A comparative study of biotechnological approaches for producing valuable flavonoids in *Prosopis farcta*

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Abstract The callus and hairy root cultures of *Prosopis farcta* were established to develop effective strategies to enhance its valuable and medicinally important flavonoid compounds. For callus induction, the hypocotyl, cotyledon and shoot explants were subjected to different plant hormones, naphthalene acetic acid (NAA), benzylaminopurine (BAP), kinetin and dichlorophenoxyacetic acid (2,4-D). Greater callus induction was obtained from hypocotyl explants on MS medium containing 3.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP. With the addition of 0.5 mg L^{-1} asparagine to this medium, the maximum callus growth was achieved. Hairy root culture of *P. farcta* was performed using transformation of different explants with strains of *Agrobacterium rhizogenes* LBA9404, A4, AR15834. The AR15834 strain was more effective for hairy root induction where it caused hairy root formation on 59% of the infected cotyledon explants. We compared profiles

of flavonoids isolated from seedling roots, hairy roots, and callus cultures of *P. farcta*. The colorimetric analysis showed that the content of total flavonoids of hairy roots was 1.54 and 2.52 times higher than in seedling roots and callus, respectively. The presence of flavonoids was verified by LC/MS in positive ion mode. The results showed that flavonoid composition was different in the roots and callus. Naringenin was the major constituent in callus, whereas resveratrol, quercetin and myricetin were the most abundant compounds found in hairy roots. The main objective of this research was to establish hairy roots in *P. farcta* to synthesize flavonoids at levels comparable to in vitro-grown roots. The present study also opens up a way to further improve the production of pharmaceutically valuable flavonoids and to produce desired metabolites using the hairy root culture system.

Keywords *Prosopis farcta* · Hairy root · *Agrobacterium rhizogenes* · Callus culture · Flavonoids · HPLC · LC/MS

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Abbreviations

NAA	Naphthalene acetic acid
BAP	Benzylaminopurine
Kin	Kinetin
2,4-D	Dichlorophenoxyacetic acid
2,4,5-T	Trichlorophenoxy acetic acid
DW	Dry weight
CTAB	Cetyltrimethylammonium bromide
MeOH	Methanol

Introduction

Prosopis farcta belongs to family Fabaceae and subfamily Mimosoideae, distributed from India to Iran (Asadollahi et al. 2010). The plant has been used as a folk medicine for treatment of some diseases and disorders. For example, it may be used in the treatment of inflammation, measles, diabetes (Henciya et al. 2017) and also reduce cardiac or chest pain (Asadollahi et al. 2010). Furthermore, antitumour activity and antioxidant capacity have been reported for *Prosopis* species (Robertson et al. 2011). The medicinal value of these plants can be observed from the variety of chemical agents they possess. The most important bioactive constituents of *Prosopis* species are phenolic compounds such as flavonoids (Seturaman Prabha et al. 2014). In recent years, there has been an increasing attention in research on flavonoids because of their beneficial pharmacological properties (Brahmachari and Gorai 2006). To produce the desired chemicals and pharmaceuticals from plants, the micropropagation approaches have vast potential as a complement to conventional agriculture in the commercial production of bioactive plant compounds (Rao and Ravishankar 2002). Also these strategies provide significant alternative sources that can face the fluctuations of environmental factors, abiotic constraints and cultivation problems (Matkowski 2008). Moreover, plant cell and tissue culture as the emerging fields of biotechnology have been widely studied for the production and understanding the basic mechanism involved in the synthesis of desired compounds (Khanpour-Ardestani et al. 2015). Several attempts have been made to demonstrate applicability of tissue culture technology for *Prosopis* species (Caro et al. 2002). When plant explants are cultured on the appropriate medium containing balanced concentrations of auxin and cytokinin, they can induce unorganized, growing, and masses of actively dividing cells (Zawawi et al. 2013). A successful approach was reported by Trejo-Espino et al. (2011), who established *Prosopis laevigata* callus by hypocotyl explants, cultured on MS medium supplemented with trichlorophenoxy acetic acid (2,4,5-T) and kinetin (Kin). Compared to wild-growing plants, undifferentiated cell cultures serve as an important source for the production of bioactive flavonoids (Grzegorzczak-Karolak et al. 2016). Along with this, transgenic hairy root culture of this plant by transformation techniques

