P. Nartop et al. / Hacettepe J. Biol. & Chem., 2019, 47 (2), 185–192

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Astragalus trojanus Stev. Batch Cultures: Cycloartane-type Metabolite Accumulation in Response to pH, Sucrose and Casein Hydrolysate

Astragalus trojanus Stev. Kesikli Kültürleri: pH, Sükroz ve Kazein Hidrolizat Etkisi ile Sikloartan-tip Metabolit Birikimi

Pınar Nartop[1*], Aynur Gürel[2], İsmail Hakkı Akgün[2], Erdal Bedir[3]

[1]Deparment of Biomedical Engineering, NKU Çorlu Engineering Faculty, Çorlu, Tekirdağ, Turkey.

[2]Department of Bioengineering, Engineering Faculty, Ege University, Izmir, Turkey.

[3]Department of Bioengineering, Engineering Faculty, Izmir Institute of Technology, Izmir, Turkey.

ABSTRACT

In this study, two grams of callus regenerated from stem and leaf explants of *Astragalus trojanus* Stev. were cultured in Woody Plant Medium (WPM) supplemented with 1 mg/L 2,4-D for four weeks and used as inoculum in order to investigate the effects of working volume and media composition. The highest biomass was obtained in 250 mL flask with astragaloside IV (1.66 µg/mg) and cycloastragenol (0.19 µg/mg) accumulation. Different concentrations of sucrose and casein hydrolysate (1 and 2 g/L) were also tested and the effect of pH was also investigated. Biomass accumulation cannot be enhanced, however, astragaloside IV and cycloastragenol content was ascended. The highest astragaloside IV (95.23 µg/mg) and cycloastragenol (5.93 mg/mg) accumulations were obtained at pH 6.8 and 2 g/L casein hydrolysate, respectively.

Key Words

Astragalus trojanus, kesikli kültür, astragalozit IV, sikloastragenol.

ÖZ

Bu çalışmada, *Astragalus trojanus* Stev. gövde ve yaprak eksplantlarından elde edilen iki gram ağırlığında kalluslar 1 mg/L 2,4-D içeren Woody Plant Medium (WPM) besin ortamında dört hafta boyunca kültüre alınmışlar ve çalışma hacminin ve besin ortamının etkilerinin incelenmesi için başlangıç materyali (inokulum) olarak kullanılmışlardır. En fazla biyokütle astragaloside IV (1.66 µg/mg) ve cycloastragenol (0.19 g/mg) içeriğiyle 250 ml'lik erlenden elde edilmiştir. Farklı konsantrasyonlarda sükroz ve kazein hidrolizat (1 ve 2 g/L) denemeleri yapılmış ve pH'ın etkisi de incelenmiştir. Biyokütle birikiminde artış sağlanamamış ancak astragaloside IV ve cycloastragenol içerikleri artmıştır. En yüksek astragaloside IV (95.23 µg/mg) ve cycloastragenol (5.93 µg/mg) içerikleri pH 6.8'de ve 2 g/L kazein hidrolizat denemesinde elde edilmiştir.

Anahtar Kelimeler

Astragalus trojanus, kesikli kültür, astragaloside IV, cycloastragenol.

Article History: Received: Mar 03, 2018; Revised: Feb 12, 2019; Accepted: Mar 18, 2019; Available Online: Sep 15, 2019.

DOI:

Correspondence to: P. Nartop, Deparment of Biomedical Engineering, NKU Çorlu Engineering Faculty, Çorlu, Tekirdağ, Turkey.

E-Mail: pinarnartop@gmail.com

INTRODUCTION

Higher plants are the rich source of secondary medicinal metabolites. Medicinal plants are mostly harvested from the wild and valuable secondary metabolites are generally extracted from these plants' roots, leaves, flowers, etc. After 1970's, it was realized that plant cell cultures could produce the secondary metabolites at high concentrations [1]. Today, plant cell and tissue cultures are the promising source for secondary metabolite production at high concentrations and these techniques are also used to investigate secondary metabolism pathways and plant physiology [2]. In order to enhance production of secondary metabolites via plant cell culture techniques, many biotechnological strategies such as manipulation of nutrients, modification of liquid media, optimizing the culture environment, biotransformation and immobilization have been used [3-5].

