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Influence of Different strains of *Agrobacterium rhizogenes* on Hairy roots Induction and phenolic acids content in *Astragalus hamosus*

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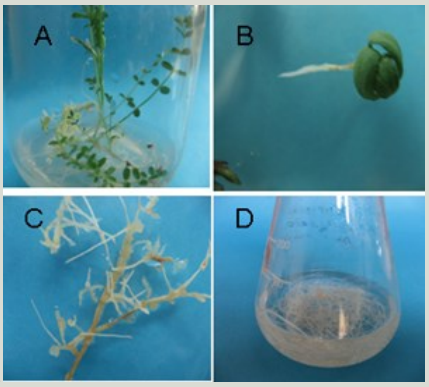
Abstract

Different species of the genus *Astragalus* are sources of phenolic compounds, which are used in ancient Chinese medicine as antiperspirant, antihypertensive, diuretic, tonic, and anti-cancers. In the last two decades, there has been considerable interest in the production of phenolics by genetically transformed root cultures because hairy root cultures display stable production of secondary metabolites, which are often comparable to plant roots. In this study, hairy roots were induced using different explants of *in vitro-grown Astragalus hamosus* L. seedlings following inoculation of *Agrobacterium rhizogenes* strains *15834*, *2659* and *WT*. The *2659* proved to be more competent than other strains and the highest transformation rates were observed in leaf explants (67%). The transformed roots appeared after 14-24 d of incubation on hormone free MS (Murashige and Skooge) medium. Growth of hairy roots was assessed on the basis of total root elongation, lateral root density and biomass accumulation. Hairy root lines were further established in Gamborg (B_5) medium and the biomass increase was maximum from 17 to 30 d. PCR confirmed integration and expression of left and right termini-linked R_i T-DNA fragment of the R_i plasmid from *2659* into the genome of *Astragalus hamosus* hairy root All the clones showed a higher growth rate than non- transformed root. Phenolic acids were analyzed quantitatively and qualitatively by gas-liquid chromatography.



Keywords: Astragalus, Hairy root, Agrobacterium rhizogenes, Phenolics

1.Introduction

The large and diverse group of chemicals produced by plants, which include alkaloids, anthraquinones, anthocyanins, flavonoids, saponins, and terpenes, has played an essential role in the pharmaceuticals, cosmetics, perfumeries, dyeing, and flavor industries. Plants produce many of these compounds through secondary metabolism. Secondary metabolites are compounds belonging to extremely varied chemical groups that are not essential to plant cell growth and are produced in small amounts(Kim et al., 2002 b). Even though secondary metabolism generally accounts for less than 10% of the total plant metabolism, its products are the main plant constituents with pharmaceutical properties. Plants that fight cancer.

A new route to enhancing secondary metabolite production in a tissue culture system is the transformation of desirable plant species using the natural vector system of *Agrtobacterium rhizogenes*. It is the causative agent of hairy root disease in plants (Giri and Narasu, 200; Bourgand *et al.*, 2001).

Astragalus is generally considered the largest genus of vascular plants, with an estimated 2,500–3,000 species (Podlech, 1986). The roots of various *Astragalus* species represent an ancient and well-known drug used in traditional Chinese medicine as an antiperspirant, diuretic tonic, and as anticancer agent (Tang *et al.*, 1992). The natural plant population of this essential medicinal plant is over-exploited for pharmaceutical preparations, and therefore, *in vitro* production as to alternative sources has been attempted (Yamamoto *et al.*, 1986; Morimoto *et al.*, 1995).

However, the cultures tend to be genetically unstable and synthesize very low beneficial secondary metabolites (Rhodes et al., 1990). *A.rhizogenes*, the causative agent of the hairy root syndrome, is a common soil organism capable of entering a plant through a wound and causing a proliferation of secondary roots (Heijden et al., 1994; Wordragene t al., 1992). The underlying mechanism of hairy root formation is the transfer of several bacterial genes into the plant genome. The observed morphologenic effects in the plants after infection have been attributed to the transfer of part of a large plasmid known as the Ri (root-inducing) plasmid. *Agrtobacterium rhizoghenes-mediated* hairy root cultures exhibit a stable and fast growth rate compared to cell suspension culture, genetic and biochemical stability, and increased production of secondary compounds (Giri and Narasu 2000). These transformed hairy roots, can be produced by inoculation of plants with atropine strains of *A. rhizogenes* containing a T-DNA, divided into two regions, T_L and T_R (Huffman et al., 1984). The T_L region of R_i plasmid contains eighteen open reading frames (ORF) (Slightom *et al.*, 1986). ORF_S 10, 11, 12, and 15 represent the root locus, i.e., rol A, rol B, rol C and rol D, respectively. Root loci (rol) have been found to be essential for hairy root induction (White *et al.*, 1985; Jouanin *et al.*, 1987). The *rolA* gene corresponds to ORF 10 of the TL-DNA of *A. rhizogenes (*Slightom *et al., 1986).* Transgenic *rolA* plants of tobacco *(*Vilaine et al., 1987*),* tomato (van Altvorst, et al., 1992) and potato (HHnisch ten Cate, et al., 1988), share common developmental changes, including the formation of adventitious roots and wrinkled leaves. These symptoms are similar to plants expressing the entire TL-DNA, but the roots excised from *rolA-transformed* plants grow slower in vitro compared to roots bearing the entire TL-DNA (Vilaine et al., 1987).