can be a potent way for the large-scale production of flavonoids. Hairy roots are transgenic roots obtained by infection of wounded sites of plant with the *Agrobacterium rhizogenes*. Such roots usually grow faster than plant cell cultures in the basic culture media, without the need for additional phytohormones (Giri and Narasu 2000). To establish tissue and organ culture systems successfully, several essential factors should be considered such as the type and age of explants, plant growth regulators and certain chemical additives (to induce callus) and strains of *A. rhizogenes* (Shekhawat et al. 1993; Rama Krishna and Shashree 2015). The major advantage of using cell culture and hairy root technology is high biosynthetic rate and growth potential in producing important metabolites as do the mother plants (Estrada-Zúñiga et al. 2009).

There are no reports available on induction of hairy root culture in *P. farcta*. Realizing the importance of *P. farcta* extracts in medicinal applications, we applied biotechnological methods to induce callus and hairy root of *P. farcta* as sources for the production of various metabolites. In addition, we address about the issue of possible differences in the profiles of flavonoid compounds among seedling roots, hairy roots and callus cultures.

Materials and methods

Chemicals and standards

All chemicals, culture media, plant growth regulators, flavonoid standards and antibiotics were purchased from Merck and Sigma-Aldrich (Taufkirchen, Germany) at the highest purity available.

Plant material

Prosopis farcta L. seeds were collected in western Ilam province, Iran. The healthy seeds of uniform size were selected and scarified with 98% sulphuric acid for 13 min and surface sterilized with 2% of sodium hypochlorite solution, thoroughly rinsed in distilled water. Seeds were placed on Murashige and Skoog (1962) medium (MS) containing 3% sucrose and incubated at 24–25 °C in the dark for 3 days. Then the germinated seeds were maintained at 25 °C in a

growth chamber with a daily 16 h photoperiod ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). The seedlings were used for the callus induction and transgenic hairy root production. Also, the seedling roots were used to study flavonoid accumulation and comparison with that in hairy root and callus cultures.

Callus establishment

For callus induction, the different plant explants: hypocotyl, cotyledon, shoot (1–2 cm pieces) were excised from 12 day-old seedlings. The segments were cultured on MS medium supplemented with 3% sucrose, 0.8% agar containing different combinations of NAA (1.0, 2.0 and 3.0 mg L^{-1}), BAP (1.0, 2.0 mg L^{-1}), Kin (0.5, 1.0 mg L^{-1}) and of 2,4-D (1.0, 2.0 mg L^{-1}) at pH 5.8. Samples were replicated ten times (10 replicate Petri dishes with four explants per dish), and experiments were repeated twice. All cultures were maintained in darkness at 25°C . The calli were initiated from the explants after 15–17 days of inoculation and data were collected for callus induction rate (percentage), callus status and then the optimum medium for callus culture was determined based on the data.

To improve the callus growth, the optimum medium (3 mg L^{-1} NAA and 2 mg L^{-1} BAP) was supplemented with asparagine (0.5 mg L^{-1}). Callus were subcultured for 1 month, then, extracted to measure flavonoid content.

Inoculation of *Agrobacterium rhizogenes*

The single colony from each strain, *LBA9404*, *AR15834* and *A4* was grown in liquid LB medium (Park and Facchini 2000) supplemented with rifampicin stock (50 mg L^{-1}) and incubated at 28°C for 2 days. Optical density (O.D) of 2 days old bacterial cultures, 0.8–1.0 O.D in a 1 cm cuvette, was determined at 600 nm.

Plant transformation

Different explants from in vitro grown *P. farcta* (hypocotyl, cotyledon and shoot explants) were cut into small pieces (0.5–1 cm). Briefly, the 12-day-old explants were infected by contaminated syringe needles with *Agrobacteria* strains *LBA9404*, *A4* and *AR15834*, separately. This experiment was performed

with 100 segments from each explants in all strains in three independent steps. After infection, the explants were transferred to solid MS medium. Furthermore, the uninfected explants were used as negative controls. All media were maintained in culture room at 25°C in darkness for a co-cultivation period (between 48 to 72 h). After 2–3 days, the infected explants were then put on the fresh solid MS medium supplemented with antibiotic (cefotaxime 200 mg L^{-1}) and incubated at 25°C in dark. The roots which appeared on the explants were cut and transferred to the same medium. The transformation frequency of explants was calculated by the average number (as percentage) of hairy roots obtained from the infected segments. The transgenic status of these roots was approved by PCR using *rolC* specific primers. The fast growing line of hairy roots (line No. C2) were collected, dried at 60°C and used for the measurement of flavonoids.