Astragalus trojanus Stev. (Fabaceae) is an endemic medicinal plant and member of the Astragalus genus which is represented by 445 species (224 are endemic) in the flora of Turkey [6,7]. It has been found as one of the notable species with high cycloartane-type glycoside content [8-10]. Aqueous root extracts of some Astragalus species are used in Turkish folkloric medicine as an antiperspirant, diuretic, tonic, wound-healing drug and for the treatment of diabetes mellitus, nephritis, leukemia, and uterine cancer [11,12].

Astragalus genus contains triterpene saponins which are thought to be effective in immune system stimulation, and have anticancer and wound healing effects [13,14]. Astragalus genera are the richest source of cycloartanes, the unique triterpenoids with a characteristic 9,19-cyclopropane ring, and cycloastragenol is a key intermediate in the biosynthesis of different phytosterols [15]. It has been shown that cycloastragenol (CA), the main aglycon of many cycloartane-type glycosides, extends T cell proliferation by increasing telomarase activity which helps the delay the onset of cellular aging and pharmacological telomerase-based therapy enhances immune function [16]. CA has been introduced to the dietary supplement market as a new antiaging entity. As the content of this compound in Astragalus species is very low, ranging between 0.1% and 0.5%, and transformation of the glycosides into their aglycone (CA) is problematic due to acid labile property of the cyclopropane ring, new methodologies must be established to satisfy the increasing demand for these high value-added compounds. It is also reported that AST IV was listed in the 2005 edition of Pharmacopoeia of the People's Republic of China [17,18].

Despite their importance, there has been only a limited number of reports about plant tissue cultures of Astragalus genus and most of them are about shoot and organ regenerations and hairy root cultures [19-22]. In our previous study, we have found 3.5 µg/mg astragaloside IV (AST IV) and 4.8 µg/mg CA in callus tissues regenerated from the stems of *Astragalus trojanus* in dark conditions [18].

In this study, we investigated the effects of pH and different sucrose and casein hydrolysate (CH) concentrations on AST IV and cycloastragenol accumulation in batch cultures of *A. trojanus.*

MATERIALS and METHODS

Stems and leaves of 4-week-old in vitro micropropagated plantlets grown in semi-solid Woody Plant Medium (WPM) [23] medium supplemented with 1 mg/L 6-benzyladenine (BA), 3% sucrose and 0.7% agar were cut into 1 cm segments and placed into semi-solid WPM media supplemented with 1 mg/L 2,4-D, %0.7 agar and %3 sucrose (WD3). The pH of the media was adjusted to 5.8. The media were autoclaved for 15 minutes at 121°C and a pressure of 1.2 kg/cm^3. Stem and leaf explants were incubated under dark conditions at 24±1°C. 2 g of four-week old calli obtained from stem and leaf explants on WD3 media were inoculated into 50 mL liquid medium, with the same composition except for agar, in 250 mL flasks with three replicates. They were cultivated on an orbital shaker at 100 rpm and 24±1°C under dark conditions. After four weeks, suspension cultures were diluted to their half concentration with liquid WD3 medium and subcultured two times with the intervals of 14 days. After the second subculture, suspension cultures grown in WD3 medium were inoculated, with 1/2 ratio, into the modified WD3 media listed in Table 1. Suspension of the yellow and red cells was obtained from leaf and stem explants, respectively. In order to determine the effect of working volume in batch cultures, 10 and 20 mL suspension cultures in WD3 medium were cultivated in 50 and 100 mL flasks, respectively. Only yellow cells were used in this experiment.

Table 1. Contents of media inoculated with suspension cultures grown in WD3 medium.

