	-	-
3	-	-
$1/_2$ MS + $1/_2$ sucrose + 2,4-D (mg/l)		
0.1	-	-
0.5	-	-
1	-	-
0.5 GA_3 + IAA, IBA, NAA (mg/l)		
0.5	-	-
1	-	-
3	-	-
0.5 GA_3 + 2,4-D (mg/l)		
0.1	-	-
0.5	-	-
1	-	-
0.5 mg/l ascorbic acid + IAA, IBA, NAA (mg/l)		
0.5	-	-
1	-	-
3	-	-
0.5 mg/l ascorbic acid + 2,4-D (mg/l)		
0.1	-	-
0.5	-	-
1	-	-
$1/_2$ MS + $1/_2$ sucrose + activated charcoal (mg/l)		
0.5	-	-
1	-	-
2	-	-

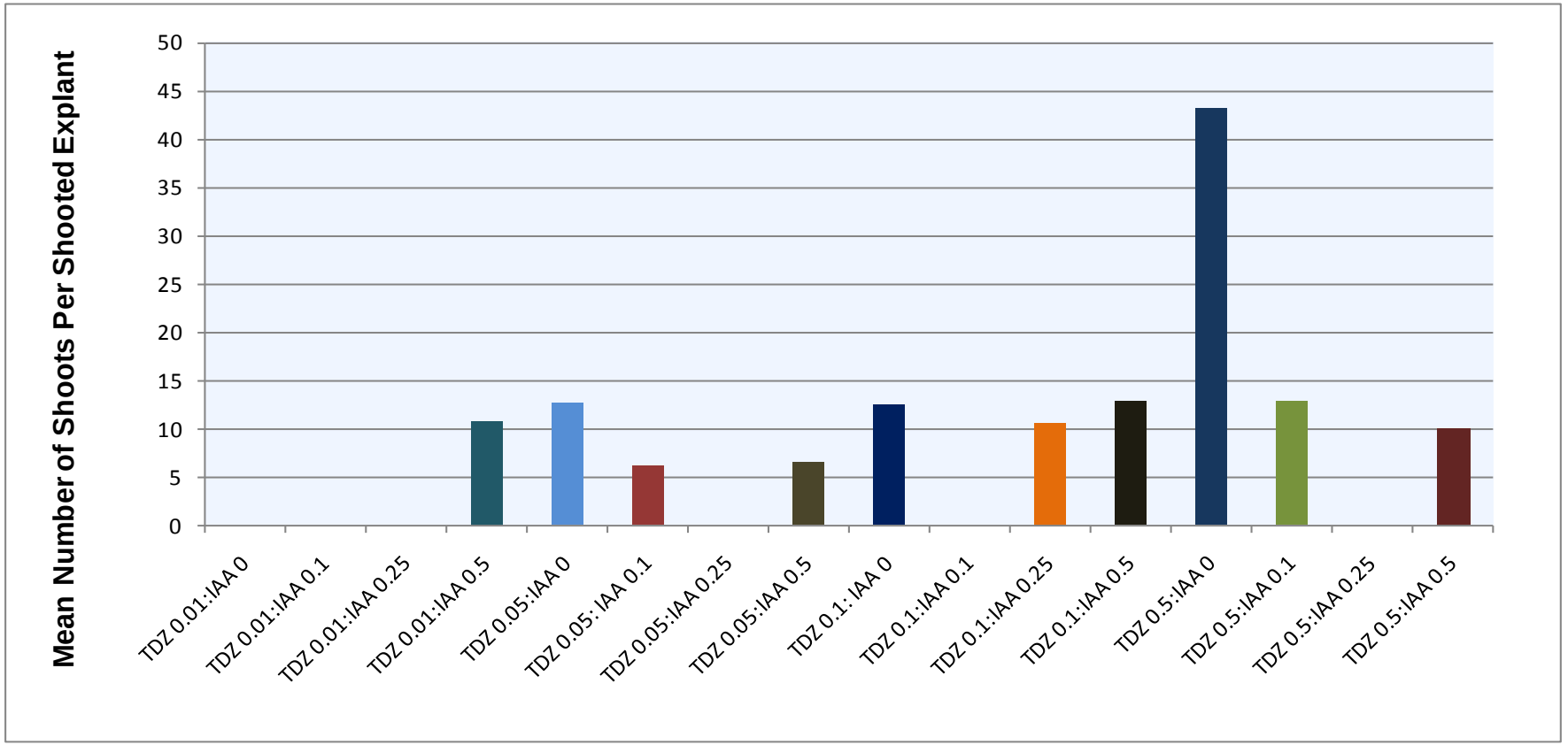

Figure 4.1. Shoot development from leaf explants incubated on media containing different concentrations of TDZ and IAA.

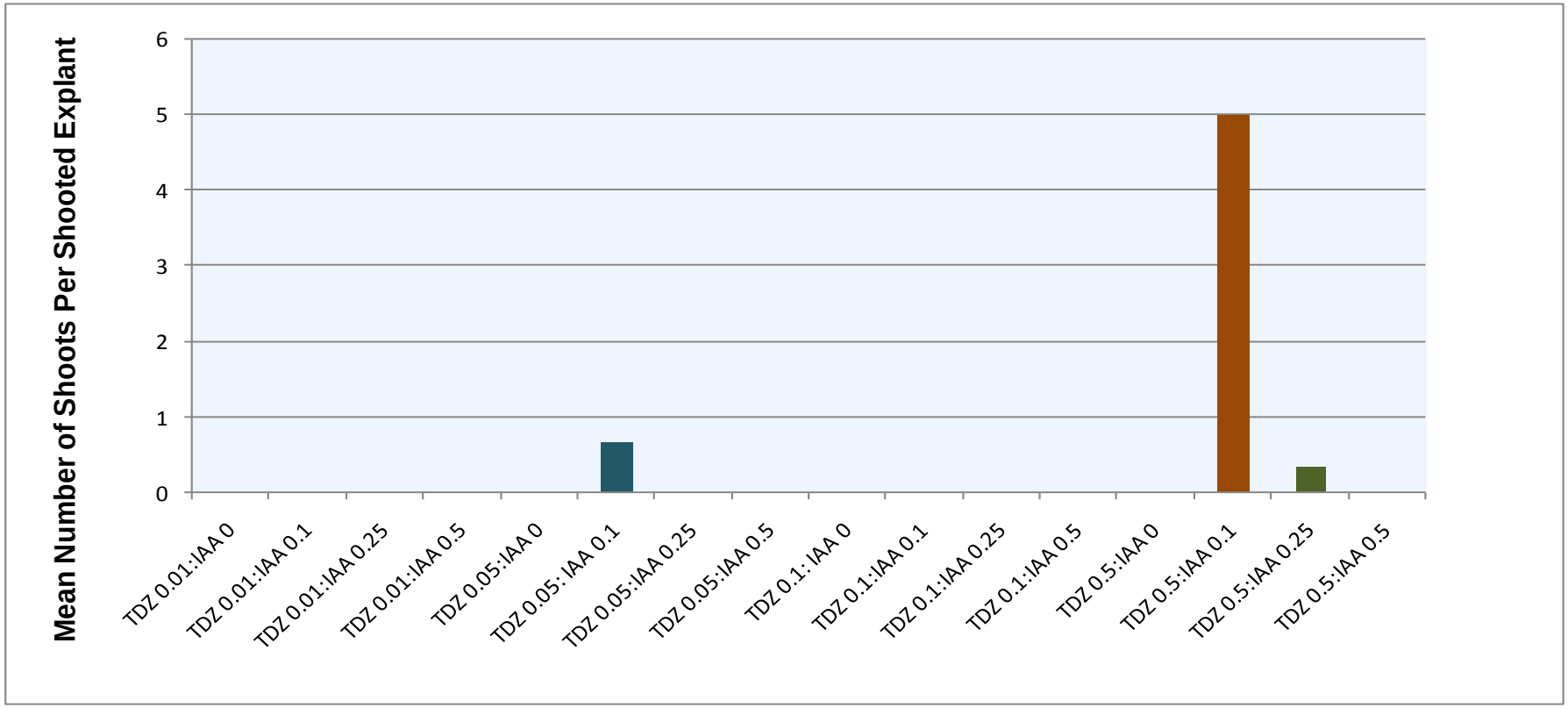

Figure 4.2. Shoot development from petiole explants incubated on media containing different concentrations of TDZ and IAA.

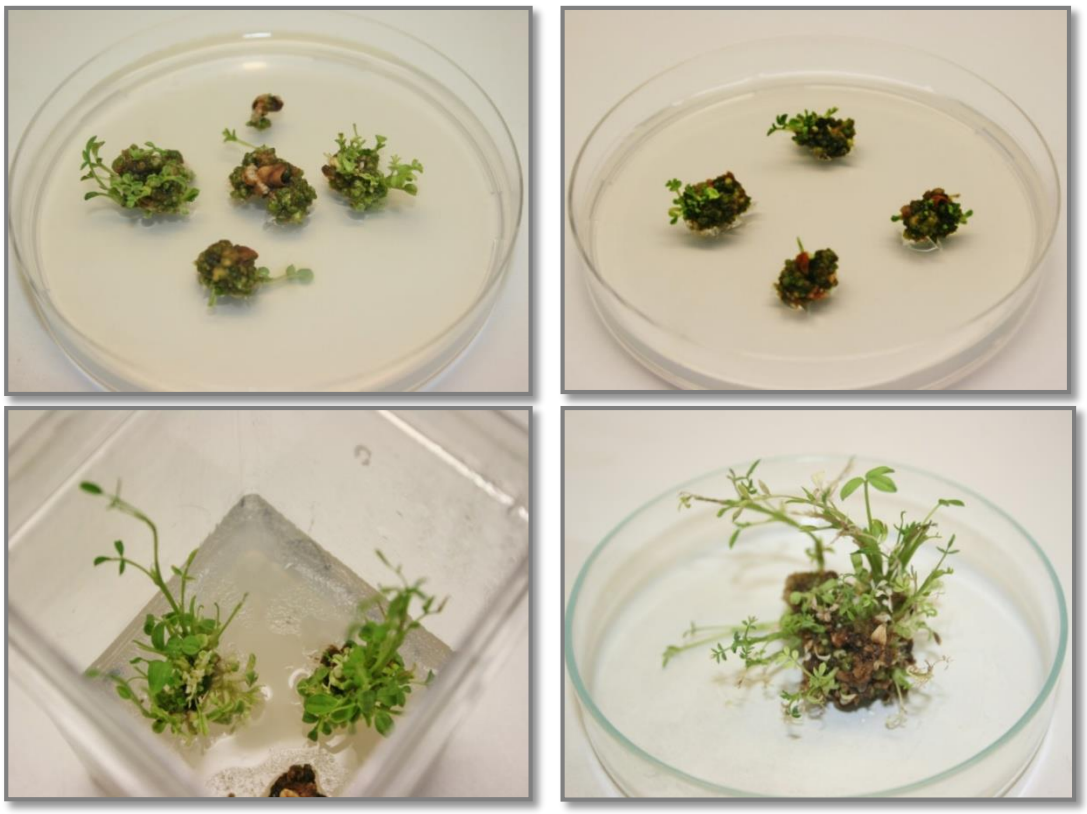

Figure 4.3. Shoot formation from leaf explants with 0.05 mg/l TDZ.