Among these, rol B plays the central and most important role while rol A, rol C, and rol D promote root formation synergistically (Aoki and Syono 1999). the T_R-DNA corresponds to the tms loci of the Ti plasmids (White et al., 1985), and can directly synthesize auxin (Capnone et al., 1989).

The atropine synthase (age) gene responsible for opine biosynthesis in the transformed tissue is also located in T_R region of R_i plasmid (Binns and Tomashow 1998). Hairy roots have been induced in many dicotyledonous plants

by transformation with A. rhizogenes Ri T-DNA (Costamino et al., 1994). Hairy roots are able to regenerate whole viable plants with high genetic stability. Most have an altered phenotype, including hairy root syndrome, dwarfing, altered flowering, wrinkled leaves, and increased branching, which have proven useful in ornamental plant breeding programs (Giovanni et al., 1997). The roots produced by the subsequent transfer of T-DNA have been recognized as a potential alternative source of many secondary compounds in medicinally important plants as the genetic transformation does not affect the natural root synthetic capacities (Flores and Medino-Bolivar 1995).

The aim of this study was to compare of virulence of different *Agrtobacterium rhizogenes*, and to determine the best strain for genetic transformation in *Astragalus hamosus* analysis of phenolic compounds in hairy roots.

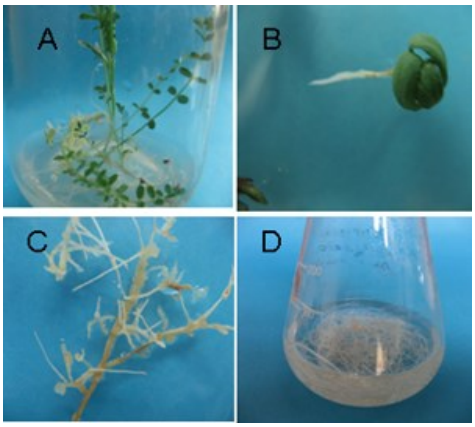


Fig.1: Hairy root induction in *Astragalus hamosus* . A: seedling of *A. hamosus* in vitro, B:Hairy root induction in infected explants, C: hairy root in semi solid culture medium, D: liquid culture of hairy roots.

2. Methods and Materials

2.1. Materials

2.2.1 Bacterial Strains

Agrtobacterium rhizohenes Strains *2659*, *15834,* and *WT* (gift from Dr. M. Karimi, Gent, Belgium) were used to determine the transformation efficiency. Bacterial Strains *2659*, *15834,* and *WT* were maintained on solid LB (Tie-He *et al.*, 2006) medium as required. The bacterial strains were grown for 36 h in liquid LB medium at 28º C on a rotary shaker at 120 rpm in the dark before inoculation.

2.2.2 Hairy root induction

The cotyledon leaf (1-week-old), radicle, stem, and the young leaf explant from 2-week-old sterilized seedlings were cut into small pieces and infected with the bacterial cultures separately for 5 min. Explants were blotted dry on sterile filter paper to remove excess bacteria and co-culture onto hormone-free MS Murashige and Skooge, 1962) media with 0. .8% agar and containing 0.3% sucrose (Akramian *et al.*, 2008).

In addition, many explants were incubated in the same conditions as control. After 48 h of co-culture, the explants were transferred onto hormone -free MS media containing 250 mg dm^{-3} cefotaxime to eliminate bacteria and then incubated at 25± 2 ºC under 16 h light and 8 h dark photoperiod. After 21 d, hairy roots appeared on the cut ends of explants. The frequency of genetic transformation was investigated during a five-week period. Explants were detached and cultured onto fresh 1/2 strength B_5 media containing 70 mg dm^{-3} cefatoxime in 25±2 ºC under dark. Hairy root cultures were transferred to fresh media every 2 weeks and maintained as separate independent clones.