Media Code	Basal Medium	2,4-D (mg/L)	Sucrose (g/L)	pH	Content
WD3*	WPM	1	30	5.8	0 mL/L-CE
WD3+CE*	WPM	1	30	5.8	100 mL/L-CE
WD4*	WPM	2	30	5.8	0 mL/L-CE
WD5*	WPM	4	30	5.8	0 mL/L-CE
WD3**	WPM	1	30	5.8	0 mL/L-CE
WD3P48***	WPM	1	30	4.8	0 g/L CH
WD3P68***	WPM	1	30	6.8	0 g/L CH
WD3S15***	WPM	1	15	5.8	0 g/L CH
WD3S45***	WPM	1	45	5.8	0 g/L CH
WD3CH1***	WPM	1	30	5.8	1 g/L CH
WD3CH2***	WPM	1	30	5.8	2 g/L CH

*Suspended yellow cells only
**Suspended red cells only
***Suspended yellow and red cells

In our study, CE was prepared similarly as mentioned by Ziebur and Shrift (1971) [24]. 175 g fresh sweet corn was grinded in a mortar and boiled in 100 mL reverse-osmosis water for 5 min. The liquid was first filtered through cheesecloth then a filter paper under vacuum filtration. 5 mL of the filtrate was added to 45 mL liquid medium (10% v/v) (Table 1) and autoclaved for 15 minutes at 121°C and a pressure of 1.2 kg/cm^3.

Living cell and total cell numbers of suspension cultures grown in WD3 medium were determined using trypan blue with an interval of 7 days. The viability percentage of culture (%) (living cell number per mL x 100/ total cell number per mL) was also determined. At the end of culture, the biomass was filtered through normal filter paper to identify the total fresh weights of biomass for each flask. After drying, dry weights (g/L) and dry weight percentages (%) (dry weight x 100 /fresh weight) were calculated.

In order to determine settled cell volume percentages, suspended cells in 50 mL culture media were allowed to settle for 60 min in a graduated glass tube and the fraction of the whole culture volume occupied by cells was determined as the settled cell volume (%) (settled cell volume – mL x 100/ total volume of the culture – mL) [25].

All the samples were extracted for 15 minutes with 5 mL HPLC grade methanol for 3 times. After sonication, samples were centrifuged until clear extracts were obtained. All of the clear extracts were combined and evaporated under vacuum. Evaporated samples were lyophilisated to get dry extracts. Dried extracts were dissolved with HPLC grade methanol to obtain concentration of 5 mg/mL. All the sample solutions were passed through 0.45 nylon membrane filters prior to injections.

HPLC-ELSD analyses were performed on a Thermo Surveyor Plus instrument, equipped with a quaternary pump, autosampler, column oven, diode array (Thermo Fisher Scientific, MA, USA) and Softa 300S ELSD detector (SofTA Corporation, CO, USA). For all separations, a Thermo Hypersil GOLD RP (100x4.6 mm, 5 µm particle size; Thermo Fisher Scientific, MA, USA) HPLC column was used. LC separations were carried out using following solvents: water (A) and acetonitrile (B) and gradient elution was performed as: 0 min 72A/28B, in 5 min to 70A/30B, in 4 min to 38A/62B, in 3 min to 30A/70B hold for 3 min. Additionally, column was washed with 5A/95B for 2.5 min and prior to the next injection, the column was equilibrated for 2.5 min with the beginning conditions. Detection was performed with ELSD detector with the settings as: 40°C at spray chamber, 70°C at the operating chamber, 105°C at drift tub and N2 pressure 50 psi. Flow rate was 2 mL/min, column temperature was 30°C and the injection volume was 10 µL.

Two main compounds of Astragalus species, astragaloside IV (AST IV) and cycloastragenol (CA), were calibrated for quantitative analysis of samples. Standard stock solutions were prepared with methanol (2000 µg/mL) and additional six levels were prepared by dilution

of stock solutions (1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 62.5 µg/mL, 25 µg/mL) with methanol. Retention times for AST IV was 7.22 min and CA was 11.23 min. Regression coefficient for AST IV was 0.9958 and for CA was 0.9973.

All experiments were repeated three times. Experiments were implemented in a factorial randomized plots design with one factor (media). Data were analysed with one way ANOVA test and post hoc LSD tests were performed.

