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ARTICLE

Determination of triterpenoid saponin and polysaccharide content from *in vitro* cultures of *Astragalus mongholicus* Bunge

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Abstract: In this study, the efficient micropropagation protocol of *Astragalus mongholicus* Bunge was established and also triterpenoid saponin and polysaccharide content in ethanol, methanol and aqueous extracts of different samples were determined by using spectrophotometric methods to investigate whether the content of biologically active compounds depends on the stage of development of the plant during *in vitro* culture. The content of total saponins and polysaccharides in different cultures of *A. mongholicus* grown *in vitro* was higher (990 and 505 µg/ml) in ethanol extracted 14-day-old young shoot samples than in 28-day-old propagated shoot samples and rooted shoots.

Keywords: *Astragalus mongholicus; in vitro cultures; secondary metabolite; total triterpenoid saponin; polysaccharide;*

INTRODUCTION

There are 111 species of *Astragalus* in Mongolia, 21 of them are endemic plants that grow only in Mongolia [8, 32]. There have been determined the Saponins, flavonoids, and polysaccharides compounds, which are beneficial to human health, have been determined in *Astragalus* [18, 24, 29]. Many species have been used in traditional medicine for their immunostimulant, anti-inflammatory, antioxidative, cardioprotective, hepatoprotective, antiviral properties and ability to treat diabetes [14, 15, 17, 20, 22, 26, 30, 31, 34, 36, 38]. *Astragalus mongholicus* Bunge is a plant with rare status, which is important for sand setting, soil strengthening, and bee pastures during flowering. Soil nitrogen fixation can also be done by creating air nitrogen-accumulating bacteria through the roots, and it is used in hay pastures, hospitals and numerous medicines [12].

The study of the pharmacological role of *Astragalus* plants, we find that the demand for this plant is growing, but the natural resources have been dwindling from year by year, about which researchers from many countries have been warning [28].

This type of plant is currently being cultivated, but it takes 3-4 years for the cultivated plants to mature, while the content of biologically active compounds depends greatly on the environmental conditions of the geographical region where is the plants are cultivated [13, 19].

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Plant tissue culture system allows short-term clonal propagation of plants and year-round production of plant's biologically active compounds under controlled conditions [23]. Researchers in many countries believe that tissue culture may possibly solve the problem of field cultivation.

There are many reports on in vitro regeneration of *Astragalus* species such as *A. adsurgens* [21], *A. cariensis* Boiss [4], *A. chrysochlorus* [10], *A.cicer* L. [1], *A.maximus* [32], *A.melilotoides* [11], *A.nezaketae* [5] and *A.schizopterus* [35], but there are no reports on tissue culture of *Astragalus mongholicus*. This research was conducted to establish an effective protocol for the *in vitro* propagation of the rare medicinal plant *Astragalus mongholicus* and to determine the content of the main compounds in various cultures.

MATERIALS AND METHODS

Mature seeds of *Astragalus mongholicus* Bunge collected from Ikh Mongol Uul in Rashaant Soum, Bulgan aimag (Province), were used in the research. In order to increase the germination rate and dormancy breaking, the seeds were incubated in H_2SO_4 solution (98% v/v) for 1 hour followed by 3 rinses with sterile distilled water. They were surface-sterilized in ethanol (70% v/v) for 1 minute, followed by seed soaking in sodium hypochlorite (5.0% v/v) for 15 minutes, after which they were washed 5 times with sterile distilled water. The seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. Cultures were incubated in a growth chamber with a photoperiod of 16-hour light (24±2°C) and 8-hour dark (20±2°C).

Callus induction

Epicotyl and leaf explants were cultured in MS medium supplemented with 0.1-4.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) alone including a combination of 0.5 mg/L Kinetin and 6-Benzylaminopurine (BA). Callus formation and morphology were determined after 4 weeks. 10 explants were placed horizontally on the surface of the Petri dish with nutrient medium and incubated at 24±2°C in a dark conditions. Each experiment was repeated 3 times.