PCR analysis

Extraction of DNA from the hairy root lines was done using the cetyltrimethylammonium bromide (CTAB) extraction method (Khan et al. 2007). Moreover, non-transformed plants genomic DNA and plasmid DNA from *AR15834* strain were used as negative and positive controls, respectively. The transgenic status of hairy roots was approved by PCR using *rolC* specific primers: *rolC* 5'-TAACATGGCTGAAGAC-GACC-3' and 5'-AAACTTGCACTCGCCATGCC-3'. Also, the presence of any remaining *Agrobacterium* in hairy roots was evaluated by *virD* gene with following primers: *virD* 5'-ATGTCGCAAGGCAG-TAAGCCC-3' and 5'-GGAGTCTTTCAGCATG-GAGCAA-3'. PCR amplification was carried out in a thermal cycler (peqLab., Wilmington, DE, USA) and the conditions are shown in Table 1. PCR products were confirmed by agarose gel electrophoresis [1% (w/v)] and ethidium bromide staining (Wang et al. 2006).

Spectrophotometric determination of total flavonoid contents

Total flavonoid contents were estimated by using the aluminum chloride as reagent, according to Akkol et al. (2008). Briefly, to 1 mL of methanolic extract of the samples were added 250 μL of reagent and 250 μL of potassium acetate solution and the mixtures

Table 1 PCR amplification profile

Cycle no.	Denaturation		Annealing		Extension	
	Temperature (°C)	Time	Temperature	Time	Temperature (°C)	Time
1	95	2 min	–	–	–	–
2–34	95	30 s	50 °C <i>rolC</i> 55 °C <i>virD</i>	40 s	72	50 s
35	–	–	–	–	72	5 min

incubated at 25 °C for 35 min. The absorbance of the samples was determined at 415 nm. The calibration curve of rutin was prepared and total flavonoid contents presented as µg equal rutin in g DW.

HPLC analysis of flavonoids

Extraction of flavonoid compounds was performed following Keinänen et al. (2001). In brief, 0.2 g fine powdered samples transferred to a tube with 1.5 mL of MeOH/H₂O 40:60, 0.5% acetic acid. After 3 h shaking, samples were centrifuged for 12 min at 4 000×g and the supernatant was subsequently analyzed by HPLC (Agilent Technologies 1260 infinity, Santa Clara, CA, USA). The stationary phase was a C18 column (Perfectsil Target ODS-3 (5 µm), 250 × 4.6 mm; MZ Analysentechnik, Mainz, Germany). The separation of flavonoids compounds was carried out using the method of Gudej and Tomczyk (2004). The elution solvent was composed of 0.5% phosphoric acid in water (A) and acetonitrile (B) and facilitated by gradient system as followed: 0–30 min: B (18%); 30–60 min: B (67%); 60–65 min: B (18%); 65–70 min: B (18%). The UV detector was set at 280 nm. Chromatography was done at 25 °C at a flow rate of 0.8 mL/min. Identification of flavonoids was performed by comparison of retention times and UV spectral peaks of the sample with authentic standard. Quantitative estimation of flavonoids was done by measurement of the integrated peak areas of the standard concentrations and samples. Also, reported flavonoid compounds were identified by LC–MS method.

Liquid chromatography/mass spectrometry

A liquid chromatography (Shimadzu, Kyoto, Japan) coupled to a single quadrupole ion mass spectrometer equipped with a Phenomenex C18 column (250 mm × 4.6 mm I.D., 3.5 µm) was applied for

the analysis of flavonoids. The binary mobile phase consisted of acetonitrile (A) and 0.01% formic acid aqueous solution (B) using the gradient program as follows: 0–5 min, 40–50% (A); 5–13 min, 50–60% (A); 13–15 min, 60–68% (A); 15–20 min, 68–40% (A); and 20–30 min, 40% (A). The column temperature was maintained at 30 °C, the flow rate 0.6 mL/min and the injection volume 50 µL. The spectra were recorded in the targeted mode within the m/z mass range of 100–2000. Flavonoid profiles were identified in the positive ion modes. In this work, [M + H]⁺, [M + Na]⁺ ions of flavonoids were determined.