RESULTS and DISCUSSION

In the fourth week of batch cultures of yellow cells (Table 2), the fresh and dry weights were found the highest in 250 mL flask. The fresh weight in 250 mL flask was found 0.463 g/L, 2.7 times higher than the 100 mL flask and nearly 3.3 times higher than the 50 mL flask. The dry weight in 250 mL flask was also found two times higher than 100 mL flask and 7.8 times higher than 50 mL flask. In dry weight percentages, 100 and 250 mL flasks were found very close to each other (11.262% and 10.439%), whereas 50 mL flask was found nearly three times lower. Fresh and dry weights and dry weight percentages are the parameters for primary metabolism [26]. These results showed that when the working volume was increased to 250 mL, primer metabolism was triggered and as a consequence of this biomass accumulation was ascended.

Increase in working volume affected both primary and secondary metabolism. AST IV accumulation was only found in 250 mL flask at 1.66 µg/mg concentration and cannot be detected in 50 and 100 mL flasks. However, in CA accumulation, the higher concentration (5.67 µg/mg) was detected in 50 mL flask. In 250 mL flask, CA concentration was found at lower concentration (0.19 µg/mg) compared to 50 mL flask, whereas CA cannot be detected in 100 mL flask.

Both in yellow and red cell suspension cultures in WD3 medium in 250 mL flask, living and total cell number/mL started to decrease after the fourth week of culture (Table 3). Similarly, viability percentages were also found higher in the first four weeks of the culture period. The biomass accumulated in yellow cell suspension cultures were very low (0.013 g/L) when compared to red cell suspension cultures (2.025 g/L) (Table 4b). In order to enhance the biomass of yellow cell culture in WD3 medium, concentration of 2,4-D was increased and corn extract (CE) was also used. However, the highest biomass was accumulated in suspension cultures of red cells in WD3 medium.

According to SCV percentages (Table 4a), the values were started to decrease in the fourth week of the culture period, which also showed correlation with the values of cell numbers/mL shown in Table 3. As a result of these experiments, the culture period of batch cultures, mentioned in Table 5, was terminated in the fourth week.

CE is used to stimulate cell division activity in various plant species and known to contain zeatin and zeatin riboside [27]. CE was used as callus regeneration stimulator in some Astragalus species by Ziebur and Shrift (1971) [24]. In yellow cell suspension cultures, fresh and dry weights and dry weight percentage were found higher in CE added medium (Table 4b). However, in Table 4a, at the beginning of culture period (day 0), SCV of WD3+CE medium was higher more than three times of other yellow cell suspension cultures (WD3, WD4, and WD5). CE is not soluble in water and suspends like cells in cell cultures. As a consequence, CE settled like suspended cells, enhanced the SCV, fresh and dry weights and dry weight percentage.

The change at pH from 5.8 to 4.8 and 6.8 reduced biomass accumulation are higher than two times in WD3 medium. pH is one of the most effective factors

Table 2. Fresh (FW) and dry (DW) weights, dry weight percentages (DWP) (%), AST IV and CA concentrations of yellow cell suspension cultures in WD3 medium.

Flask Volume (mL)	50 mL	100 mL	250 mL
FW (g/L)	0.141±0.023	0.172±0.033	0.463±0.169
DW (g/L)	0.005±0.002	0.019±0.005	0.048±0.020
DWP (%)	3.791	11.262	10.439
AST IV (µg/mg)	0.00	0.00	1.66
CA (µg/mg)	5.67	0.00	0.19

Table 3. Living and total cell numbers/mL and viability of yellow and red cell suspension cultures in WD3 medium.

Days of Culture Period		0	7	14	21	28	35	42
Yellow cells	Living Cell Number/mL	$22x10^3$	$30x10^3$	$52x10^3$	$56x10^3$	$54x10^3$	$46x10^3$	$38x10^3$
	Total Cell Number/mL	$38x10^4$	$31x10^4$	$60x10^4$	$64x10^4$	$72x10^4$	$51x10^4$	$55x10^4$
	Viability (%)	80.00	95.24	86.42	82.56	74.23	89.86	68.92
Red cells	Living Cell Number/mL	$17x10^4$	$23x10^4$	$28x10^4$	$28x10^4$	$27x10^4$	$26x10^4$	$25x10^4$
	Total Cell Number/mL	$20x10^4$	$27x10^4$	$32x10^4$	$32x10^4$	$32x10^4$	$32x10^4$	$33x10^4$
	Viability (%)	84.74	84.47	85.51	87.52	83.21	79.73	76.42