Shoot proliferation

For shoot proliferation, apical buds were cultured in MS medium supplemented with BA, Thidiazuron (TDZ) and Zeatin (0.5, 1.0, 2.0, 4.0 mg/L) alone and combined with 0.5 mg/L 1-Naphthaleneacetic acid (NAA). In each experiment, 10 explants were performed with 3 replicates. Cultures were grown in the culture room with 16-hour light and 8-hour dark-light period and a temperature of 24±2°C. After 4 weeks, cultures were subcultured in optimal nutrient medium. Four weeks after the start of the experiment, the number of shoots and plant height in each explant were counted and recorded.

Rooting

Proliferated shoots (1-2 cm tall) were used for rooting experiments. Rooting effect in 1/2 MS medium supplemented with 0.5-4.0 mg/L Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) combined with 0.5 mg/L NAA and 1/2 MS without hormone were tested. Cultures were grown in the culture room with 16-hour light and 8-hour dark-light period and a temperature of 24±2°C. Each rooting experiment was replicated 3 times using 10 shoots. Rooting percentage, root number and root length parameters were evaluated and recorded after 4 weeks of culture.

Determination of total saponin and polysaccharide content in plant samples grown *in vitro*

Sample extraction

A. mongholicus in vitro grown shoot culture initiation period (14 days old), proliferated shoots, rooted shoots, adventitious root culture and callus culture were weighed 2 g each and placed in three solvents: 20 ml of 70% ethanol, methanol and distilled water for 72 hours on a shaker. Before starting the phytochemical analysis, the shoots, root and callus culture was filtered through filter paper.

Calibration curve

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Individual standard solution was prepared in each test solvent (ethanol, methanol, aqueous extract).

Determination of polysaccharide

Preparation of blank solution

1 ml of 5% phenol followed by 5 mL of concentrated H_2SO_4 were added to each 1 mL of 70% ethanol, methanol and distilled water.

Preparation of standard solution

A stock solution of 1000 µg/ml of glucose was prepared in 96% ethanol, methanol and distilled water. Aliquots were taken from this solution to obtain sugar concentrations of 50-1000 µg/ml. 1 ml of 5% phenol solution was added to 1 ml of glucose solution followed by 5 ml of concentrated H2SO4. The absorbance was measured after 10 minutes at 488nm against blank.

Determination of polysaccharide content in samples

Phenol-sulfuric acid method for polysaccharide determination, 1 ml of the extract in the above 3 solvents was used for polysaccharide analysis. 1 ml of 5% phenol solution was added to 1 ml of extract followed by 5 ml of concentrated H_2SO_4. The absorbance was measured after 10 minutes at 488nm against blank. The experiment was repeated 3 times.

Determination of total saponin

Preparation of blank solution

0.25 mL of 8% vanillin, followed by 2.5 mL of 72% sulfuric acid was added to each 0.25 mL of 96% ethanol, methanol, and distilled water.

Preparation of standard solution

A stock solution of 1250 µg/ml of astragaloside-IV was prepared in 96% ethanol, methanol and distilled water. Dilutions were made from this solution to obtain astragaloside-IV concentrations of 50–1250 µg/mL. 0.25 ml of 8% vanillin was added to 0.25 ml astragaloside-IV solution followed by 2.5 ml of 72% sulfuric acid.

Determination of total saponin content in samples

In order to determine the content of total saponins by the vanillin-sulfuric acid method, 0.25 ml of 8% vanillin was added to 0.25 ml of the extract in the above 3 solvents, after which 2.5 ml of 72% sulfuric acid was added, following which it was placed min in shaking water bath at 60°C for 15 minutes.. Also, the standard and blank solutions were incubated together in the water bath. After cooling at an ambient temperature for 5 min, the absorbance of the standards and extracts are measured at 560 nm using a spectrophotometer. The experiment was repeated 3 times.

Statistical analyses

The one-way ANOVA analysis of variance was performed on the data. The statistical significance of differences between the means was assessed at the 5% level with the Tukey HSD test using JMP statistical software (SAS, 2008). Glucose and astragaloside-IV standard curves and regression equations were generated using Microsoft Excel software.