Data analysis

The statistical analyses were performed using SPSS software (Statistical Package for Social Science; version 21). A one-way ANOVA was performed to identify significant differences among the treatment–control groups ($P \leq 0.05$) and measured by using Duncan's multiple range. All data were presented as mean ± standard deviation.

Result and discussion

Development of callus culture

For callus induction the seedling cotyledon, hypocotyl and shoot explants were used. The results related to callus formation from the explants have been summarized in Table 2. Although, calli were observed from the second week of culture, callus induction seems to be highly affected by the type of explants (Chaudhry et al. 2007). Higher callus induction occurred on hypocotyl explants under all hormonal combinations tested compared to other explants. The same results were observed in callus induction of *Levisticum officinale* (Khodashenas et al. 2015) and *Beta vulgaris*

Table 2 Combined effect of auxins and cytokinins on callus proliferation

Hormone (mg L ⁻¹)	Cotyledon		Hypocotyl		Shoot	
	Percentage of response	Nature of callus	Percentage of response	Nature of callus	Percentage of response	Nature of callus
Control						
NAA	Kin					
1	1	41 ± 3.0 ^{cd}	WC	44 ± 5.5 ^{de}	WC	17 ± 1.5 ^c
2	0.5	34 ± 4.1 ^{ef}	WOC	41 ± 4.1 ^{def}	WOC	13 ± 1.5 ^d
2	1	45 ± 2.0 ^{cd}	WOC	45 ± 4.0 ^{de}	WC	30 ± 3.0 ^a
3	1	51 ± 3.5 ^b	WOC	52 ± 3.6 ^c	WC	23 ± 1.9 ^b
NAA	BAP					
1	1	46 ± 3.7 ^c	WC	52 ± 3.0 ^c	WC	21 ± 2.4 ^b
2	1	40 ± 5.1 ^{cd}	WC	43 ± 3.6 ^{de}	WC	14 ± 2.5 ^{cd}
2	2	42 ± 4.3 ^{cd}	WC	47 ± 3.0 ^{cd}	WC	14 ± 2.3 ^{cd}
3	1	54 ± 4.9 ^{ab}	WC	69 ± 7.6 ^b	WC	27 ± 3.0 ^a
3	2	57 ± 4.4 ^a	WC	90 ± 4.2^a	WC	29 ± 2.8 ^a
24-D	Kinetin					
1	1	39 ± 2.1 ^{de}	WOF	36 ± 2.8 ^{fgh}	WOF	17 ± 1.6 ^c
2	1	26 ± 2.5 ^{gh}	WOF	52 ± 4.5 ^c	WOF	22 ± 1.5 ^b

Data collected after 15 days of inoculation callus on MS medium supplemented with different concentrations and combinations of auxins and cytokinins for callus proliferation

WOC white organogenetic compact, WC white compact, WOF white organogenetic friable

Means sharing the same letter in each column do not differ significantly at $P < 0.05$ (Duncan's test). Best explant and hormonal combination are indicated in bold

(Taghipour et al. 2013), in which callus could be produced successfully from different plant segments.

The percentage of calli formed under different concentrations and combinations of auxins, NAA and 2,4-D, and cytokinins, BAP and Kin are summarized in Table 2. There were no callus induction in MS without the plant growth regulators or with auxin and cytokinin alone. Callus induction was promoted in media with intermediate ratio of auxin and cytokinin, while an inappropriate ratio of these growth regulators favours root or shoot regeneration, as reported by other researchers (Nandwani and Ramawat 1992; Mathur and Shekhawat 2013). The highest callus frequency (90%; $P \leq 0.05$) was obtained from hypocotyl explants on the medium supplemented with 3 mg L⁻¹ NAA and 2 mg L⁻¹ BAP. The percentage of callus formed on cotyledon explants followed a similar trend on this medium, but with a lesser extent (57% respectively, Table 2) than hypocotyl explants. The present finding supports the earlier finding that the greater callus formation is achieved when a

suitable concentration of NAA and BAP is applied (Toker et al. 2003).