in biomass and secondary metabolite accumulation in plant cell and tissue cultures. In hairy roots of Ansodus acutangulus, two times higher biomass was obtained at pH 6.5 when compared to pH 4.5, however alkaloid production was found higher at pH 4.5 [28]. In our study, pH 4.8 and 6.8 enhanced AST IV accumulation in yellow cell suspension cultures. At pH 6.8, AST IV accumulation was detected at 95.23 µg/mg, whereas 11.30 µg/mg at pH 4.8. Accumulation of CA was also stimulated at pH 6.8, however it cannot be detected at pH 4.8. In red cell suspension cultures, CA accumulation was stimulated at pH 6.8 (0.21 µg/mg) and AST IV cannot be detected.

Sucrose concentrations were also found effective on both biomass and secondary metabolite accumulation. Biomass was decreased at both concentrations of sucrose, however AST IV was enhanced from 1.66 µg/mg to 76.65 µg/mg at 45 g/L sucrose concentration compared to control in yellow cell suspension cultures. Except from WD3S45 medium, AST IV and CA cannot be detected at both sucrose concentrations. Increase of sucrose concentration at plant cell cultures generally causes higher biomass [29]. However, in our study, both increase and decrease at sucrose concentration caused lower biomass accumulation. When compared to 15 g/L sucrose concentration, biomass accumulation was found higher at 45 g/L concentration. Sucrose may cause osmotic stress in plant cell and tissue cultures. The lower osmotic strength may result in improved response in growth of cultures [30].

Table 4. (a) Settled cell volume (SCV) (%) and (b) fresh and dry weights (g) and dry weight percentages (%) of yellow/ and red cell suspension cultures.

Table 4a

Medium Code	0.Day	7.Day	14.Day	21.Day	28.Day	35.Day	42.Day
WD3*	1.59±0.21	2.60±0.49	2.13±0.41	2.37±0.15	1.90±0.20	1.58±0.06	1.72±0.28
WD3+CE*	6.26±0.08	6.68±0.26	5.02±0.40	4.54±0.43	4.87±0.62	4.41±0.40	4.21±0.61
WD4*	2.00±0.24	2.12±0.41	2.16±0.05	1.93±0.26	1.46±0.84	1.48±0.03	1.36±0.13
WD5*	1.56±0.23	1.79±0.16	1.90±0.20	1.63±0.22	1.63±0.22	1.65±0.23	1.37±0.12
WD3**	7.37±1.45	9.82±1.73	11.75±0.80	11.79±0.97	11.91±0.73	11.72±0.28	11.69±0.88

Table 4b

Medium Code	WD3*	WD3+CE*	WD4*	WD5*	WD3**
FW(g/L)	0.013±0.00 b	0.178±0.075 b	0.018±0.003 b	0.016±0.005 b	2.025±0.045 a
DW(g/L)	0.0043±0.0008 c	0.0239±0.0094b	0.0008±0.0004d	0.0008±0.0005d	0.2222±0.0065 a
DW (%)	33.94	13.47	4.55	4.94	10.98

Values within column followed by different small letters are significantly different at the 0.01 level by LSD's test.
*Yellow cells
**Red cells
FW=p<0.01;F=513.798;MSE:0.005;LSD:0.175
DW=p<0.01;F=354.448;MSE:0.000;LSD:0.023

Table 5. Fresh (FW) and dry (DW) weights (g) and dry weight percentages (DWP) (%) of yellow and red cell suspension cultures in the fourth week of culture.