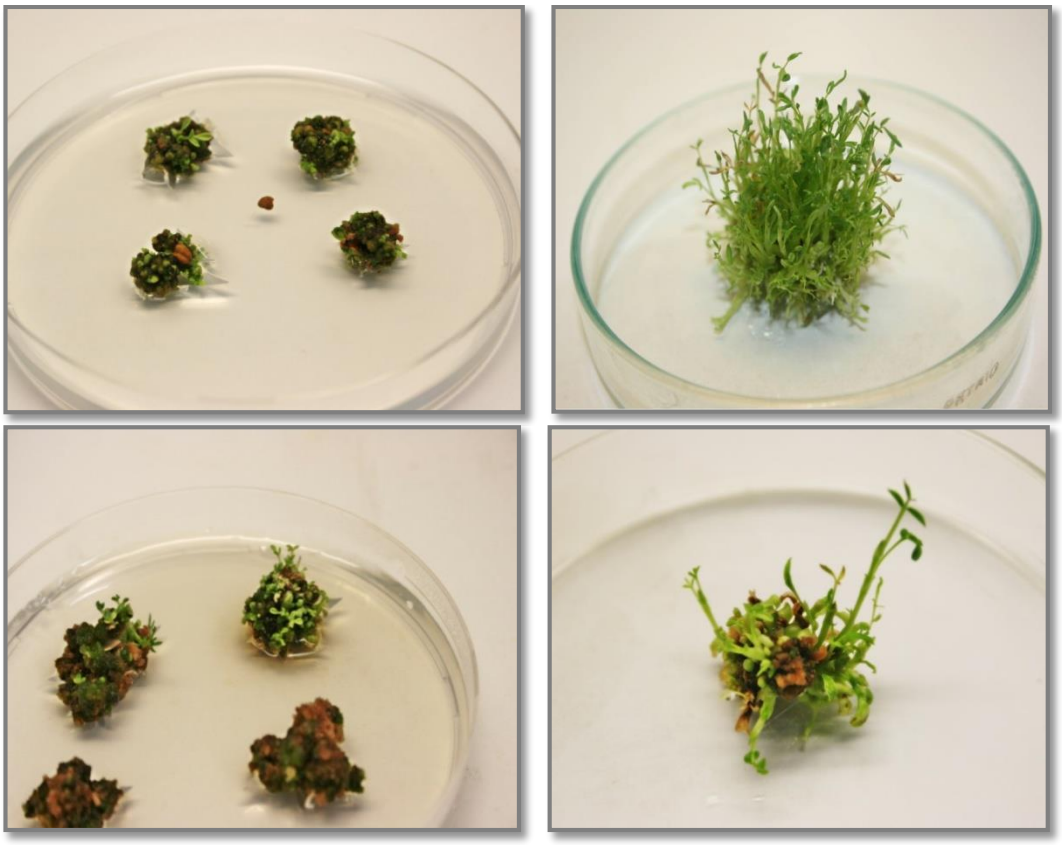

Figure 4.4. Shoot formation from leaf explants with 0.5 mg/l TDZ.

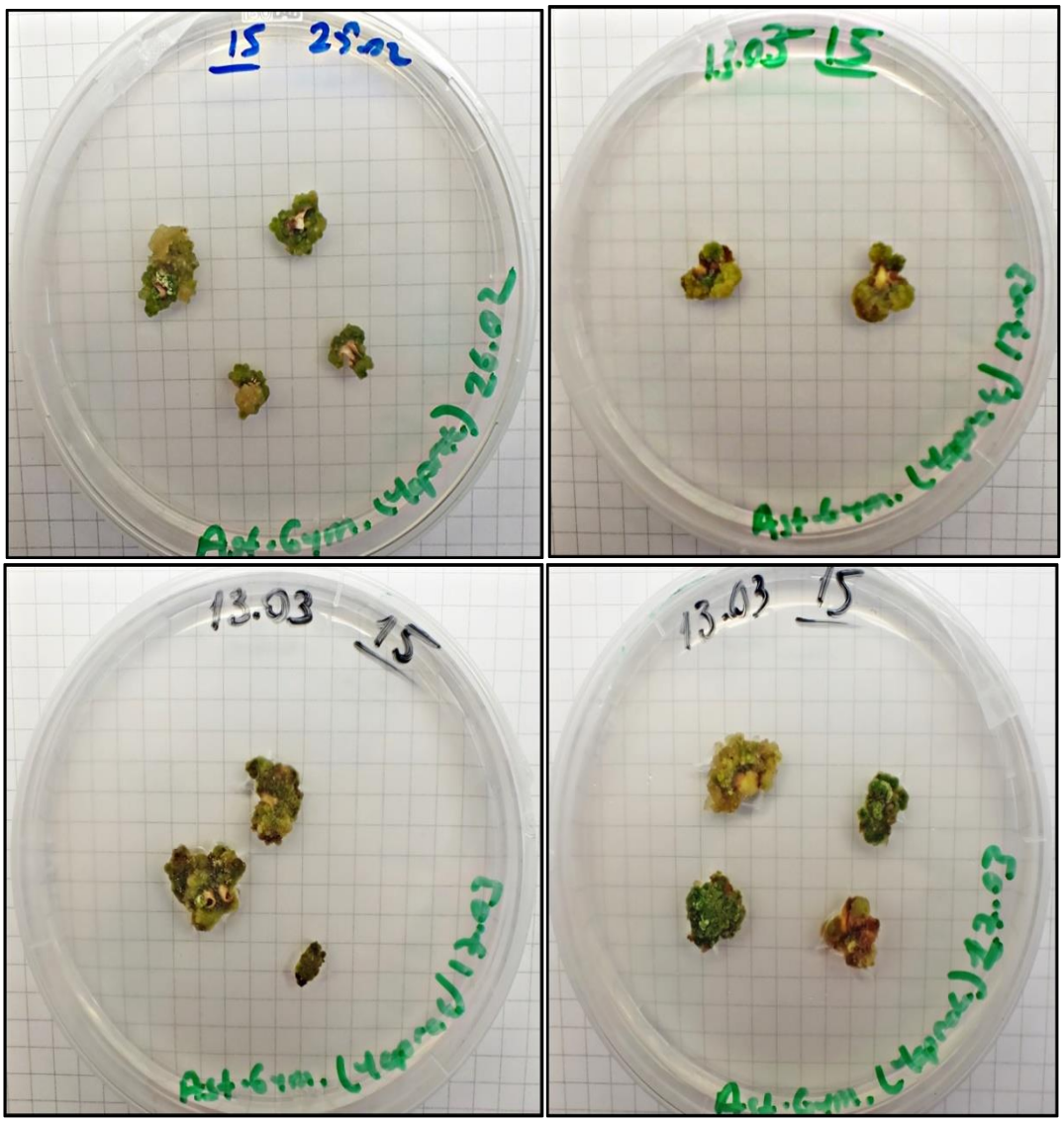

Figure 4.5. Callus formation in leaf explants of *A. gymnolobus* on MS medium containing 3 mg/l BA + 0.5 mg/l 2,4-D.

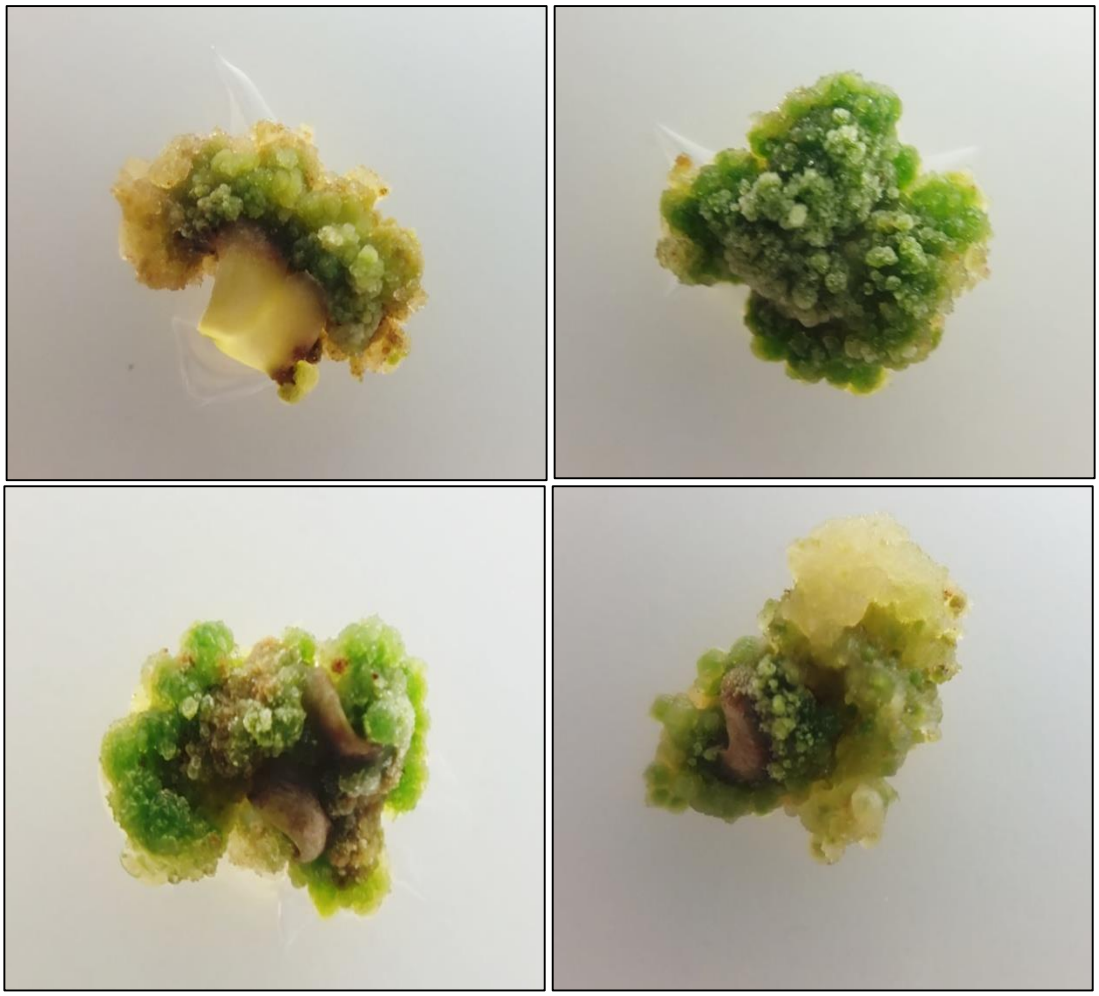

Figure 4.6. Callus formation in leaf explants of *A. gymnolobus* on MS medium containing 3 mg/l BA + 0.5 mg/l 2,4-D.