RESULTS AND DISCUSSION

Callus formation from leaf and epicotyl explants of A. mongholicus

The purpose of the experiment was to direct shoot regeneration in explants, but most of the explants were enlarged and callus was formed within 2-3 weeks after culture initiation. Therefore, we evaluated the callus and also classified it into two types - light yellow and light green, according to their morphology. The frequency of callus induction varied depending on the cytokinin types, 2,4-D concentration and explants in culture media. The percentage of callus formation was highest (58.3%) in MS medium with 2.0 mg/L 2,4-D and 0.5 mg/L BA (Table 1). Moreover, light yellow and light green callus were formed depending on the explants in MS medium with 2.0 mg/L 2,4-D alone (50%). Light green callus was formed when the concentration of BA was high. The percentage of callus formation was lower in the MS medium with 2,4-D and Kinetin. Leaf explants were more effective than epicotyl explants on callus formation. Further, light green compact callus formed in MS medium with 2.0 mg/L 2,4-D and 0.5 mg/L BA was used for phytochemical experiments.

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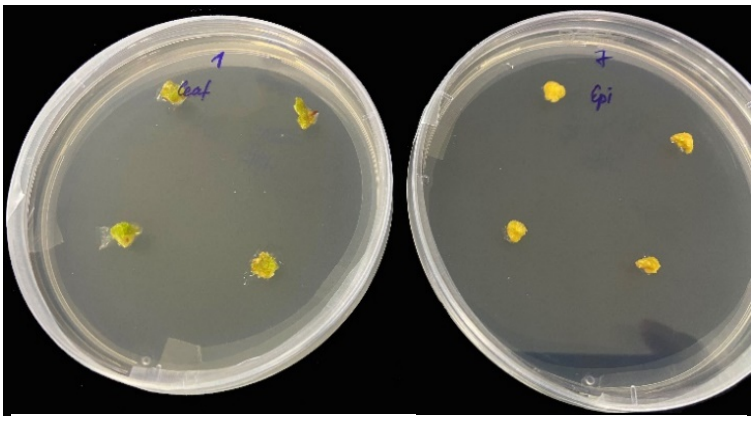
Table 1. Effects of different auxin and cytokinin hormones on callus formation from leaf and epicotyl explants of A. mongholicus

Hormone (mg/L)		Callus formation %		Appearance	
Auxin	Cytokinin	Epicotyl	Leaf	Epicotyl	Leaf
0.1 2,4-D	-	0	0	-	-
0.5 2,4-D	-	8.3	41.6	light yellow, compact	light yellow, friable
1.0 2,4-D	-	0	8.3	-	light yellow, friable
2.0 2,4-D	-	33.3	50	light yellow, compact	light yellow, compact
4.0 2,4-D	-	0	33.3	-	light yellow, compact
1.0 2,4-D	0.5 Kinetin	0	0	-	-
2.0 2,4-D	0.5 Kinetin	8.3	25	light yellow, compact	light yellow, compact
4.0 2,4-D	0.5 Kinetin	8.3	0	light yellow, compact	-
1.0 2,4-D	0.5 BA	16.6	16.6	light yellow, compact	light green, compact
2.0 2,4-D	**0.5 BA**	25	58.3	light yellow, compact	light green, compact
4.0 2,4-D	0.5 BA	33.3	0	light yellow, compact	-



a) light green callus *b) light yellow callus*

Figure 1. Effects of hormones on callus formation from leaf and epicotyl explants of A. mongholicus. a) Light green callus formed from leaf explants in MS medium with 2.0 mg/L 2,4-D and 0.5 mg/L BA, b) Light yellow callus formed from epicotyl explants in MS medium with 2.0 mg/L 2,4-D and 0.5 mg/L Kinetin

Shoot proliferation from apical bud of A. mongholicus

An efficient proliferation was developed through apical bud. The results of the experiment showed that depending on the hormones there were significant differences in shoot number and plant height. ($P<0.05$) (Table 2). The highest number of shoots was induced in MS medium with 2 mg/L TDZ and 0.5 mg/L NAA (15.3 shoots) and MS medium with 2 mg/L BA had a good effect on shoot proliferation (10.3 shoots). There was no shoot proliferation in the medium without auxin and cytokinin.