Medium Code	Yellow Cells			Red Cells		
	FW(g/L)*	DW(g/L)	DWP(%)	FW(g/L)**	DW(g/L)***	DWP(%)
WD3(Control)	0.0859±0.0295a	0.0097±0.000	11.249	0.463±0.169a	0.048±0.020a	10.49
WD3P48	0.0325±0.008 b	0.0043±0.001	13.23	0.0087±0.008b	0.0004±0.0001b	4.598
WD3P68	0.0391±0.0128b	0.0044±0.0023	11.24	0.0070±0.0025b	0.0003±0.0001b	4.286
WD3S15	0.0181±0.0073b	0.0010±0.0004	5.51	0.0029±0.0005b	0.0001±0.0000b	3.448
WD3S45	0.0116±0.0018b	0.0006±0.0001	5.46	0.0033±0.0014b	0.0003±0.0000b	9.091
WD3CH1	0.0122±0.0052b	0.0006±0.0003	4.9	0.0077±0.0029b	0.0004±0.0001b	5.195
WD3CH2	0.0231±0.0109b	0.0015±0.0011	6.49	0.0118±0.0019b	0.00078±0.0000b	6.780

Values within column followed by different small letters are significantly different at the 0.01 or 0.05 level by LSD's test.
*p<0.05;F=3.622;MSE:0.001;LSD:0.04(Fresh weight of yellow cells)
**p<0.01;F=7.312;MSE:0.012;LSD:0.268(Fresh weight of red cells)
***p<0.01;F=5.801;MSE:0.000;LSD:0.032(Dry weight of red cells)

Addition of CH at 1 and 2 g/L concentrations did not enhance biomass accumulation in batch cultures of yellow cell suspension cultures (Table 5). However, 2 g/L CH increased AST IV accumulation approximately 13 times (21.49 µg/mg). CA accumulation was also enhanced 31 times in WD3CH2 medium (5.93 µg/mg) (Table 6). CH is generally used in order to induce growth in plant cell and tissue cultures, especially in plant regeneration [31,32]. It is a source of calcium, phosphate, several microelements, vitamins, and 18 amino acids. Moreover, it is more effective than the addition of the major amino acids which it provides in plant tissue cultures [33,34]. In our study, CH did not stimulate growth, however AST IV and CA contents were enhanced.

In red cell suspension cultures, AST IV cannot be detected any of the samples tested, however, CA accumulation was detected in two samples obtained from WD3P68 (0.21 µg/mg) and WD3S15 (2.12 µg/mg). In our study, sucrose, pH, and CH did not enhance biomass accumulation, however, AST IV and CA contents increased. The highest AST IV content was detected at 95.23 µg/mg concentration in WD3P68 medium, whereas maximum CA content was 5.93 µg/mg in WD3CH2 medium in yellow cell suspension cultures. In our study, AST IV contents of the native plant were found 36.2 µg/mg in roots and 30.9 µg/mg in stems, whereas CA contents were found 3 µg/mg in roots and 2.3 µg/mg in stems. Ionkova et al. (1997) reported that astragaloside contents (Astragalosides I, II and III) of hairy root cultures of Astragalus mongholicus were approximately 2% of dry weight (20 µg/mg) [21]. In Astragalus membranaceus hairy root cultures, AST IV amount was found 1.4 mg/g [22]. In Radix Astragali, the average content of AST-IV was found to be 0.016% (0.16 µg/mg) [35].

According to our data, it was proved that cell suspension cultures of A. trojanus have a potential of AST IV and CA production, however, biomass of the batch cultures were very low. Therefore, different medium compositions and techniques can be used in order to increase biomass accumulation in further studies.

Table 6. AST IV and CA concentrations of yellow and red cell suspension cultures.

Medium Code	Yellow Cells		Red Cells	
	AST IV(µg/mg)	CA(µg/mg)	AST IV(µg/mg)	CA(µg/mg)
WD3-control	1.66	0.19	ns	ns
WD3P48	11.30	ns	ns	ns
WD3P68	95.23	4.24	ns	0.21
WD3S15	ns	ns	ns	2.12
WD3S45	76.65	ns	ns	ns
WD3CH1	ns	ns	ns	ns
WD3CH2	21.49	5.93	ns	ns

ns: non-significant

Acknowledgments

The financial support of The Scientific and Technical Research Council of Turkey (TUBITAK) (Project No. TOVAG-109O627) and Ege University Science and Technology Centre (EBILTEM) (BAP-2012/BİL/017) are gratefully acknowledged.