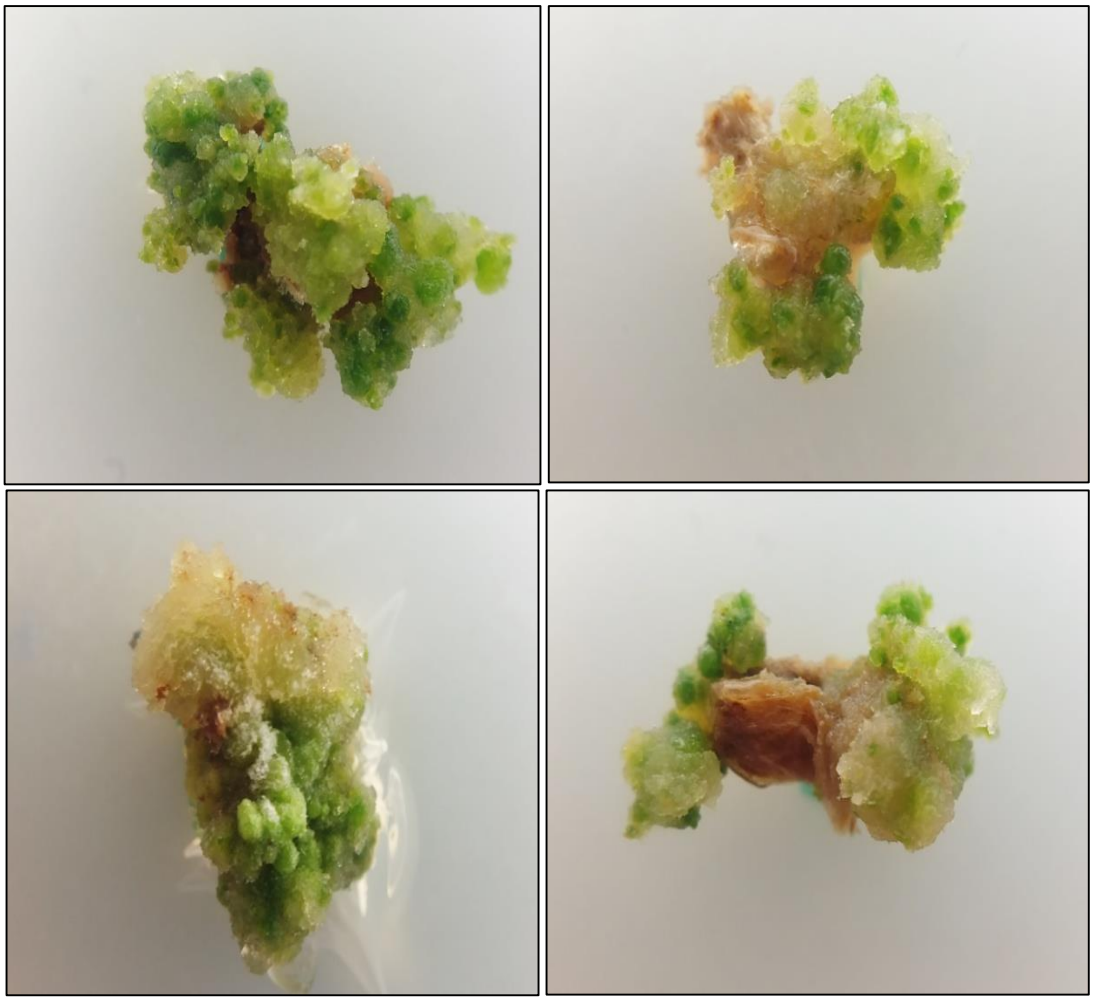

Figure 4.7. Callus formation in petiole explants of *A. gymnolobus* on MS medium containing 3 mg/l BA + 0.1 mg/l 2,4-D.

4.2 Biological Activity and Constituents of *Astragalus gymnolobus*

4.2.1 Antibacterial Assay

Four different extracts [methanol extract of field-grown leaves (AM) and *in vitro*-grown callus (IM), water extract of field-grown leaves (AW) and *in vitro*-grown callus (IW)] were used to screen for antibacterial and antioxidant effects of *A. gymnolobus* (Table 3.1, 4.6, 4.7 and 4.8).

Generally, field-grown leaves showed better antibacterial activities than *in vitro*-grown callus. Among field-grown leaf extracts, methanol extract was better than water extract. Best inhibitory activity was observed with methanol extract of field grown leaves (Table 4.6; Figure 4.8, 4.9, 4.10, 4.11 and 4.12).

Water extract of field-grown leaves did not show any antibacterial activity against to used bacteria. Methanol extracts of field-grown leaves exhibited a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. This activity against both types of bacteria may be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins (McCutcheon et al.,1992). Methanol extracts of field-grown leaves showed antibacterial activity against *S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa* and *P. vulgaris* (Table 4.6; Figure 4.8, 4.9, 4.10, 4.11 and 4.12). Antibacterial activity of methanol extracts of field-grown leaves against *S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa and P. vulgaris* may explain why *Astragalus* spp. is used in folk medicine to treat nephritis, immunologic diseases (cause by *S. pyogenes*), sepsis, urinary tract infections (caused by *S. aureus, S. epidermidis, P. aeruginosa* and *P. vulgaris*) (Table 3.2 and 4.6; Figure 4.8, 4.9, 4.10, 4.11 and 4.12). Best antibacterial activity was obtained with methanol extract of field-grown leaves against *S. pyogenes* (Table 4.6; Fig. 4.9 and 4.13).

Turker and Koyluoğlu (2011) showed that alcoholic extracts of *A. gymnolobus* exhibited little inhibition against only *S. pyogenes*. Bisignano et al. (1994) investigated that *A. siculus* showed antibacterial activity against *S. aureus, S. epidermidis, Streptococcus faecalis, Proteus mirabilis, Citrobacter freundii, P. aeruginosa* and *Klebsiella oxytoca.A. brachypterus* showed strong inhibition against only *S. pyogenes* (Turker and Yıldırım, 2013).

Teyeb et al. (2012) reported that three extracts (methanol, chloroform, and ethyl acetate) from aerial parts and two extracts (water and methanol) from roots of wild *A. gombiformis* exhibited diameters of inhibition zone equal or above 12 mm (at 150µ/disk) and minimal inhibitor concentrations ranging between 233 and 1250 microg/ml. Antibacterial activities of some members of *Astragalus* spp. (*A. siculus, A. gummifer, A. membranaceus, A. malanophrurius* and *A. verrucosus*) were recorded against Gram-positive and Gram-negative bacteria (Pistelli et al., 2002; Bisignano et al.,1994).

Methanol extract of field-grown leaves did not show antibacterial activity against *S. marcescens, S. typhimurium, K. pneumoniae, E. cloacae* and *E.coli* (Table 4.6; Figure 4.8, 4.9, 4.10, 4.11 and 4.12). *S. aureus, S. epidermidis, P. aeruginosa and P. vulgaris* were inhibited by methanol extract of only field-grown leaves (Table 4.6; Fig. 4.8, 4.10, 4.11 and 4.12).

Generally, the Gram-positive bacteria (*S. pyogenes, S. epidermidis* and *S. aureus*) seem to be more susceptible to the inhibitory effects of the plant extracts than Gram-negative bacteria (*S. typhimurium, S.marcescens, P. aeruginosa, P. vulgaris, K. pneumoniae, E. cloacae* and *E.coli*). Susceptibility of Gram-positive bacteria may come from their cell wall structure consisting of a single layer. However, the Gram-negative cell wall is a multi-layered structure and quite complex (Essawi and Srour, 2000). Bacterial growth was generally sensitive to the reference antibiotics tested (Table 4.6). Positive controls (reference antibiotics) generally showed antibacterial activity to our test organisms. Since final concentrations of all extracts were adjusted with distilled water, it was used as a negative control and no inhibition was observed with water. Methanol extract of field-grown leaves did not show greater antibacterial activity than reference antibiotic chloramphenicol, ampicillin, carbenicillin, erythromycin and tetracycline against both Gram-positive and Gram-negative bacteria (Table 4.6).

Generally, *in vitro*-grown callus extracts did not show better antibacterial activities than field-grown leaves. Methanol extract of *in vitro*-grown callus exhibited a little antibacterial activity against only *S. pyogenes*. Similarly, water extract of *in vitro*-grown callus showed a little antibacterial activity against only *S. pyogenes* (Table 4.6).

Antibacterial activities of field-grown leaves were better than *in-vitro* grown callus against used bacteria. Sometimes the level of the detected secondary products

in shoot cultures is lower than donor plants (Stafford, 1991). For example, production of steroids obtained from shoot tip cultures of *Digitalis* species was much lower than those found in the donor plant (Seidel and Reinhard, 1987). If the secondary product synthesis is low, there are some procedures for enhancing productivity. Optimization of hormone regime is often effective. The type and concentration of phytohormones available to cultured cells is probably the most important factor influencing their potential for secondary product synthesis. For example 2,4-D can stimulate both cell division and cell expansion in many systems. However, it can also bring about a dramatic suppression of secondary product synthesis in cell cultures. Alterations in other environmental factors such as nutrient levels, light regime and temperature may also be effective in increasing productivity and reduced phosphate levels often stimulate product accumulation (Parr, 1989).

Table 4.6. Antibacterial activity of *A. gymnolobus* extracts. Data presented as zone of inhibition of bacterial growth in mm. Means with the same letter within columns are not significantly different at $P>0.05$.

	Mean diameter of inhibitory zones (mm ± SE)									
Treatments	***S. auerus***	***S. epidermidis***	***S. marcescens***	***S. pyogenes***	***S. typhimurium***	***P. aeruginosa***	***P. vulgaris***	***K. pneumonia***	***E. cloacae***	***E. coli***
AW	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
AM	13,3 ± 0.21d	13,3 ± 0.34d	- -	16,2 ± 0.29e	- -	10,0 ± 0.41bc	8,7 ± 0.21c	- -	- -	- -
IW	- -	- -	- -	10,8 ± 0.48f	- -	- -	- -	- -	- -	- -
IM	- -	- -	- -	11,5 ± 0.22ef	- -	- -	- -	- -	- -	- -
Ampicillin	35,0 ± 0a	27,0 ± 0c	- -	48,0 ± 0ab	27,9 ± 0c	- -	23,0 ± 0b	8,0 ± 0c	26,0 ± 0c	20,0 ± 0c
Carbenicillin	35,0 ± 0.58a	36,5 ± 2.02a	25,0 ± 2.89a	50,0 ± 7.51a	28,5 ± 0.87b	15,5 ± 0.75a	30,0 ± 2.89a	21,0 ± 6.93b	31,5 ± 0.29a	30,0 ± 1.16a
Chloramphenicol	25,0 ± 1.30c	32,5 ± 0.87b	25,0 ± 0a	35,0 ± 0d	30,0 ± 0a	8,0 ± 0c	22,0 ± 0b	28,0 ± 0a	28,0 ± 0b	27,0 ± 0b
Erythromycin	30,0 ± 0b	37,0 ± 0a	7,0 ± 0c	44,0 ± 0bc	10,0 ± 0e	8,0 ± 0c	11,0 ± 0c	11,0 ± 0c	9,0 ± 0d	11,0 ± 0d
Tetracycline	31,0 ± 0.58b	- -	15,0 ± 0.58b	39,5 ± 2.29cd	23,0 ± 0.58d	13,0 ± 0ab	27,5 ± 0.29a	26,5 ± 0.29ab	26,0 ± 0c	26,5 ± 0.29b
Water	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