The combination of NAA with other cytokinins negatively impacted on shoot proliferation and increased the growth of callus formation. The combination of NAA with TDZ had a positive effect on the number of shoots and leaves, but leaf chlorosis had a negative effect on shoots. Yorgancilar and Erisen et al. (2011) suggested MS medium with 1 mg/L BA for the proliferation of *Astragalus shizopterus* because TDZ induced the highest number of shoots, but it had problems with abnormal leaf morphology and chlorosis. According to our research, the number of shoots was the best in the medium with TDZ and NAA. However, similar to the results of the above researchers, chlorosis occurred and the color of the leaves turned yellow.

Therefore, MS medium with 2 mg/l BA is considered suitable for obtaining healthy shoots (10.3 shoots) in the micropropagation of *A. mongholicus*. BA is a widely used cytokinin in the *in vitro* culture of *Astragalus*, such as *Astragalas adsurgens* Pall [21], *A. melilotoides* Pall [11], *A. aquilonius* Barneby, *A.*

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amblytropis Barneby, *A. columbianus* Barneby [3] and *A. cariensis* Boiss [5]. Rout (2005) reported that *Clitoria ternatealinn* (*Fabaceae*) increased shoot proliferation in MS medium with BA and NAA. *A. maximus* willd showed maximum shoot regeneration in a medium with 0.5 mg/L zeatin riboside (ZR) [32]. Erişen (2005) reported that maximum shoot formation was observed in *A. Duranii* in medium with 0.2 mg/L TDZ. In *A. nitidiflorus*, the best proliferation was found at 0.1 mg/L BA [35]. From these experiments, it appears that the cytokinins, which show the best results, are different, depending on the plant species.

Table 2. Effect of different hormones on shoot proliferation of A. mongholicus

Hormone	Concentration (mg/L)	Shoot number	Plant height	Hormone	Concentration (mg/L)	Shoot number	Plant height
BA	0.5	8.25±3.16^{c-e}	4.51±1.56^{ab}	BA+0.5 mg/L NAA	0.5	5.17±1.49^{f-h}	3.95±1.63^{a-d}
	1	7.00±3.02^{d-h}	4.40±1.33^{a-c}		1	6.71±1.55^{e-h}	3.48±1.45^{a-f}
	2	**10.3±3.61^{b}**	4.37±1.24^{a-c}		2	7.31±2.05^{d-g}	3.87±1.21^{a-e}
	4	7.64±2.23^{c-g}	3.84±1.84^{a-e}		4	7.80±1.79^{c-f}	3.55±1.28^{a-f}
TDZ	0.5	6.12±2.47^{e-h}	3.12±1.40^{b-f}	TDZ+0.5 mg/L NAA	0.5	10.7±3.84^{bc}	3.77±0.92^{a-f}
	1	4.28±2.28^{h}	2.42±1.02^{e-f}		1	12.8±5.39^{ab}	3.32±1.01^{a-f}
	2	4.75±1.77^{gh}	2.43±1.09^{f}		2	**15.3±3.90^{a}**	2.95±1.29^{b-f}
	4	6.47±1.83^{e-h}	2.86±1.15^{d-f}		4	14.0±3.87^{ab}	2.66±0.98^{d-f}
Zeatin	0.5	5.70±1.97^{e-h}	4.35±1.61^{a-c}	Zeatin+0.5 mg/L NAA	0.5	4.37±1.14^{gh}	3.21±0.83^{a-f}
	1	7.29±2.14^{d-h}	4.84±1.18^{a}		1	5.28±1.32^{e-h}	3.85±0.92^{a-f}
	2	6.50±2.34^{e-h}	4.76±1.26^{ab}		2	5.16±2.65^{e-h}	2.69±0.97^{c-f}
	4	4.66±1.74^{gh}	3.79±1.97^{a-e}		4	5.33±2.06^{e-h}	4.11±1.37^{a-e}

Results were expressed as a mean±standard deviation. Tukey's (P<0.05) test shows that consecutive means with the same letters are not significantly different. For other letters separated by hyphens, "a-d" means group abcd with the same meaning.