Plant material: The mature seed of wild plants of *A. hamosus* was collected from Kurdistan in Iran. The dormancy of seeds was broken by scarification. Mechanical scarification can be done without damaging seeds (Miklas et al., 1987; Townsend and McGinnies, 1972). The seeds were germinated in half-strength MS in the dark and then they transferred to growth chamber under 16 h light and 8 h dark photoperiods.

2.2.3. GLC analysis of phenolic acids

2.1.3.1. Plant material

Samples of different explants of seedling *A.hamosus* included root, leaf, and both the line of hairy root air dried in the shade. The explants of plant specimens were separated and then ground to a fine powder in a small mortar and pestle.

2.1.3.2 Extraction of phenolic compounds:

The powder of plant samples (0.4 gr of explants powder) was extracted by 4 ml of methanol 80% (Merk) for 5 minutes in some glass vials and three times repeated. Each time, extracts were blotted dry on filter- paper. Then, each extract was separately collected. extracts were evaporated to dryness in free air.

2.1.3.3 Derivatization and GLC

In the next step, residues redissolved in 7 ml acetonitrile and 5 ml n- hexane (Merk) into decanter and two phase upon and lower observated. Phenolic compounds is presented in lower phase. Extracts were re-evaporated to dryness in free air. The residues were treated with 1 ml anhydrous pyridine (Merk) and then trimethylsilylated with 0.2 ml hexamethyldisilasane (Fluca) and 0.1 ml trimethylclorosilane (Fluca) for 30 seconds.Thereafter, the solution were ready for the analysis.

A Shimadzu GLC-16A equipped with a flame ionization detector and 1.6 m × 3.2 mm i.d. glass column packed with 5% SE-30 was used. Operation temperatures were for the oven and 270°C for the injector and 280°C for detector block. Nitrogen was used as carrier gas at a flow rate of 50 ml min^{-1}. Flow rates of hydrogen and air were 55 and 400 ml min^{-1}, respectively. The peacks were identified by comparison of the retention times with authentic standards.The peack areas were determined automatically by the Shimadzu C-R5A data processor. 0.2 ml of the mixture of original phenolic compounds and 0.2 ml of extracts were used for injection. Three injection were made for each replication. The results are the mean of three replication.

3. Results

In this research, genetically modified roots *Astragalus hamosus* were produced in order to comparable ability virulent different strains of *Agrtobacterium rhizogenes*. Visible roots were formed after 14-24 d at the site of bacterial inoculation of leaves discs. The hairy roots were initiated on the cut surface of the infected leaves explants. No roots formation was observed on cotyledon leaves, stems and pedicle explants and in control explants, too.

Different types of *A. rhizogenes* had great influence on the induction of hairy roots. The transformation efficiency of *2659* strain was more efficient than that of *15834* and *wt* Strains.

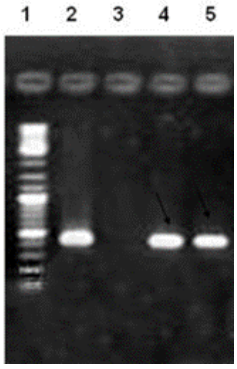


Fig. 2: PCR analysis of genomic DNA isolated from *Astragalus hamosus* hairy roots. 1: marker, 2: positive control, 3 negative control, 4: line 1, and 5: line 2 of the hairy root.

Strain *15834* was benefical but not as effective as strain *2659* (Fig. 1). In contrast to these two strains, strain *WT* did not induce hairy roots in any of the used explants. Thus, strain *WT* seem to be totally avirulent for *Astragalus hamosus*.
Similarly, more roots were obtained with leaves infected by strain *2659* (Table 1). Among the various bacterial strains examined in this study, *2659,* was found to be the virulent. *A. rhizogenes* strains for hairy root formation 67% of leave explants of *Astragalus hamosus*. Strain *2659* induced 7/13±0.92 roots in leaf explant of *A. hamosus.*