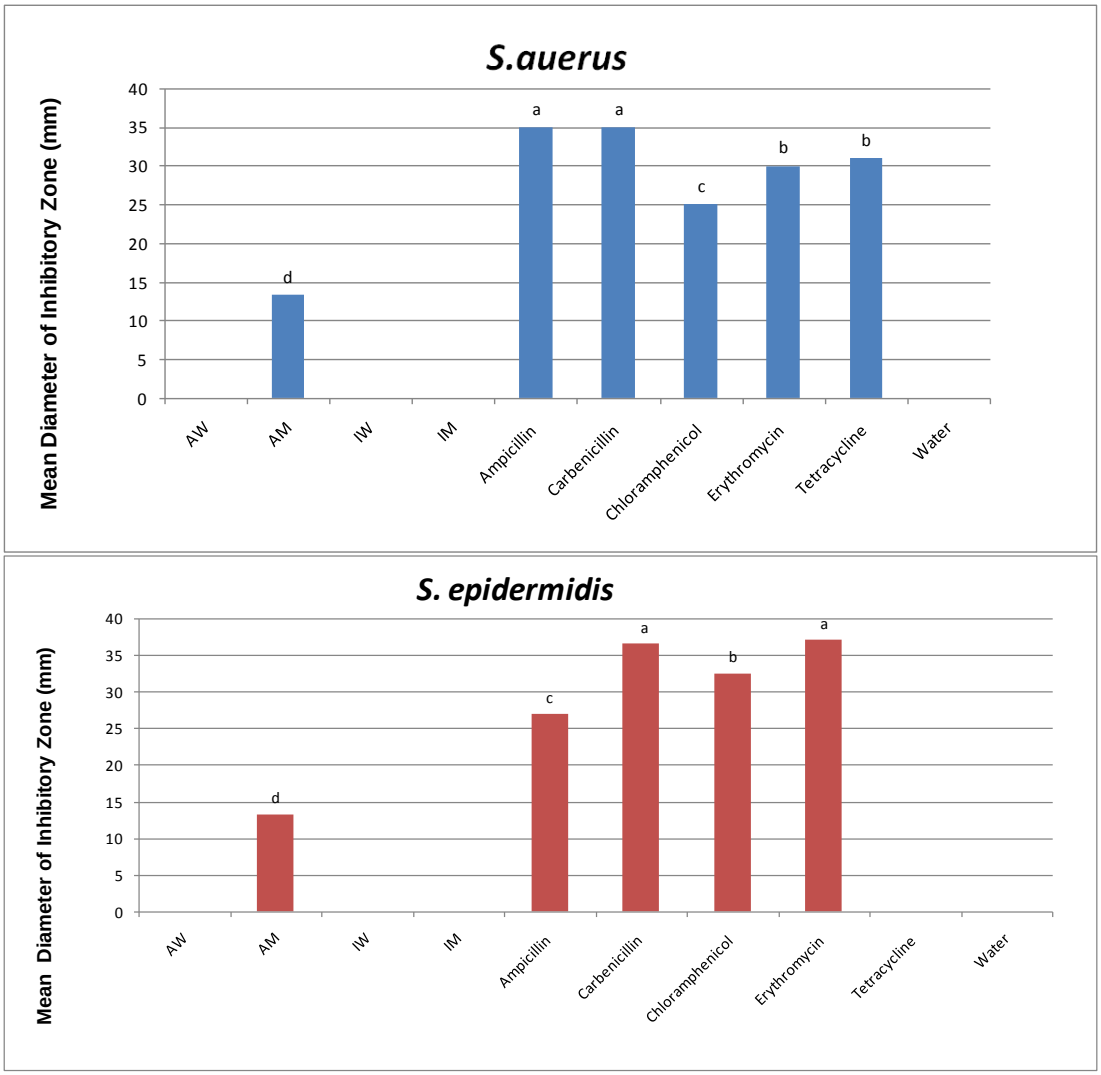

Figure 4.8. Antibacterial activity of *A. gymnolobus* extracts and controls against *S. auerus* and *S. epidermidis*.

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

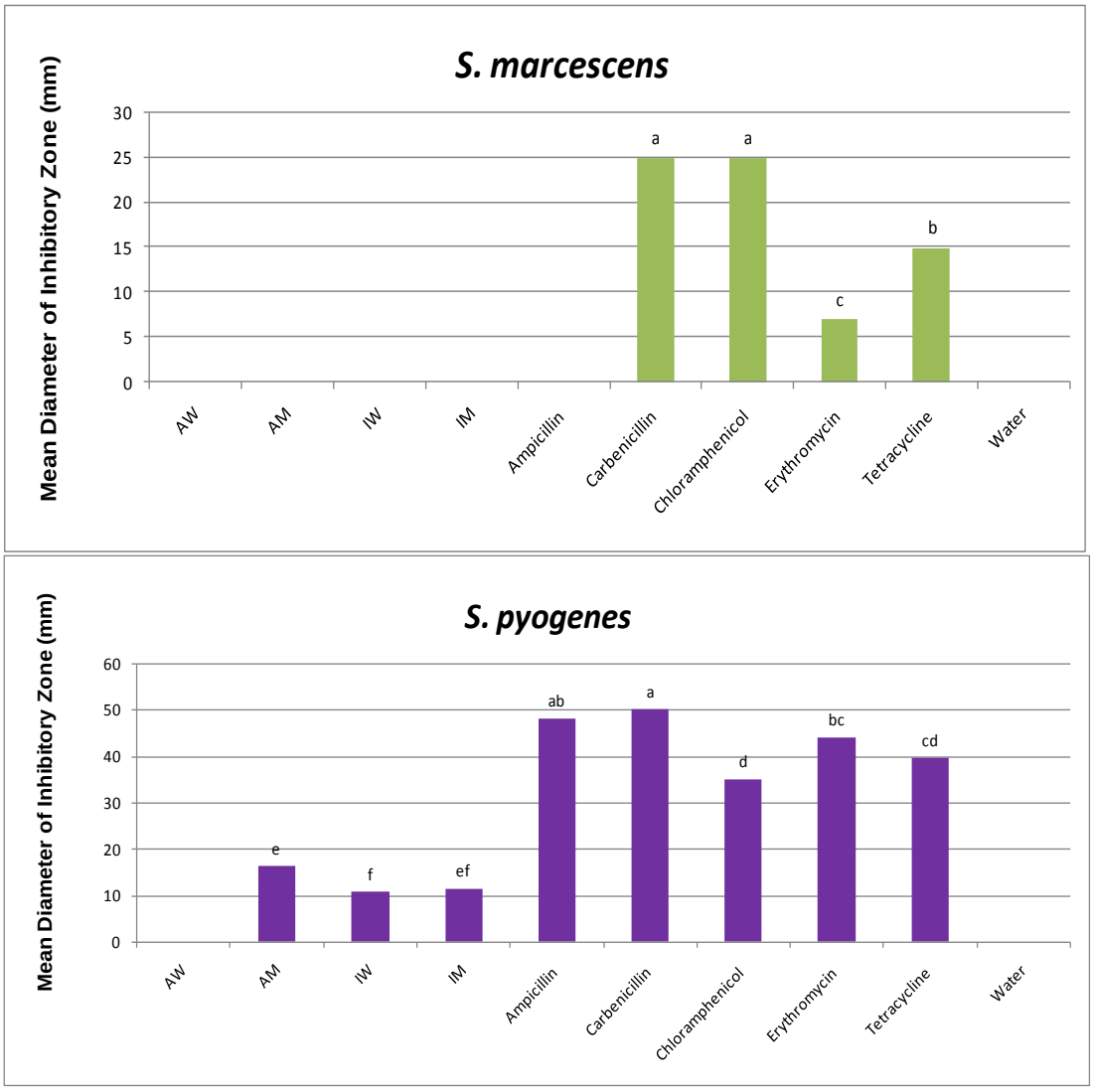

Figure 4.9. Antibacterial activity of *A. gymnolobus* extracts and controls against *S. marcescens* and *S. pyogenes.*

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

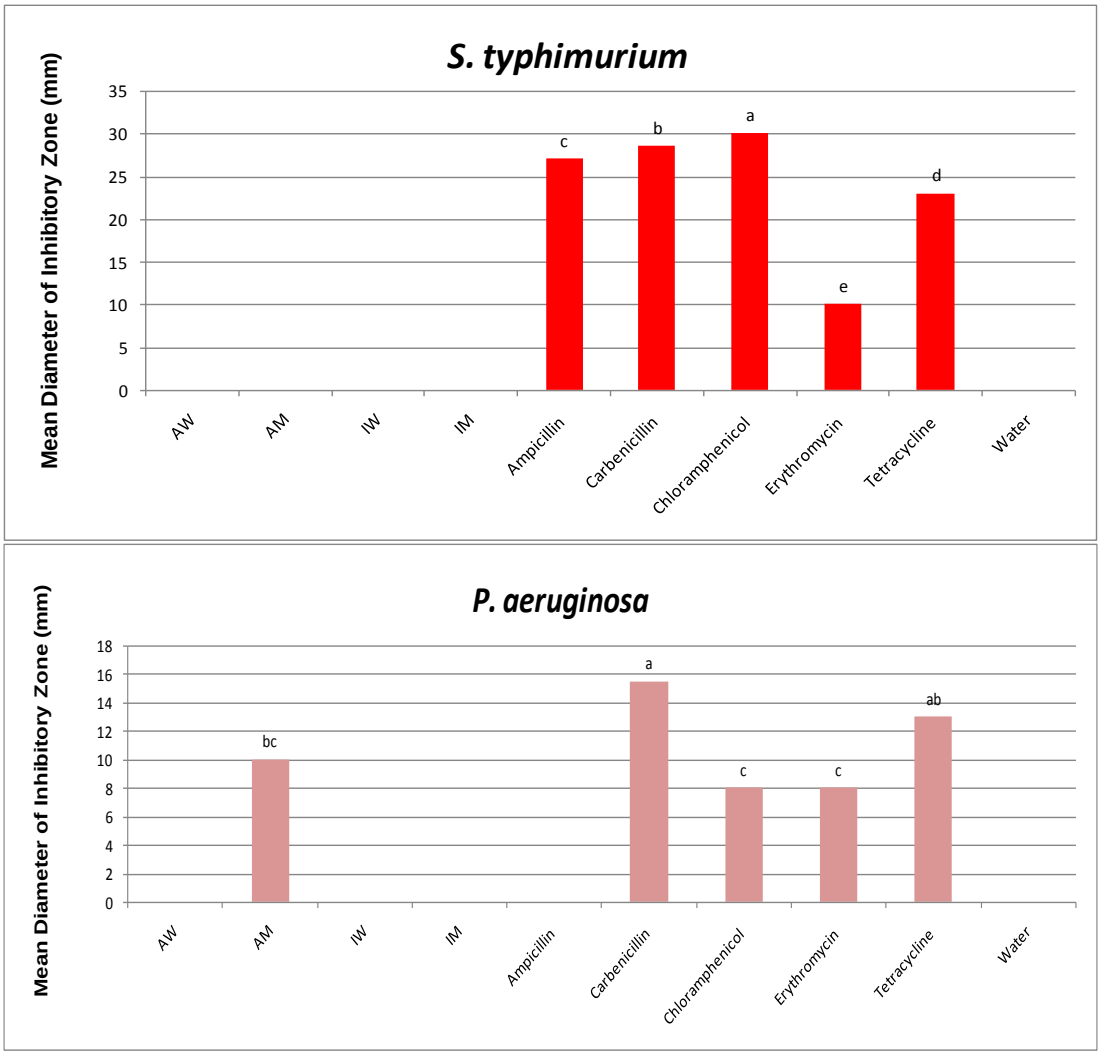

Figure 4.10. Antibacterial activity of *A. gymnolobus* extracts and controls against *S. typhimurium* and *P. aeruginosa*.