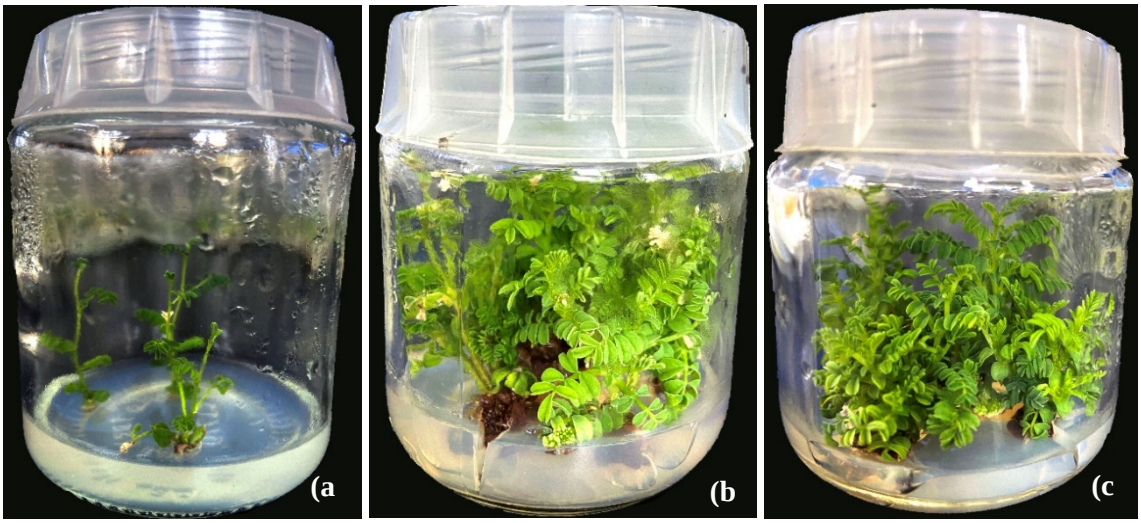

Figure 2. Effects of hormones on shoot proliferation of A. mongholicus.
a) Shoot formation in MS medium without hormones,
b) Shoot formation in MS medium with 2 mg/L TDZ and 0.5 mg/L NAA, and
c) Shoot proliferation in MS medium with 2 mg/L BA

Rooting of propagated shoots of A. mongholicus

No roots were formed when the shoots were tested for rooting in ½ MS medium with different concentrations (0.5-4.0 mg/L) of auxin-type hormones (IAA, IBA and NAA) alone and without hormones. Therefore, the proliferated shoots (after 4 weeks) were tested in ½ MS medium with 0.5-4.0 mg/L IAA and IBA combination with 0.5 mg/L NAA. The results showed that 30% root induction, 13 roots, and 0.4 cm root length were induced in ½ MS medium with 4.0 mg/L IAA and 0.5 mg/L NAA, which was the most suitable medium compared to other mediums (Fig. 3 c). According to the other studies, MS medium

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without hormone was effective for rooting of Astragalus species *A. maximus* [32], *A. adsurgens* [21], *A. amblytropis* and *A. aquilonius* [3]. For the *A.adscendens* plant [6], shoots were successfully rooted in MS medium with NAA.