3.1 Growth study and initiation of hairy root liquid culture

The growth of clones transformed by *A. rhizogenes* Strain *2659* on solid 1/2 B_5 medium was studied. Roots excised from intact non-transformed (NT) *in vitro* plantlets were used as controls since the unifected controls did not produce roots. Two root tips [approximately 2 cm long; 0.2 g fresh mass[of each clone, harvested from approximately 5-week-old cultures were transferred to the same medium in 9 cm petri dishes and cultured for 30 d. The number of lateral branches (number of roots per each centimeter on the primary roots) was recorded and expressed as the lateral root density and then fresh mass (FM) of the roots from each sample were determined.
To determine the effect, fastest growing hairy roots (0.2 g) of clone emerged from *2659* Strain were transferred into MS, B_5, half- strength MS and half-strength B_5 liquid media containing 250 mg dm^{-3} cefotaxime on rotary shaker (80 rpm) at 28º C in dark. Data were taken after 10 and 15 d of culture. To time course for biomass accumulation an increase in FM was recorded at 7 d intervals for 5 weeks. The concentration of cefatoxime was gradually lowered and finally concentration of cefatoxime received to 70 mg dm^{-3}.

3.*2. DNA extraction and PCR amplification*

Total genomic DNA from the different clones of hairy roots was extracted by CTAB (Khanujo et al., 1999) with minor modifications and subjected to PCR analysis. Plasmid DNA from *A. rhizogenes s*trains was isolated by alkaline lysis methods (Sambrook et al., 1989). Approximately 10 ng of genomic DNA was used as a template for PCR. In order to show the integration of T-DNA of R_i plasmid in the transformed roots, a segment from rol B gene regions was amplified. The gene-specific primers used for amplifying rol B gene region was: 5′-GCT-CTT-GCA-GTG-CTA-GAT-TT-3′ and primer pair 5′- GAA-GGT-GCA-AGC-TAC-CTC-TC -3′. Non-transformed *A. hamosus* root DNA was used as a negative control for PCR analysis. The amplification protocol for 450 bp rol B fragment was: a 4-min melting at 94 ºC followed by 35 cycles of a 60s melting at 94 ºC, a 1 min annealing at 55 ºC and a 1 min elongation at 72 ºC and final elongation for 7 min at 72 ºC. PCR products were analysed by electrophoretic separation on 1% agarose gels (m/v) in 0.5 X TBE buffer and staining with etidium bromide.

3.3. Result of GLC analysis

The composition of phenolic compounds in different explants of *Astragalus hamosus* investigated. Results showed that composition explants differed. Fig. 3, shows the analysis of mixture of authentic sterols as TMS ether derivatives by GLC in the same conditions as used for the analysis of phenolic compounds from the different explant of *Astragalus hamosus*. As seen in Fig. 3, vaniline, synamic acid, dihydroxybenzoic acid, syrengic acid, coumaric acid, gallic acid, feroulic acid, cafeic acid, naringinine and clorogenic constitue a homologus series, and consequently elute at gradually about by increasing retention times, reflecting the decrease in vapour pressure brought about by increasing carbon number. Phenolic compounds are separated and derivatization before determination by GLC. As shown in Fig. 4, five peaks of authentic phenoli acids (synamic acid, dihydroxybenzoic acid, syrengic acid and coumaric acid) presented in all of explants this species. Vaniline also presented in all explants except hairy root (line 2). Feroulic acid, cafeic acid, naringinine and clorogenic presented only in both the line of hairy root of *Astragalus hamosus*. Number of unknown peaks are observed only in both the lines of hairy roots. Two peaks presented between authentic dihydroxybenzoic acid and syrengic acid. Between syrengic acid and coumaric acid also presented three unknown peaks that was not in another explants. A clear unknown peak between gallic acid and feroulic acid are observed only in hairy root

explants, too

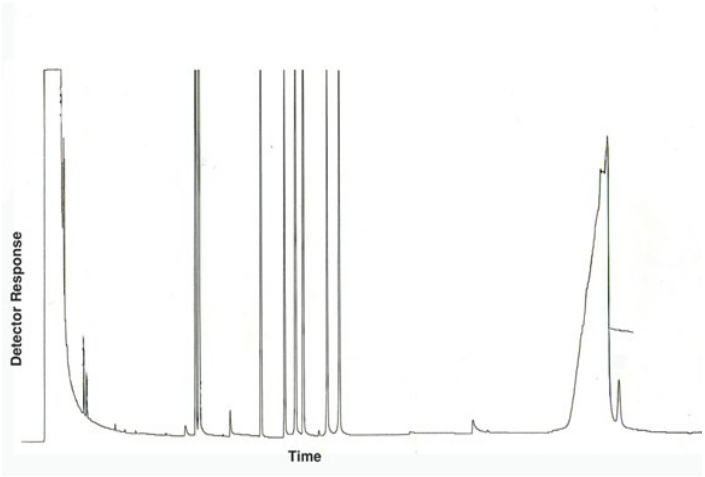


Fig. 3. GLC analysis of authentic phenolic acids as their TMS ether derivatives, performed on a 5% SE-30 glass column. For conditions see Section 2 . Peak identifications are (1)vaniline; (2) synamic acid; (3)Dihydroxyben zoic acid;(4) syrengic acid; (5) coumaric acid; (6) gallic acid; (7) feroulic acid; (8) cafeic acid; (9) naringinine.