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

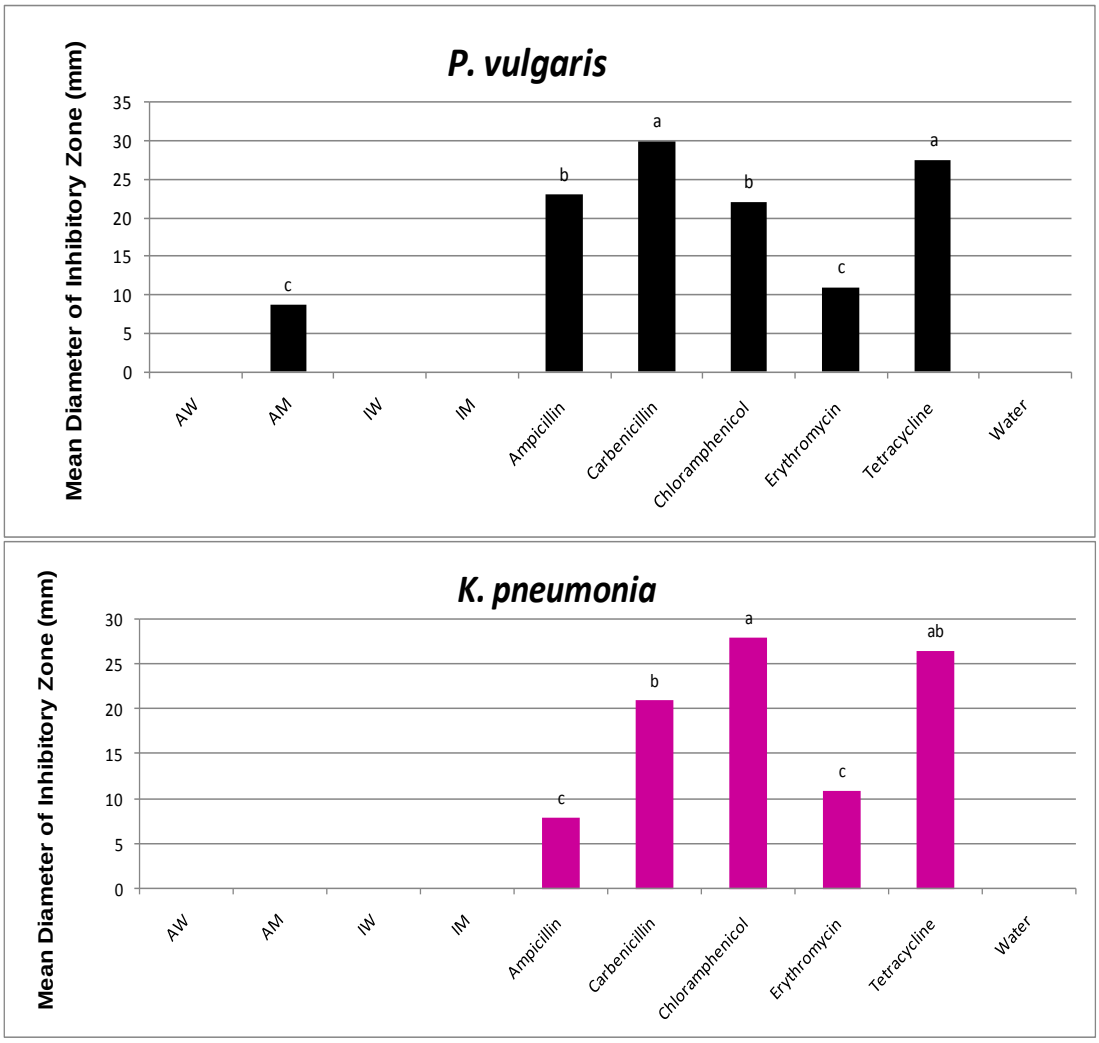

Figure 4.11. Antibacterial activity of *A. gymnolobus* extracts and controls against *P. vulgaris* and *K. pneumonia*

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

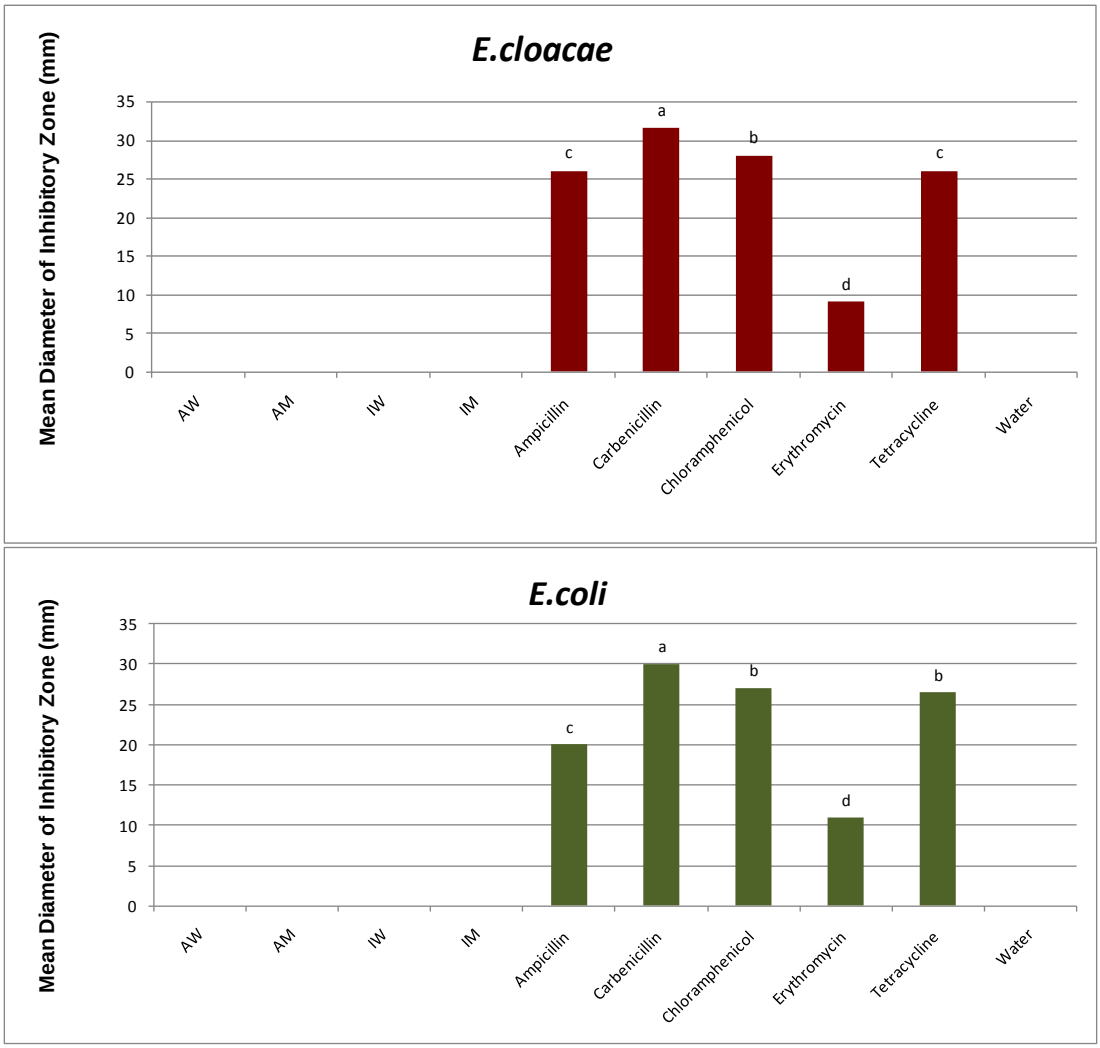

Figure 4.12. Antibacterial activity of *A. gymnolobus* extracts and controls against *E. cloacae* and *E. coli.*

AW: Water extract of field-grown leaves;
AM: Methanol extract of field-grown leaves;
IW: Water extract of *in vitro*-grown callus;
IM: Methanol extract of *in vitro*-grown callus.

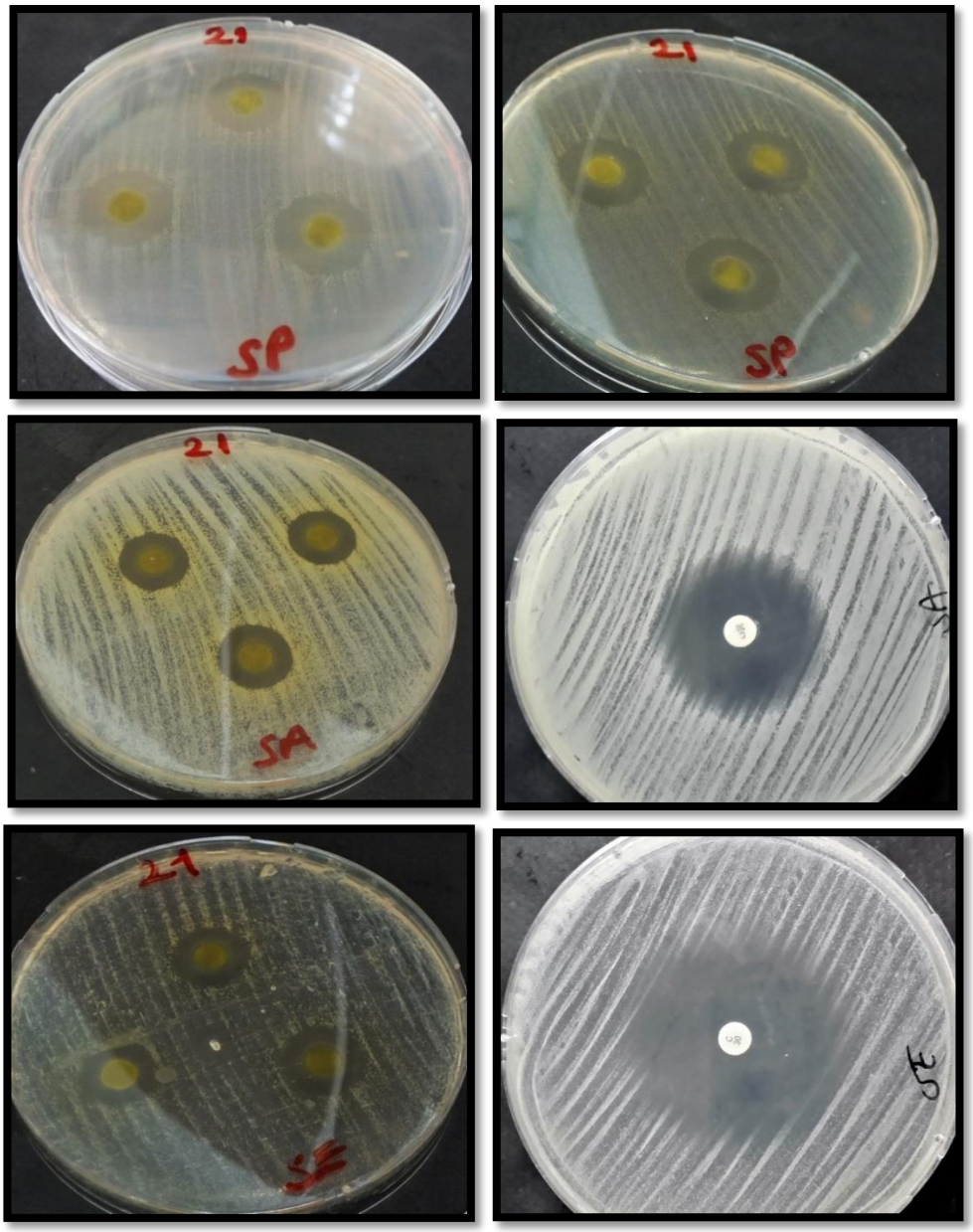

Figure 4.13. Antibacterial activity of AM (methanol extract of field-grown plants) against *S. Pyogenes* (above); antibacterial activity of AM (methanol extract of field-grown plants) against *S. aureus* with control (carbenicillin) (below, left); antibacterial activity of AM (methanol extract of field-grown plants) against *S. epidermidis* with control (carbenicillin) (below, right).