References

1. I. Smetanska, Production of secondary metabolites using plant cell cultures, Adv. Biochem. Eng. Biotechnol., 111 (2008) 187-228.
2. M.Singh, B.Roy, V. Tandon, R. Chaturvedi, Extracts of dedifferentiated cultures of Spilanthes acmella Murr. possess antioxidant and anthelmintic properties and hold promise as an alternative source of herbal medicine, Plant Biosyst., 148 (2014) 259-267.
3. M.S. Hussain, S. Fareed, S. Ansari, M.A. Rahman, I.Z. Ahmad, M. Saeed, Current approaches toward production of secondary plant metabolites, J. Pharm. Bioallied. Sci., 4 (2012) 10-20.
4. P. Nartop, A. Gürel, Immobilization of Plant Cells and Its Effects on Secondary Metabolite and Biomass Production, Biotechnology and Genetic Engineering II of Recent Progress in Medicinal Plants Series, Vol.39:1-933699-99-X Series, ISBN:0-9656038-5-7, 2014, Studium Press.
5. P. Nartop, Ş. Akay, A. Gürel, Immobilization of Rubia tinctorum L. suspension cultures and its effects on alizarin and purpurin accumulation and biomass production, Plant Cell Tiss. Org. Cult., 112 (2013) 123-128.
6. P.H. Davis, Flora of Turkey and East Aegean Islands, University Press, Edinburgh, 1970, 3, 164.
7. E. Bedir, I. Çalış, S. Piacente, C. Pizza, IA. Khan, New flavonol glycoside from the aerial parts of Astragalus vulneraria, Chem. Pharm. Bull., 48 (2000a) 1994-1995.
8. E. Bedir, I. Çalış, R. Aquino, S. Piacente, C. Pizza, Secondary metabolites from the roots of *Astragalus trojanus*. J. Nat. Prod., 62 (1999) 563-568.
9. E. Bedir, I. Çalış, R. Aquino, S. Piacente, C. Pizza, Trojanoside H: A cycloartane-type glycoside from the aerial parts of *Astragalus trojanus*, Phytochemistry, 51 (1999) 1017-1020.
10. E. Bedir, I. Tatlı, I. Çalış, I.A. Khan, Trojanosides I-K: New cycloartane-type glycosides from the aerial parts of *Astragalus trojanus*, Chem. Pharm. Bull., 49 (2001) 1482-1486.
11. T. Savran, D. Gülcemal, M. Masullo, T. Karayıldırım, E. Polat, S. Piacente, Ö. Alankuş-Çalışkan, Cycloartane Glycosides from Astragalus erinaceus, Rec. Nat. Prod., 6 (2012) 230-236.
12. Y. Cai, Q. Luo, M. Sun, H. Corke, Antioxidant activity and phenolic compounds of 112, traditional chinese medicinal plants associated with anticancer, Life Sci., 74 (2004) 2157-2184.
13. E. Bedir, N. Pugh, I. Çalış, D.S. Pasco, I.A. Khan, Immunostimulatory effects of cycloartane-type triterpene glycosides from Astragalus species, Biol. Pharm. Bull., 23 (2000b) 834-837.
14. S. Hasançebi, N. Turgut-Kara, Ö. Çakır, Ş. Arı, Micropropagation and root culture of Turkish endemic Astragalus chrysochlorus (Leguminosae), Turk. J. Bot., 35 (2011) 203–210.
15. A. Nalbantsoy, T. Nesil, Ö. Yılmaz-Dilsiz, G. Aksu, S. Khan, E. Bedir, Evaluation of the immunomodulatory properties in mice And ın vitro anti-Inflammatory activity of cycloartane-type saponins from Astragalus species, J. Ethnopharmacol., 139 (2012) 574–581.
16. H.F. Valenzuela, T. Fuller, J. Edwards, D. Finger, B. Molgora, Cycloastragenol extends T-cell proliferation by increasing telomerase activity, J. Immunol., 182 (2009) 90–30.
17. S. Wang, H. Huang, W. Gao, C. Zhuang, B. Li, P. Zhou, D. Kong, Anti-hepatitis B virus activities of astragaloside IV isolated from Radix Astragali, Biol. Pharm. Bull., 32 (2009) 132–135.