The data on callus induction were recorded after 15–17 days of inoculation. Table 2 shows that all produced calli on the media containing NAA and BAP were compact with least shoot and root regeneration; whereas friable calli were observed in media supplemented with 2,4-D. Consistent with our results, it has been reported that the friable callus showed organogenesis capacity while these observations had not been recorded for compact calli (Ritchie et al. 1989). The nature of calli after the 1st subculture was compact, of brown colour with poor growth. In this study, to minimize the extent of explant browning as well as to accelerate callus growth, the chemical compound asparagine was added to the medium with higher callus induction capacity (3 mg L⁻¹ NAA and 2 mg L⁻¹ BAP). After the 2nd subculture, the obtained results clearly indicated that the culture treated with asparagine showed pronounced positive responses, i.e. less browning and better growth (Fig. 1). To achieve successful tissue culture of *P. laevigata*, Buendía-

Fig. 1 Callus development in *P. farcta* explants. **a** calli induction in the seedling cotyledon, hypocotyl and shoot explants after 2 weeks on MS medium containing 3 mg L^{-1} NAA, 2 mg L^{-1} BAP, **b** callus from shoot explants, **c** callus

from hypocotyls explants, **d** callus from cotyledon explants after 28 days on MS medium containing 3 mg L^{-1} NAA, 2 mg L^{-1} BAP and 0.5 mg L^{-1} asparagine

González et al. (2007) optimized the medium with amino acids such as asparagine and glutamine.

Transformed hairy root

It has been showed that various species, organs and tissues of plants show various degrees of susceptibility to infection with *A. rhizogenes* (Carvalho et al. 2004). However different explant sources can be used for hairy roots production, some tissues perform more successful than others. In this work, three parts of *P. farcta* (hypocotyl, cotyledon and shoot explants) were infected with *Agrobacterium* strains: *LBA9404*, *A4* and *ARI5834*, in three separate stages. The cotyledon segments showed the maximum transformation after inoculation with *ARI5834* and *LBA9404* strains, with a frequency of 59 and 23%, respectively (Table 3). A high percentage of hairy root production in cotyledon explants in the present study may be related to the more competence for transformation and more sensitivity of these explants to *A. rhizogenes* than other explants, which this sensitivity depends on the physiological status of tissues (Pawar and Maheshwari

2004). This is in agreement with some previously reported results for other plants (Moghadam et al. 2014). The shoot segments showed no response to transformation.

Virulence of the *Agrobacterium* varies according to the strains that affected the frequency of the hairy root formation (Giri et al. 2001). The transformation efficiency of each strain was investigated in all types of inoculated explants, after 16 ± 1 days of co-cultivation on MS medium. The maximum transformation frequency occurred in strain *ARI5835* with 24.7 percent, while the percentage of hairy root formation from the explants inoculated with *LBA9404* and *A4* was 11.6% and 4.8% respectively. *ARI5834* is one of the most popular and efficient *Agrobacterium* strains used for the hairy root induction of various explants (Sharifi et al. 2014). The transgenic roots of strains *A4* turned brown and their growth were arrested. Uninfected explants were not able to show any induction of roots even after 2 months on MS medium.

After three passages on MS medium containing cefotaxime, hairy root cultures were free of bacteria

Table 3 Frequency of hairy root induction on hypocotyl (H), cotyledon (C) and shoot (S) explants of *P. farcta* infected with different strains of *A. rhizogenes* and the maximum transformation frequency of each strain

Explant	Hypocotyl	Cotyledon	Shoot	Maximum transformation frequency of each strain = (H + C + S)/3
Strain				
A4	0	14.6 ± 1.1	0	4.8
LBA9404	12 ± 2.0	23 ± 2.6	0	11.6
AR15834	15.3 ± 2.5	59 ± 3.4	0	24.7