4. Discussion

Hairy roots appeared from deeply wounded sites after 14-24 d of inoculation from different explants with *2659*, *15834* and *WT* strains of *A. rhizogenes*. Hairy roots, in contrast to untransformed roots, grow autonomously in hormone-free media. No adventitious root was formed from control explants.

Strain *WT* failed to induce roots from any of the explants used (Table 1) whereas the transformation efficiency with *15834* was very low compare to *2659* strain. The *2659* strain proved to be more competent than *15834*. The highest transformation rates was observed in leaf explant with strain *2659* (67%) and with Strain *15834* (31.3%), 14 and 24 d, respectively, was necessary for root emergence. Influence of bacterial strains on transformation frequency has been documented earlier in different plant species (Zehra et al., 1999). Strains *15834* and *2659* behave differently in present study because they have other chromosomal virulence genes. It was observed that most hairy roots emerged from the deep wounded sites rather than mere scratching but this phenomenon was not investigated further. . It was hypothesized that the cellcells containing high auxin and sucrose contents targets for hairy root induction (Nilsson and Olsson 1997). Results of GLC analysis showed an increase in the amount of phenolic composition in transformed plants by *Agrobacterium rhizogenes*. Moreover, in this study, some of phenolic compounds presented only in two line of hairy root of this species. As seen infection *Astragalus hamosus* by *Agrobacterium rhizogenes* are caused increase this protective composition in transformed plants.

Table 1. Frequency of hairy root induction on the site of infection on different explants by different *A. rhizogenes* strains in *A. hamosus*. Means ± SE, n= 30. No response was found with *WT* strain.

Strain	Explant	Hairy roots number	Root emergence (d)	Hairy root induction (%)
2659	leaf	7.13±0.92	15±1.3	67%
	cotyledon	0	0	0
	pedicle	0	0	0
	stem	0	0	0
15834	leaf	2.13±0.74	22±0.6	31.3%
	cotyledon	0	0	0
	pedicle	0	0	0
	stem	0	0	0

Molecular analysis of transgenic hairy roots: Initially two lines produced by *2659* was maintained. Further study was carried out on hairy root clones of *2659* Strain and the transformation rate of *2659* was found to be higher than *15834.* Randomly selected 2 lines of *2659* were subjected to molecular analysis for confirmation of transformation event, and growth dynamics. The integration of the T-DNA region was confirmed by showing the presence of the 450 bp rol B segments. PCR results showed the integration of T-DNA in both clones except non- transformed root. No amplification of either genos fragments was achieved from non-transformed root DNA. Positive control showed amplification of the expected 450 bp vir D_1 fragment (Fig. 2). Plasmid DNA was taken as positive control, and DNA of non-transformed root as negative control. Hairy roots result from the integration of root loci (rol), present at T-DNA, in the plant genome (White et al., 1985; Jouanin et al., 1987).

Different Strains of *A. rhizogenes* are used for determine the best strain for hairy root formation in *A. hamosus*. Our results in this study confirm the results of previous investigation that different bacterial strains had various hairy roots-generating capacity. The best strains for induction of hairy root in *A. hamosus* leaf explants were determined as *2659* and *15834*, respectively.