4.2.2 Antioxidant Assay

4.2.2.1 Free Radical Scavenging Activity-DPPH (2,2-diphenyl-1-picrylhydrazil) Method

The scavenging activity of DPPH radical caused by antioxidants was determined by measuring the decrease in its absorbance at 517 nm. Ascorbic acid was used as the antioxidant standard in this experiment. In the present study, antioxidant activity of methanolic extract of field-grown leaves, and *in vitro*-grown callus was assessed. The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteau) and total flavonoid content (aluminum chloride colorimetric) were used in this assessment.

In DPPH assay, the best antioxidant activities were obtained by methanolic extract of field-grown leaves (Table 4.7; Figure 4.14). In methanol extract of field-grown plants, DPPH radical scavenging activity was observed strong enough at 25 µg/ml concentrations (91.47 %). Methanol extract of field-grown leaves at 25 µg/ml concentration showed the similar radical scavenging activity in that of ascorbic acid. The scavenging activity of DPPH radical of methanol extract of field-grown leaves decreased when its concentration increased. Good antioxidant activities were observed with gradually prepared methanolic extract of field-grown leaves (Table 4.7; Figure 4.14).

The free radical scavenging activity of *in vitro*-grown callus has little tendency to scavenge the free radicals according to methanolic extract of field-grown leaves. The scavenging activity of DPPH radical of methanol extract of *in vitro*-grown callus increased when its concentration increased, the free radical scavenge tendency increased. The best antioxidant activities of methanol extracts of *in vitro*-grown callus showed at 200 µg/ml concentrations (51.75 %) that it did not show high active radical scavenge capability as much as ascorbic acid (Table 4.7; Figure 4.14).

Table 4.7. % inhibition of DPPH by *A. gymnolobus* extracts.

	% inhibition of DPPH Concentrations			
Treatments	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**
Ascorbic acid	97.15	96.69	96.13	78.32
AM	91.47	86.34	83.50	71.72
IM	18.83	20.62	36.60	51.75

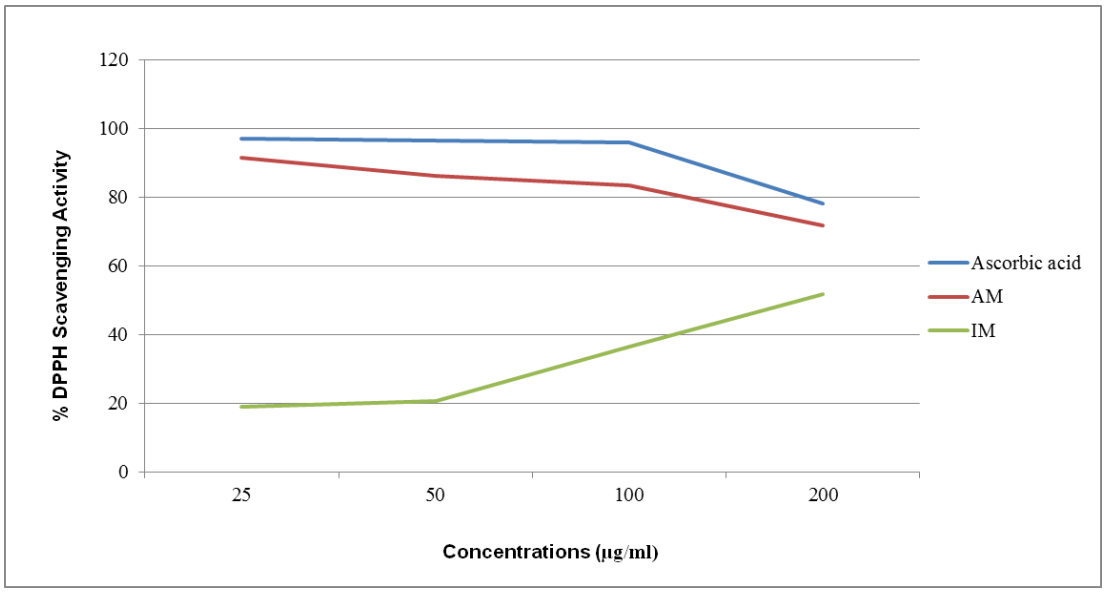

Figure 4.14. Free radical (DPPH) scavenging activity (%) of methanolic extract of field-grown leaves and *in vitro*-grown callus from *A. gymnolobus* and ascorbic acid (positive control).

4.2.2.2 Total Phenolic Assay (Folin-Ciocalteau Method for Total Phenolic Content)

Hydroxyl groups on phenolic compounds have scavenging ability so they are very important plant constituents. A number of studies reported a significant relation between the phenolic contents of plant extracts and their antioxidant properties (Gülçin et al., 2004). The most phenolic content is included in methanolic extract of field-grown leaves (271.484 ± 0.000 mg gallic acid equivalent/g dried extract). Methanol extract of *in vitro*-grown callus contained lower phenolic content than in methanol extract of field-grown leaves (19.144 ± 0.000 mg gallic acid equivalent/g dried extract) (Table 4.7 and 4.8; Fig. 4.14).

4.2.2.3 Total Flavonoid Assay-Aluminum Chloride ($AlCl_3$) Colorimetric Assay for Total Flavonoids

When these extracts were compared depending on having flavonoid content, the most flavonoid content was found in methanol extract of field-grown leaves (144.693 ± 0.000 mg catechol acid equivalent/g dried extract). Methanol extract of *in vitro*-grown callus contained lower flavonoid content than in methanol extract of field-grown leaves (27.360 ± 0.000 mg catechol acid equivalent/g dried extract (Table 4.8). Therefore, methanol extracts of field-grown leaves contain more flavonoid and phenols than *in vitro*-grown callus.

Sokmen et al. (2009) reported that antioxidant activities of methanol extracts of collected four wild type *Astragalus* species (*A. microcephalus, A. lagurus, A. macrocephalus* and *A. galegiformis*) in Erzurum have been investigated. Extract obtained from the roots of *A. macrocephalus* exhibited the strongest antioxidant properties among the studied *Astragalus* species.

The antioxidant capacity of methanol extract of the flowering aerial parts of *A. squarrosus* was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric thiocyanate methods. The phenolic and flavonoid content was also measured. *A. squarrosus* showed weak free radical scavenging activity with the DPPH method which might be due to its low phenolic constituents (23.3 mg/g) and flavonoid content (26.0 mg/g) (Asgarpanah et al., 2011). In another studies, antioxidant effects with ethanol extracts of *A. membranaceus* was investigated and

showed that *A. membranaceus* have higher antioxidant effects. Therefore, *A. membranaceus* can be used as a data to develop more efficient material in functional food (Kim et al., 2009). Lim et al. (2011) reported that antioxidant activity of various extracts of *Astragalus sinicus* L. seed was evaluated *in vitro* and *in vivo*. These results suggest that the acetone extract of *A. sinicus* L. seed might be a valuable antioxidant of natural source applicable in the medicine, cosmetics and the food industry.

This study indicated that methanol extracts of field-grown leaves of *A. gymnolobus* have better antioxidant activity and are rich from the point of view of phenolics and flavonoids. They can be used as accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. The antioxidant mechanisms and their phenols or flavonoids may be attributed to strong hydrogen donating ability, and their effectiveness as scavengers of free radicals. Phenolic compounds appear to be responsible for the antioxidant activity of extracts of methanol extracts of field-grown leaves of *A. gymnolobus* (Table 4.7 and 4.8).

Table 4.8. Total phenol and total flavonoid contents of *A. gymnolobus*. Gallic acid equivalent was used as mg GAE/g extracts. Catechol equivalent was used as mg CE/g extracts.

Treatments	Total Phenolics mg GAE/ g dried mass	Total Flavonoids mg CE/ g dried mass
AM	271.484 ± 0.000	144.693 ± 0.001
IM	19.144 ± 0.000	27.360 ± 0.000

4.2.3 HPLC-DAD Analysis of Phenolic Compounds

Two different methanolic extracts of *A. gymnolobus* were subjected to HPLC analysis [Methanol extracts of field-grown leaves (AM) and *in vitro*-grown callus (IM)] (Table 4.9; Figure 4.15, 4.16, 4.17 and 4.18). A mixture of 10 standard phenolic compounds (Gallic acid, caffeic acid, rutin, luteolin, kaempferol, rosmarinic acid, myricetin, quercetin, coumarin and apigenin) was eluted using HPLC and a chromatogram for the standards was obtained after elution with suitable solvent (Figure 4.16). Then, the phenolic contents of methanol extracts were compared with the standard chromatogram and the amounts (mg/g extract) of each phenolic compound in the extracts were determined (Table 4.9; Figure 4.15, 4.17 and 4.18).

According to HPLC results, concentrations of phenolic compounds in field-grown leaves were higher than the *in vitro*-grown callus (Table 4.9). The phenolic compounds found in methanol extracts of field-grown leaves were gallic acid, apigenin, caffeic acid, luteolin, myricetin, quercetin and rutin. The concentration of these compounds in methanol extracts of field-grown leaves was higher than *in vitro*-grown callus. Rutin was dominant compound in methanol extracts of field-grown leaves (101.23 mg/g) (Table 4.9; Figure 4.15, 4.17 and 4.18). Methanol extracts of field-grown leaves contained from the highest to lowest amount rutin (101.23 mg/g), luteolin (3.13 mg/g), apigenin (1.49 mg/g), caffeic acid (0.45 mg/g), gallic acid (0.41 mg/g), quercetin (0.30 mg/g) and myricetin (0.03 mg/g). Kaempferol, rosmarinic acid and coumarin were not found in field-grown leaf extract (Table 4.9; Figure 4.15, 4.17 and 4.18)

The phenolic compounds found in methanol extracts of *in vitro*-grown callus from the highest to lowest amount were apigenin (1.17 mg/g), quercetin (0.40 mg/g) and kaempferol (0.30 mg/g). Apigenin was dominant compound in *in vitro*-grown callus extract. Although kaempferol was found high in *in vitro*-grown callus extract, methanol extracts of field-grown leaves did not contain kaempferol. Quercetin and apigenin were found in two extracts (Table 4.9; Figure 4.15, 4.17 and 4.18).