Data are mean ± SD. Values within rows followed by the same letter(s) are not significantly different at $P < 0.05$ (Duncan's test)

cells. Moreover, the presence of *rolC* gene confirmed the transgenic nature of hairy roots. DNA extracted from hairy roots after PCR amplification demonstrated the product of expected size 534 bp with used *rolC* primers (Supplementary material 1). It was not observed in PCR product of untransformed root DNA, which was used as negative control. PCR amplification was also performed using *virD* gene-specific primers, 743 bp, to check *Agrobacteria* contamination in hairy roots. Supplementary material 1 clearly showed the absence of contamination of roots with *Agrobacterium*. A hairy root line (C2) obtained from cotyledon transformed with AR15834 strain showed fast growing and transgenic nature. This hairy root exhibited the characteristic plagiotropic growth and high laterally branched nature (Fig. 2); so this hairy root line was used to measure flavonoid content.

Determination of flavonoids

Flavonoids are the most common group of polyphenol compounds that are widely found in plants and are involved in many functions. These compounds contribute to promote health and alleviate illness because of possessing considerable antiallergic, anti-inflammatory, antitumor, anti-proliferative, and anticancer activities (Kumar and Pandey 2013). The growing commercial importance of flavonoids and an increasing demand for renewable resources of precious metabolites has paved the way to develop alternative routes for their production. Various in vitro approaches have been used for flavonoid production, e.g., hairy root, callus, and cell suspension cultures. In this study, the best callus line (cultured on MS supplemented with 3 mg L⁻¹ NAA and 2 mg L⁻¹ BAP + asparagine) were processed through a colorimetric method to evaluate the amount of flavonoid

metabolites. Moreover, the amount of flavonoids was compared with that in hairy root line C2 and seedling root. Total flavonoid was found to be higher in hairy root (4800 µg g⁻¹ DW) as compared to seedling root (3100 µg g⁻¹ DW) and callus (1900 µg g⁻¹ DW).

HPLC analysis was then performed to identify and quantify flavonoids. Quantitative estimation of flavonoids was done based on the peak areas of the standard concentrations and samples (Fig. 3). In particular, the levels of 7 flavonoids derivatives (quercetin, naringenin, daidzein, resveratrol, myricetin, kaempferol, luteolin 3'-glucoside) were measured. Since the present study is the first report on the establishment of *P. farcta* hairy root cultures, the flavonoid compounds were identified by LC/MS method as well. Several reports have been cited in the literature demonstrating the applicability of MS and MS/MS coupled with liquid chromatography in the analyses of flavonoids and their derivatives (Staszko et al. 2011). In this work, [M + H]⁺ and [M + Na]⁺ ions of flavonoids were determined (Supplementary material 2).

As shown in Table 4, the content of naringenin in callus culture was prominently higher than in hairy roots and seedling root by 6.9 and 1.4 times, respectively. Naringenin is one of the flavonoids produced in the beginning of the flavonoid pathway. It can be utilized as a precursor for production of the other flavonoids as far as exogenous naringenin feeding to plant cultures resulted in increased flavonoid synthesis (Masoumian et al. 2011). On the other hand, some reports showed that cells need some degree of differentiation to produce more and complicated metabolites (Bourgaud et al. 2001). It is suggested that naringenin accumulates in undifferentiated callus of *P. farcta*, as a start point of flavonoid synthesis and it can be converted to other flavonoids in differentiated seedling and hairy roots. Likewise, some compounds

Fig. 2 Hairy root induction in *P. farcta* by using *A. rhizogenes*; AR15834 strain. **a** Young in vitro plants that used for transformation, **b** syringe transformation in different parts of

explants, **c** hairy root induction in cotyledon (Line C2) and **d** line C2 after 21 days in liquid MS free hormone medium

detected in hairy root were absent in callus cultures such as quercetin and luteolin 3'-glucoside. As a wide variety of important secondary phytochemicals in unorganized callus has been produced, while in other cases production requires more differentiated tissues. For example, *Hypericum perforatum* as a medicinal plant accumulates hypericin in foliar glands whereas the accumulation of phytochemicals in undifferentiated cells has not been demonstrated (Smith et al. 2002). In the hairy roots of *P. farcta* some of the detected compounds had higher accumulation when compared to the calli. This is due to the lack of cellular or tissue differentiation in callus cultures, as determined from observations of root and hairy root cultures. A significant accumulation was observed for resveratrol in hairy root ($4.9 \mu\text{g g}^{-1}$ DW)

compared with callus ($1.8 \mu\text{g g}^{-1}$ DW) and seedling roots ($1.1 \mu\text{g g}^{-1}$ DW). Resveratrol has crucial contribution in reactive oxygen species quenching and is considered as a beneficial flavonoid in cardiovascular health (Khurana et al. 2013).