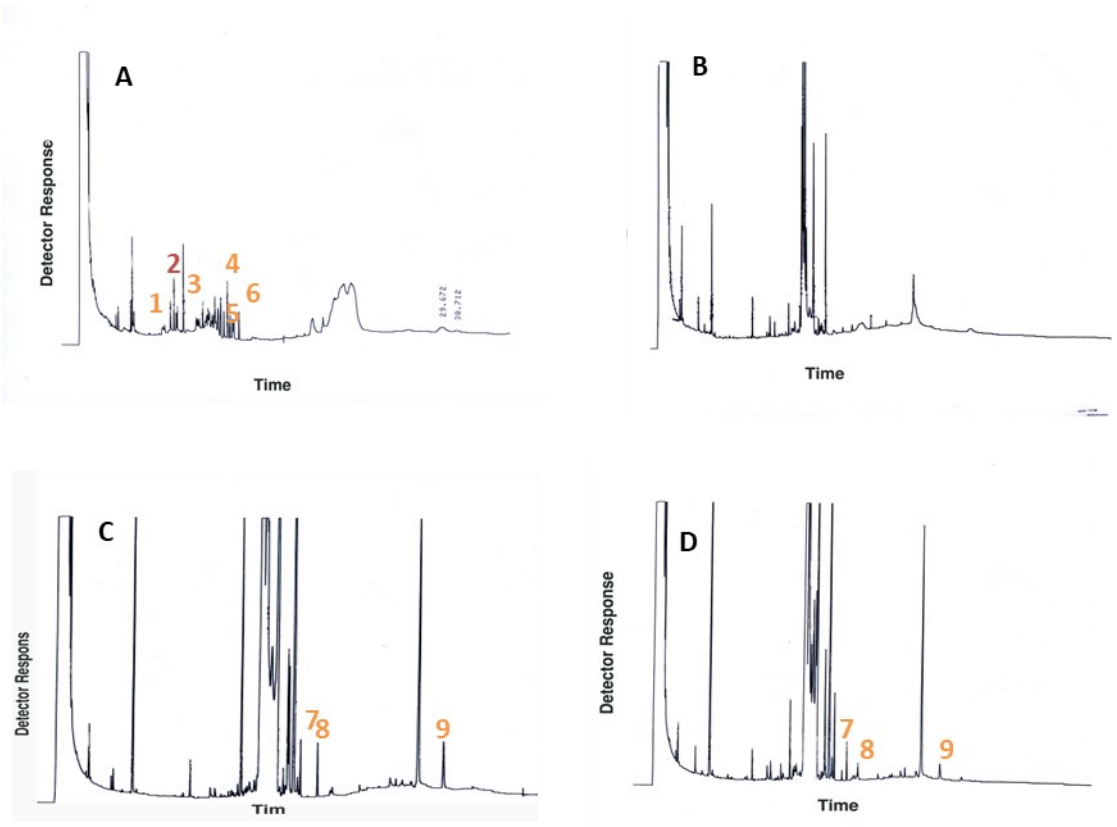


Fig. 4. Representative GLC chromatograms of total phenolic acids extracts showing phenolic acids (as their TMS ether derivatives) pattern of (A)root ;(B) shoot; (C) hairy root (line 1); (D) hairy root (line 2) of *Astragalus hamosus.*

Table 2retention times (RRT) of the TMS ether derivatives of authentic phenolic acid on 5% SE-30 glass column.

Phenolic acids	Retention time	Phenolic acids	Retention time
Vaniline	8.128	Feroulic acid	14.509
Synamic acid	8.467	Cafeic acid	15.087
Dihydroxybenzoic acid	11.369	naringinine	21.381
Syrengic acid	12.503	Clorogenic acid	27.742
Coumaric acid	13.022	-	-
Gallic acid	13.384		

Table 3 Composition of Phenolic compounds of *Astragalus hamosus*. Compasition is expressed with mg in dry weigth. Values represent the mean of three determinations

Phebolic compounds	***Shoot***	***Root***	***Hairy root (line 1)***	***Hairy root(line 2)***
vaniline	0.100	0.284	3.580	-
Synamic acid	0.126	0.057	0.092	0.086
Dihydroxybenzoic	0.273	0.067	0.105	0.145
Syrengic acid	0.823	1.281	6.824	2.274
Coumaric acid	0.203	1.850	0.126	0.910
Gallic acid	0.167	0.078	0.156	4.254
Feroulic acid	-	-	0.450	0.306
Cafeic acid	-	-	0.348	2.530
Naringinine	-	-	6.540	2.570

Table 4 Composition of Phenolic compounds of Astragalus hamosus. Compasition is expressed as both percentage of total compounds (GLC%). Values represent the mean of three determinations.

Phebolic compounds	*Shoot*	*Root*	*Hairy root (line 1)*	*Hairy root (line 2)*
vaniline	0.100	0.284	3.580	-
Synamic acid	0.126	0.057	0.092	0.086
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Gallic acid	0.167	0.078	0.156	4.254
Feroulic acid	-	-	0.450	0.306
Cafeic acid	-	-	0.348	2.530
Naringinine	-	-	6.540	2.570

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