Matkowski et al. (2003) investigated the content of isoflavones and some other polyphenolic compounds in roots and aerial parts of *Astragalus membranaceus* by means of TLC and HPLC. According to HPLC results, isoflavonoids: calycosin glucosides (7-O-D-glucoside (1), and 7-O-D-glucoside 6- O-malonate (2), ononin (3), 3-hydroxy-9,10-dimethoxypterocarpan-3-O-D-glucoside (4), calycon (5), 7,2-

dihydroxy-3,4-dimethoxyisoflavan-7-O-D-glucoside (6), formononetin-7-O-D glucoside-6-O-malonate (7), formononetin (8); and flavonols: quercetin and kaempferol were observed in the roots. Formononetin, other flavonoids (flavonols and glycosides): quercetin, rutin, quercitrin, isorhamnetin, kaempferol, luteolin; phenolic acids: chlorogenic, caffeic, ferulic, p-coumaric were observed in aerial parts. Similarly, quercetin, kaempferol, rutin, caffeic acid and luteolin were observed in methanolic extracts of *Astragalus gymnolubus* in our studies (Table 4.9; Figure 4.15, 4.17 and 4.18).

When the results of phenolic content and antioxidant activity were compared with each other, there is a significant relation between the phenolic contents and antioxidant activity of the methanol extracts of field-grown leaves and *in vitro*-grown callus. It was observed that the antioxidant activity results were high where the concentrations of phenolic compounds were found higher. The best antioxidant activities were obtained by methanol extract of field-grown leaves. Also, HPLC analysis showed that methanol extracts of field-grown leaves contained more phenolics than *in vitro*-grown callus (Table 4.7, 4.8 and 4.9; Figure 4.15, 4.17 and 4.18).

Table 4.9. HPLC analysis ofidentified phenolic compounds and their amounts in the methanol extracts of field-grown leaves and *in vitro*-grown callus of *A. gymnolobus.*Values are means ± SE of three measurements.

STANDART COMPOUNDS			**PLANT EXTRACTS (mg/g dry extract)**	
Name	**Peak number**	**RT (min)**	**AM**	**IM**
Gallic acid	1	5.51	0.41±0.01	-
Caffeic acid	2	11.40	0.45±0.04	-
Rutin	3	11.84	101.23±0.38	-
Luteolin	4	12.33	3.13±0.12	-
Kaempferol	5	13.00	-	0.30±0.02
Rosmarinic acid	6	13.77	-	-
Myricetin	7	14.51	0.03±0.01	-
Quercetin	8	16.15	0.30±0.01	0.40±0.04
Coumarin	9	16.81	-	-
Apigenin	10	17.34	1.49±0.01	1.17±0.03

RT: Retention time
AM: Methanol extract of field-grown leaves
IM: Methanol extract of i*n vitro*-grown callus

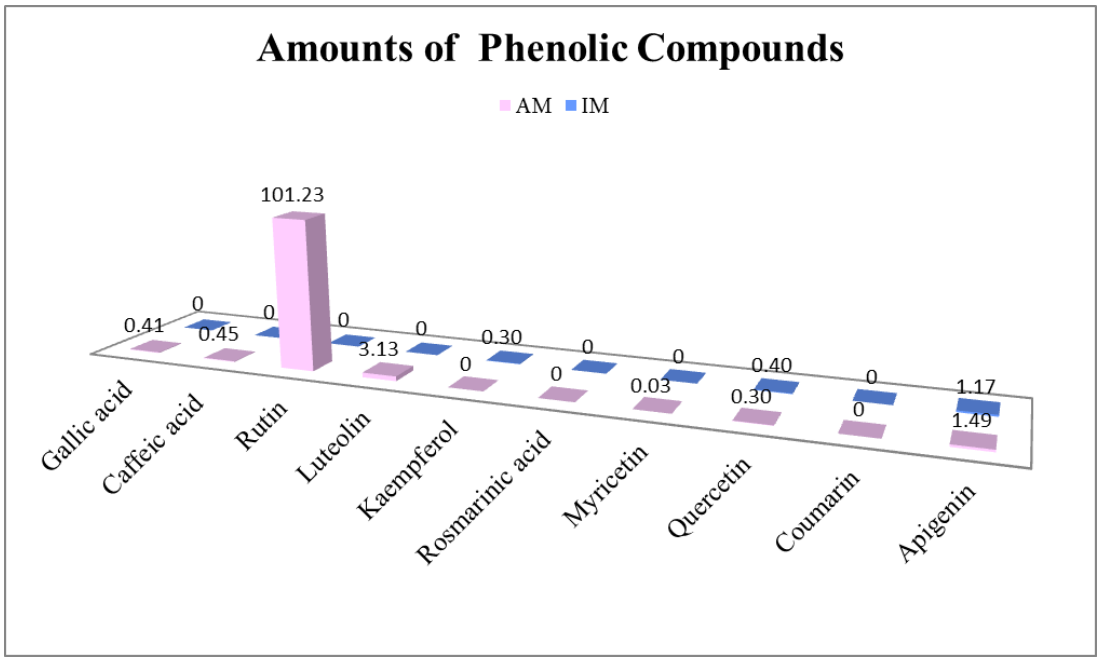

Figure 4.15. Phenolic compounds and their amounts in the methanol extracts of field-grown leaves (AM) and *in vitro*-grown callus (IM) of *A. gymnolobus.*

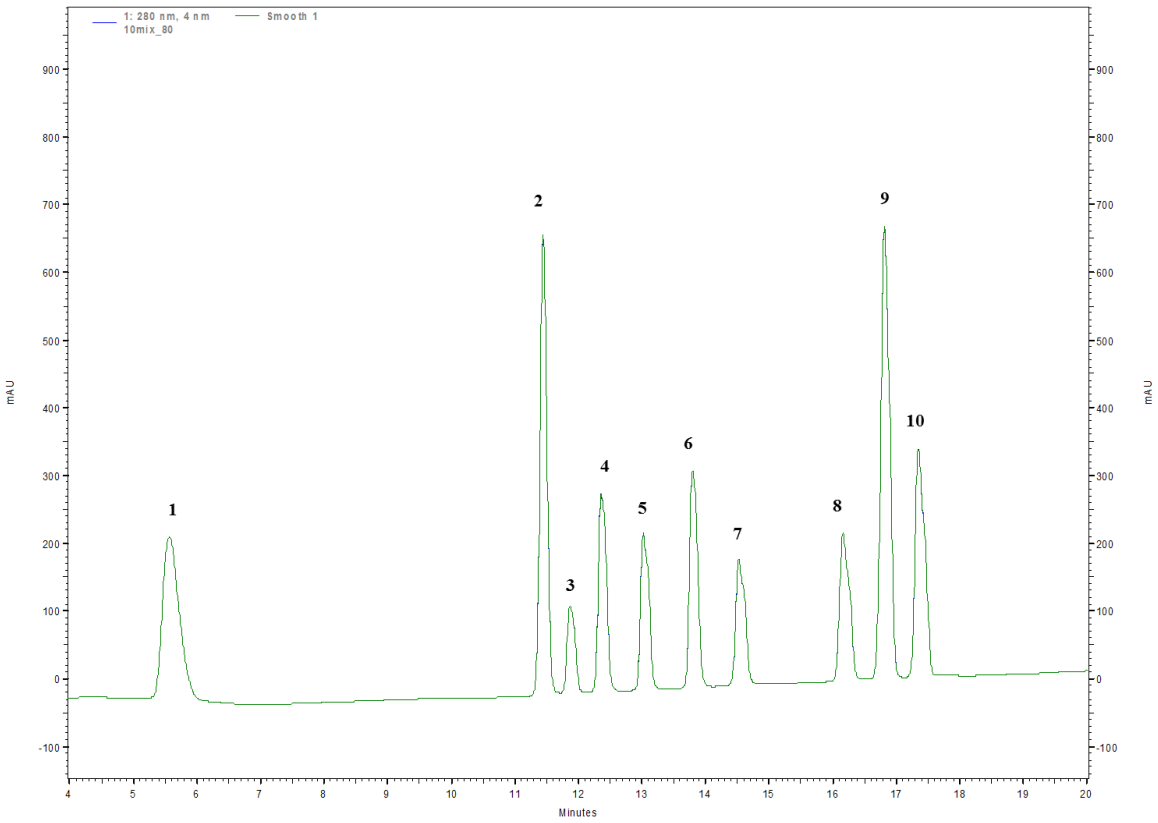

Figure 4.16. Chromatogram of the selected standarts. 1. Gallic acid, 2. Caffeic acid, 3.Rutin , 4.Luteolin, 5.Kaempferol, 6.Rosmarinic acid, 7.Myricetin, 8.Quercetin, 9.Coumarin, 10.Apigenin.