Furthermore, a significant increase was observed for quercetin, kaempferol, myricetin and luteolin 3'-glucoside in hairy roots. Currently, these compounds are of interest because of their antioxidant, anticarcinogen, antimutagen properties (Batra and Sharma 2013; Shukla and Gupta 2010). In contrast, the level of daidzein in hairy root was kept at the same level as in the seedling roots and showed ~ 1.3 -fold increase compared to that in callus culture. Daidzein as an intracellular metabolite was quantified in all the *Psoralea* root lines transformed with *A. rhizogenes*

Fig. 3 The HPLC chromatogram of the standard flavonoids (upper chromatogram) and the chromatogram of hairy root flavonoids (lower chromatogram); 1 quercetin, 2 myricetin, 3 resveratrol, 4 daidzein, 5 luteolin 3'-glucoside, 6 naringenin, 7 kaempferol

Table 4 Content of flavonoids in *Prosopis* seedling root, hairy root and callus

Samples		Root	Hairy root	Callus
Flavonoids ($\mu\text{g g}^{-1}$ DW)	Naringenin	54.1 ± 1.8^b	11.4 ± 1.1^c	78.7 ± 2.7^a
	Resveratrol	1.1 ± 0.23^c	4.9 ± 0.34^a	1.8 ± 0.14^b
	Myricetin	7.3 ± 0.97^b	12.6 ± 1.21^a	5.1 ± 0.63^c
	Daidzein	3.19 ± 0.16^a	3.31 ± 0.21^a	2.46 ± 0.11^b
	Kaempferol	13.2 ± 1.10^b	23.6 ± 1.43^a	12.8 ± 0.87^b
	Quercetin	3.1 ± 0.48^b	7.3 ± 0.76^a	Undetected
	Luteolin 3'-glucoside	4.3 ± 0.65^b	5.9 ± 0.73^a	Undetected
Total flavonoid ($\mu\text{g g}^{-1}$ DW)		3100 ± 0.71^b	4800 ± 0.91^a	1900 ± 0.45^c

Data are mean $\pm$ SD. Values within rows followed by the same letter(s) are not significantly different at $P < 0.05$ (Duncan's test)

(Rai et al. 2014). The isoflavone daidzein provides efficient protection against coronary heart disease, decrease of blood cholesterol, inhibition of hormone-related cancers, and prevention of osteoporosis (Lee et al. 2012).

Hairy roots are characterized with genetic stability because they originate from a single cell. The productivity of secondary plant compounds in hairy roots remains stable over time while the loss of their concentration has been frequently observed in callus culture (Sánchez-Sampedro et al. 2009). On the other hand, the amount of valuable flavonoids in seedling roots are considerably less than in transformed hairy roots. In addition, the hairy roots exhibited more rapid growth than the whole plants. The present study

highlights the method of hairy root culture to define flavonoid biosynthetic capacity, which makes this promising biological system for mass production of a desired flavonoid for the industrial applications or metabolic pathways studies. Previous works on *P. farcta* indicated that the plant metabolic pathways are competent and responsive to environmental agents (Zafari et al. 2017a), where the enzymes and phenolic compounds were stimulated under the application of environmental factors and elicitation by signaling molecules (Zafari et al. 2016).

Our previous observations also showed a tight correlation between elicitation by signaling molecules and increase in flavonoids because of metabolic overflowing to the flavonoid pathway (Zafari et al.

2016, 2017b). These finding together with the results of the present study suggest *P. farcta* as a new candidate for mass production of medicinally valuable flavonoid compounds by using biotechnological approaches such as cell and hairy root culture under biotic and abiotic elicitors in a bioreactor system as established previously for other plants (Ahmadi-sakha et al. 2016).

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Author contributions This research paper was accomplished with the collaboration of authors. SZ performed the experiments, analyzed data and wrote the manuscript. MS designed the study and supervised the study. NAC advised the study.

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