Table 3. Effect of different hormones on rooting of Astragalus mongholicus

Hormone (mg/L)			Root formation %	Root number	Root length
IAA	IBA	NAA			
0.5	-	0.5	25	5.80±1.64^{b,c}	0.40±0.23^{a}
1.0	-	0.5	30	3.00±1.09^{b,c}	0.35±0.13^{a}
2.0	-	0.5	30	4.70±2.73^{b,c}	0.43±0.21^{a}
4.0	-	**0.5**	30	13.0±2.16^{a}	0.40±0.15^{a}
-	0.5	0.5	20	6.00±0.00^{b,c}	0.22±0.05^{a}
-	1.0	0.5	20	2.25±0.50^{c}	0.20±0.00^{a}
-	2.0	0.5	15	3.00±0.00^{b,c}	0.50±0.14^{a}
-	4.0	0.5	10	7.50±2.12^{b}	0.45±0.07^{a}

Results were expressed as a mean±standard deviation. Tukey's (P<0.05) test shows that consecutive means with the same letters are not significantly different.

Determination of total triterpene saponin and polysaccharide content in different cultures

The aim of this study was to determine whether the content of biologically active compounds depends on the stages of development of plant during *in vitro* culture of *A. mongholicus* in different plant samples grown *in vitro* including young shoot culture, proliferated shoot, rooted shoot, adventitious root culture and leaf-derived callus. The objective of the experiment was to determine and compare the content of total saponins and polysaccharides from these samples and in natural samples.

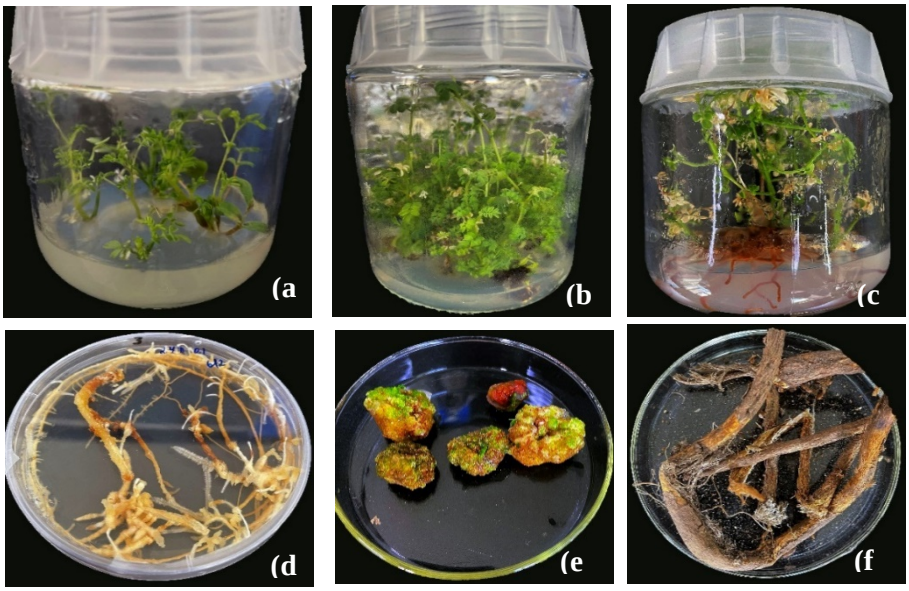

Figure 3. Samples of A. mongholicus used for phytochemical analysis. In vitro cultures: (a) 14-day-old young shoot culture, (b) 28-day-old proliferated shoots, (c) 28-day-old rooted shoot, (d) 28-day-old adventitious root, (e) leaf derived callus, and, (f) natural sample, root

In addition, the above cultures were extracted with 70% ethanol, methanol and aqueous extract to determine the suitability of the solvent. According to the test results, the content of total saponins and polysaccharides had significant differences depending on the solvents (P<0.05) (Tables 4, 5). The total saponin and polysaccharide content of *A. mongholicus* were estimated by the regression equations obtained from the standard curve. The content of polysaccharides in extracts of *A. mongholicus* cultures extracted in ethanol 14-day-old *in vitro* young shoot culture samples (505±54.6 µg/ml) was higher than 28-day-old

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proliferated shoots samples and rooted shoot samples. Young shoots and natural samples from ethanol-extracted *in vitro* cultures had higher polysaccharide content than methanol and aqueous extract samples, with 805.3±56.6 µg/ml (Standard curve equation: y=0.0052x+0.0808, r^2=0.9631) (Table 4). The total saponin content of *A. mongholicus* extract was the highest in the 14-day-old *in vitro* cultured young shoot samples (990±28.5 µg/ml) compared to other *in vitro* cultured samples. Natural samples extracted with ethanol had 1310±103 µg/ml and saponin content was higher than samples extracted with methanol and aqueous extract samples (y=0.0017x+0.2943, r^2=0.9612) (Table 5). Extracting the samples in ethanol was more suitable.