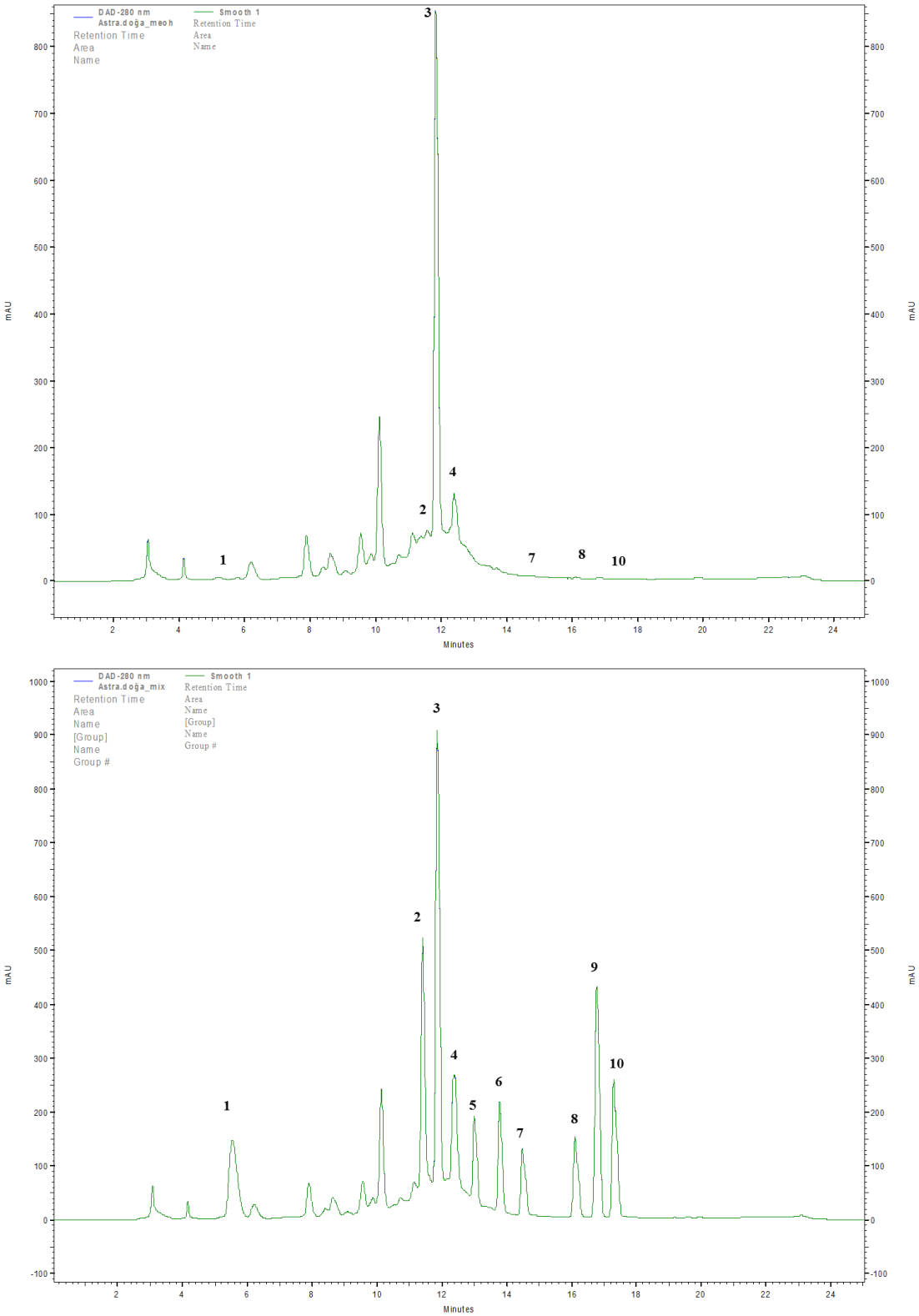

Figure 4.17. Chromatogram of field-grown methanolic leaf extract (above); Chromatogram of field-grown methanolic leaf extract with mixture of 10 phenol standards (below).1. Gallic acid, 2. Caffeic acid, 3.Rutin , 4. Luteolin, 5.Kaempferol, 6.Rosmarinic acid, 7.Myricetin, 8.Quercetin, 9.Coumarin, 10.Apigenin.

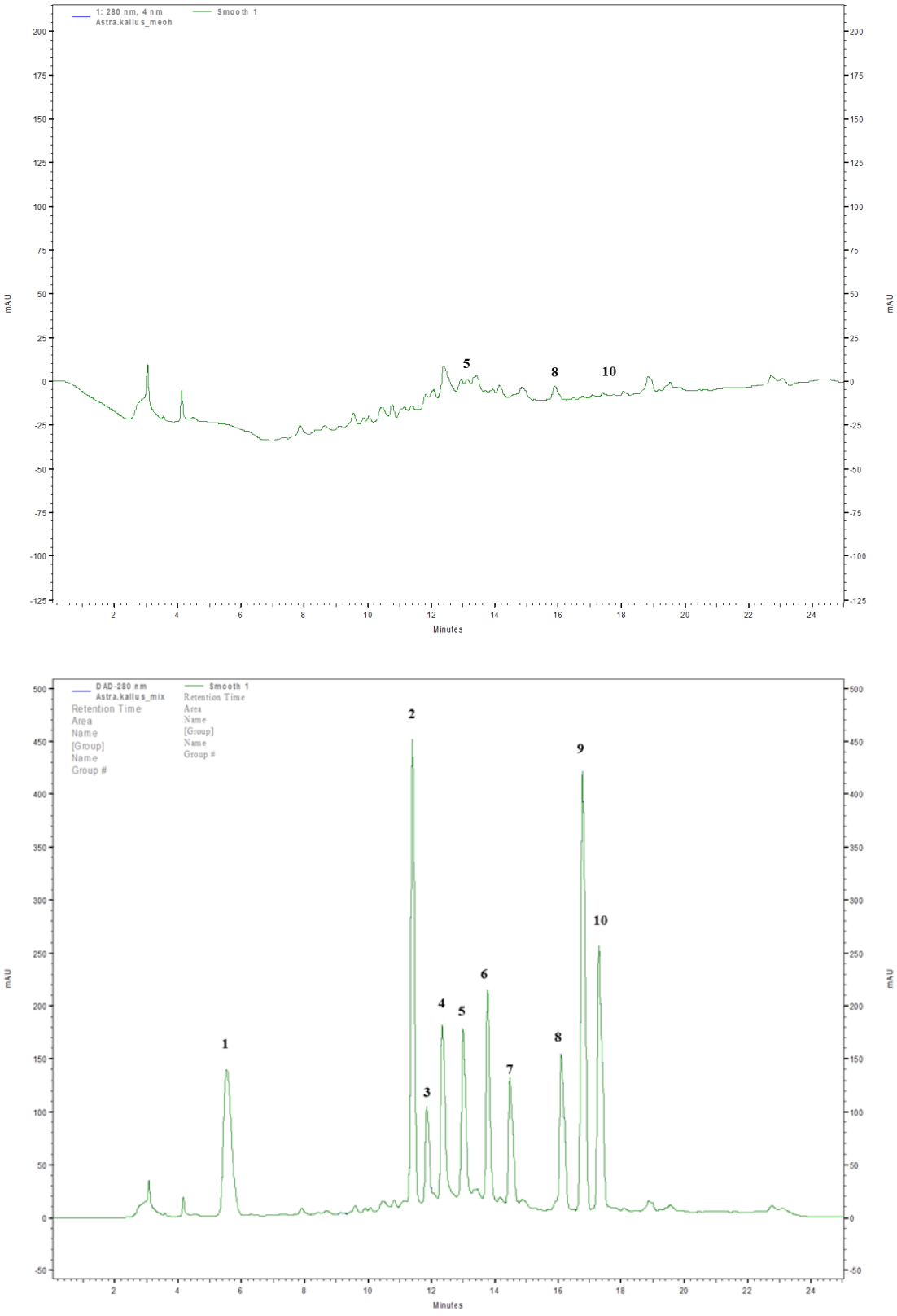

Figure 4.18. Chromatogram of in vitro-grown methanolic callus extract (above); Chromatogram of in vitro-grown methanolic callus extract with mixture of 10 phenol standarts (below). 1. Gallic acid, 2. Caffeic acid, 3.Rutin , 4. Luteolin, 5.Kaempferol, 6.Rosmarinic acid, 7.Myricetin, 8.Quercetin, 9.Coumarin, 10.Apigenin.

5. CONCLUSIONS

Astragalus species have a long history of use in folk medicine in the treatment of nephritis, diabetes, leukemia, and cancer (Tang W, 1992) and this genus are very old and well known curative plants with immunostimulant, hepatoprotective, antiperspirant, diuretic, and tonic properties (Tang and Eisenbrand, 1992). There has been no study about tissue culture of *A. gymnolobus* up to now. An *in vitro* culture protocol was established for this endemic plant with this study. With this efficient protocol, disease- and herbicide-free bulk plant material can be supplied throughout the year for pharmaceutical purposes. Two different explants (leaf and petiole) were used for tissue culture experiments. Best shoot proliferation was obtained with leaf explants. Regeneration was achieved with TDZ alone or in combination with IAA. BA or KIN in combination with different auxins were not effective for shoot regeneration with all two explants. The greatest number of shoots per shooted explant was observed in media containing 0.5 mg/l TDZ with leaf explants. There was indirect organogenesis because callus was observed for leaf and petiole explants before shoot formation. Best callus formation was obtained from leaf explants with MS medium containing 3.0 mg/l BA and 0.5 mg/l 2,4-D. Root development was not observed with different rooting media.

Two different sources of plant (field-grown leaves and *in vitro*-grown callus) were used for extractions and biological activity studies. Two different biological activities (antibacterial and antioxidant) were performed for evaluation between field-grown leaves and *in vitro*-grown callus using four different extracts [methanol extracts of *in vitro*-grown callus (IM) and field-grown plant leaves (AM), water extract of *in vitro*-grown (IW) and field-grown plant leaves (AW)].

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott et al., 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, *Enterobacter cloacae* and *Klebsiella pneumoniae* which are gram Gram-negative bacteria and *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* which are gram-positive bacteria. Generally, field-grown leaves showed better antibacterial activities than *in vitro*-grown callus. Methanol extracts of field-grown leaves showed antibacterial

activity against *S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa and P. vulgaris.* Best inhibitory activity was observed with methanol extract of field-grown leaves against *S. pyogenes*. Water extracts of field-grown leaves did not show activity against to used bacteria. Methanol and water extract of *in vitro*-grown callus exhibited a little antibacterial activity against only *S. pyogenes.* Antibacterial activity of methanol extracts of field-grown leaves against *S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa and P. vulgaris* may explain why *Astragalus* spp. are used in folk medicine to treat nephritis, immunologic diseases (cause by *S. pyogenes*), sepsis, urinary tract infections (caused by *S. aureus, S. epidermidis*, *P. aeruginosa*, *P. vulgaris*).

Methanolic extract of field-grown leaves showed better antioxidant activity than methanolic extract of *in vitro*-grown callus. The best antioxidant activity was observed with field-grown leaves at 25 μg/ml concentrations (91.47 %). Methanol extract of field-grown leavesat 25 μg/ml concentration showed the similar radical scavenging activity in that of ascorbic acid. The free radical scavenging activity of *in vitro*-grown callus has little tendency to scavenge the free radicals according to methanol extract of field-grown leaves. The best antioxidant activities of methanol extracts of *in vitro*-grown callus showed at 200 μg/ml concentrations (51.75 %) that it did not show high active radical scavenge capability as much as ascorbic acid. The most phenolic and flavonoid content is included in methanol extract of field-grown leaves.

Two different methanol extracts of *A. gymnolobus* were subjected to HPLC analysis [Methanol extracts of field-grown leaves (AM) and *in vitro*-grown callus (IM)]. A mixture of 10 standard phenolic compounds (Gallic acid, caffeic acid, rutin, luteolin, kaempferol, rosmarinic acid, myricetin, quercetin, coumarin and apigenin) was eluted using HPLC. Concentrations of phenolic compounds in field-grown leaves were higher than the *in vitro*-grown callus. Rutin was dominant compound in methanol extract of field-grown leaves. The phenolic compounds found in methanol extracts of field-grown leaves were rutin, luteolin, apigenin, caffeic acid, gallic acid, quercetin and myricetin. The phenolic compounds found in methanol extracts of *in vitro*-grown callus were apigenin, quercetin and kaempferol. Apigenin was dominant compound in *in vitro*-grown callus extract.

It was observed that the antioxidant activity results were high where the concentrations of phenolic compounds were found higher. According to our results,

methanol extract of field-grown leaves contained more phenols than *in vitro*-grown callus thereby the best antioxidant activities were obtained by methanolic extract of field-grown leaves.

With this study, in vitro culture protocol of endemic *A. gymnolobus* plant was obtained for the first time. Comparison between field-grown leaves and *in vitro*-grown callus were performed with antibacterial and antioxidant activities, total phenolic and flavonoid content, and HPLC analysis of phenolic contents of *A. gymnolobus*. Future studies should focus on fractionation of the extracts having antibacterial and antioxidant activities to identify active components of different extracts of *A. gymnolobus.*

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