18. P. Nartop, A. Gürel, I.H.Akgün, E. Bedir, Astragaloside IV and cycloastragenol production capacity of *Astragalus trojanus* calli, Rec. Nat. Prod., 9 (2015) 49-61.
19. A.M.A. Abd El-Mavla, Production of cycloartane triterpene glycosides in shoot cultures of Astragalus sieberi DC, Nat. Prod. Res., 24 (2010) 416-422.
20. S. Erişen, E. Atalay, M. Yorgancılar, The effect of thidiazuron on the in vitro shoot development of endemic Astragalus cariensis in Turkey, Turk. J. Bot., 35 (2011) 521-526.
21. I. Ionkova, T. Kartnig, W. Alfermann, Cycloartane saponin production in hairy root cultures of Astragalus mongholicus, Phytochemistry, 45 (1997) 1597-1600.
22. M. Du, X.J. Wu, J. Ding, Z.B. Hu, K.N. White, C.J. Branford-White, Astragaloside IV and polysaccharide production by hairy roots of Astragalus membranaceus in bioreactors, Biotechnol. Lett., 25 (2003) 1853-1856.
23. G.B. Lloyd, B.H. McCown, Commercial-feasible micropropagation of mountain laurel- kalmia latifolia by use of shoot- tip culture. Proc. Int. Plant Prop. Soc., 30 (1980) 421-427.
24. N.K. Ziebur, A. Shrift, Response to selenium by callus cultures derived from Astragalus species. Plant Physiol., 47 (1971) 545-550.
25. D.D.Y. Ryu, S.O. Lee, R.J. Romani, Determination of growth rate for the plant cell cultures: Comparative studies, Biotechnol. Bioengin., 35 (1990) 305-311.
26. R. Krishnan, V.S.A. Kumar, K. Murugan, Establishment of cell suspension culture in Marchantia linearis Lehm&Lindenb. for the optimum production of flavonoids, Biotech. (2013) DOI 10.1007/s13205-013-0123-7.
27. N.C. Vora, Y.T. Jasrai, Natural and low-cost substitutes of synthetic PGR for micropropagation of banana. CIBTech. J. Biotechn., 2 (2012) 9-13.
28. Q. Liu, L. Cui, Y. Guo, X. Ni, Y, Zhang, G. Kai, Optimization of nutritive factors in culture media for growth and tropane alkaloid production from Anisodus acutangulus hairy roots, J. Appl. Pharm. Sci., 3 (2013) 1-4.
29. I-S. Park, D-I. Kim, Significance of fresh weight to dry cell weight ratio in plant cell suspension cultures. Biotechnol. Tech., 7 (1993) 627-630.
30. L.A. Caro, P.A. Polci, L.I. Lindstrom, C.V. Echenique, L.F. Hernandez, Micropropagation of Prosopis chilensis (Mol.) Stuntz from young and mature plants, Biocell, 26 (2002) 25-33.
31. S. Pola, S.N. Mani, T. Ramana, Long-term maintanence of callus cultures form ımmature embryo of Sorghum bicolor, World J. Agric. Sci., 5 (2009) 415-421.
32. A.T. Roy, A. Koutoulis, D.N. De, Cell Suspension Culture and Plant Regeneration in The Latex-Producing Plant, Calotropis gigantea (Linn.) R. Br. Plant Cell Tiss. Org. Cult., 63 (2000) 15-22.

33. E.F. George, G-J. de Klerk, The Components of Plant Tissue Culture Media I: Macro- and Micro-Nutrients. In George EF, Hall MA, de Klerk G-J, eds., Plant Propagation by Tissue Culture, 3rd Edition, Vol. 1. The Background. Springer-Verlag, Dordrecht (2008) pp.65-113.
34. Z. Molnar, E. Virag, V. Ördög, Natural substances in tissue culture media of higher plants. Acta Biologica Szegediensis, 55 (2011) 123-127.
35. W. Li, J.F. Fitzloff, Determination of astragaloside IV in Radix Astragali (Astragalus membranaceus var. monghulicus) using high-performance liquid chromatography with evaporative light-scattering detection, J. Chromatogr. Sci., 39 (2001) 459-462.