Table 4. The polysaccharide content of samples extracted with different solvents

Sample	Polysaccharide µg/ml		
	Ethanol	Methanol	Aqueous
InV-young shoot	505.0±54.6^{a}	475.3±3.51^{a}	440.0±6.24^{a}
InV-multiplied shoot	484.3±23.5^{a}	424.7±16.8^{b}	394.3±11.5^{b}
InV-rooted shoot	433.6±9.29^{a}	414.3±9.50^{a}	336.7±33.5^{b}
InV-adventitious root	490.3±22.2^{a}	405.6±12.6^{b}	212.0±10.1^{c}
InV-callus	443.0±65.0^{a}	272.7±29.7^{b}	363.3±9.07^{ab}
Natural sample (Root)	805.3±98.0^{a}	485.3±5.03^{b}	621.0±31.5^{b}

Results were expressed as a mean±standard deviation. Tukey's (P<0.05) test shows that consecutive means with the same letters are not significantly different.

Table 5. Total saponin content in samples extracted with different solvents

Sample	Total saponin µg/ml		
	Ethanol	Methanol	Aqueous
InV-young shoot	990.0±28.5^{a}	601±79.5^{b}	861.0±80.5^{a}
InV-multiplied shoot	467.0±58.0^{a}	391±18.9^{a}	418.3±28.9^{a}
InV-rooted shoot	217.3±70.0^{a}	224±14.7^{a}	284±30.8^{a}
InV-adventitious root	114.0±20.0^{ab}	162±25.4^{a}	76±32.6^{b}
InV-callus	341.3±26.5^{a}	287±16.3^{b}	325±5.29^{ab}
Natural sample (Root)	1310±103^{a}	953±53.5^{b}	1257±13.6^{a}

Results were expressed as a mean±standard deviation. Tukey's (P<0.05) test shows that consecutive means with the same letters are not significantly different.

A. mongolicus roots contain more water-soluble compounds, especially polysaccharides, while *A. membranaceus* has been determined to contain more saponin glycosides [24].

The biosynthesis of secondary metabolites in plants depends on stressors and their responses [9, 7, 16]. The conditions of *in vitro* culture impose a combination of stress factors on cultured plant cells through pronounced change in the cellular environment that may be in the form of wounding of excised tissues, plant growth regulators (PGRs), salt concentrations (low or high) and high or low levels of artificial light that could generate stress effects. It has been mentioned in the studies that the type and concentration of plant hormones influence the synthesis of secondary metabolites in tissue culture [2, 37]. It is noted in previous studies that the addition of auxins and cytokinins to the medium separately, in most cases, did not give positive results in stimulating the production of secondary metabolic compounds. In general, numerousstudies showed that the addition of combinations of auxins and cytokinins stimulated the increase in the production of secondary metabolites [13, 27]. It is hypothesized that the rooting medium environment is related to lower levels of secondary metabolites associated with the use of auxin hormones alone, as well as adaptation to stress. An example of higher secondary metabolite concentrations in the medium supplemented with auxin and cytokinin hormones matches the results in callus cultures.

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CONCLUSIONS

- It has been determined that MS medium containing 2 mg/L BA alone was suitable for the propagation of shoots from the explants of the apical buds of *A. mongholicus*.
- For the rooting of *A. mongholicus* shoots, ½ MS medium with 4.0 mg/L IAA and 0.5 mg/L NAA was suitable.
- The content of total saponins and polysaccharides in different cultures of *A. mongholicus* grown in vitro was higher in ethanol extracted 14-day-old young shoot samples than in 28-day-old propagated shoot samples and rooted shoots. Extracting the samples in ethanol was more suitable.

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