Original Article:

A Comparative Study of Apigenin Content and Antioxidant Potential of *Cosmos Bipinnatus* Transgenic Root Culture

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ABSTRACT

Background: Flavonoid-derived components have been studied for their therapeutic properties.

Objectives: Apigenin has shown remarkable antioxidant and anti-inflammatory features, so we should have a reliable source of apigenin.

Methods: In this study, we used high-performance liquid chromatography method to compare the amount of apigenin in flower, root, leaf, and stem of three varieties of osmos *bipinnatus*, i.e., 'Dazzler,' 'Xanthos,' 'Sensation Pinkie', and in transgenic root culture of *C. bipinnatus* 'Dazzler'. Besides, the antioxidant activity of *C. bipinnatus* 'Dazzler' transgenic root culture was evaluated using Ferric Reducing Antioxidant Power (FRAP) assay.

Results: Dazzler variety flowers showed the highest recovery of apigenin with 0.799 mg/100 mg Dry Weight (DW). However, the Sensation pinkie variety leafs had the lowest recovery with 0.089 mg/100mg. Apigenin content in transformed roots (0.797 mg/100 mg DW) of *C. bipinnatus* 'Dazzler' was significantly higher than non-transformed roots (0.42 mg/100 mg DW). The ethanolic extract of hairy root showed the FRAP value of 668.1 µM Fe^{2+}/mg that was comparatively more than the wild root FRAP value (426.2 µM Fe^{2+}/mg).

Conclusion: In conclusion, the presence of apigenin in high amounts in hairy root cultures of C. bipinnatus 'Dazzler' indicates its great potential for the future pharmaceutical industry.

Introduction

Cosmos bipinnatus is a popular annual plant belonging to the genus Cosmos (*Asteraceae* family), composed of about 25 species mainly native to Mexico and Southwestern USA [1]. It is known as the garden Cosmos or Mexican aster, having pink, red, violet, or white-colored flowers. It is a short-day plant, and its leaves and flowers possess ornamental value [2]. It is widely distributed globally, probably

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due to its plasticity traits and strong adaption in different climates. There are different types of Cosmos flowers based on their petal colors, such as *Cosmos bipinnatus* 'Dazzler' with purple, *Cosmos bipinnatus* 'Xanthos' with yellow, and *Cosmos bipinnatus* 'Sensation Pinkie' with pink petals. In traditional medicine, the flowers and leaves of *C. bipinnatus* have been widely used to treat various diseases like headache, intermittent fever, jaundice, splenomegaly, pain, and stomach disorders [3].

The main bioactive chemical components of this plant are flavonoids such as butein, luteolin, tricin, quercetin, chrysoeriol, butin, apigenin 7-O-glucuronide, chrysoeriol 7-O-glucuronide, luteolin 7-O-glucuronide, and balanophonin as a neolignan [4, 5]. Generally, the flavonoids of *C. bipinnatus* have been extracted from the seeds and petals [4, 5].

Oxidative stress is a phenomenon caused by an imbalance between free radicals and antioxidants in cells and tissues. It naturally occurs when the production and accumulation of free radicals overwhelm the intrinsic antioxidant defenses that can negatively affect several cellular structures, such as DNA, membranes, proteins, lipids, and lipoproteins [6]. The most common sources of oxidative stress are radiation, air pollution, noise, pesticides, and household chemicals [7]. It has been proven that the antioxidant activity of a plant extract is related to its phenolic content [8]. The genus *Asteraceae* is a rich source of bioactive pharmaceutical compounds, such as phenols and flavonoids [4, 5]. Besides, the essential oils of this plant are predominantly composed of monoterpenes and sesquiterpenes [9]. Olajuyigbe and Ashafa study on its essential oil activity demonstrated that it has potential antibacterial properties against Gram-negative and Gram-positive bacteria isolates [9]. Experimental results have shown that *C. bipinnatus* leaf, root, and flower extracts possess effective larvicidal [10], anti-inflammatory [11], antioxidative, and antigenotoxic [12] activities, respectively.

These properties could be a reason to increase the demands for this medicinal plant. Therefore, the production of the desired components by hairy root cultures could be an alternative source. Hairy root cultures have attracted much attention due to their ability to high-level production and accumulation of secondary metabolites and producing necessary pharmaceuticals as the intact plants, as well [13, 14]. The main objectives of the present study were to determine and evaluate the apigenin content in three different *C. bipinnatus* varieties and establish in vitro cultures for *C. bipinnatus* 'Dazzler' with satisfactory efficiency of apigenin production and antioxidant activity.

Materials and Methods

Apigenin extraction and High-Performance Liquid Chromatography (HPLC) analysis

Apigenin standard (99.9% purity) was purchased from Sigma-Aldrich (Aldrich Division; Steinheim, Germany). *Cosmos bipinnatus* 'Dazzler', 'Xanthos', and 'Sensation Pinkie' were collected from the Botanical Garden of Zanjan University of Medical Sciences. The collected plants were prepared in duplicate and authenticated by Dr. AliReza Yazdinejad (School of Pharmacy, Zanjan University of Medical Sciences), and voucher specimens deposited in the herbarium of the School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran (herbarium No. 4114, 4115, 4116, respectively). A certain amount of air-dried and powdered plant tissues, including root, stem, leaf, and flowers of three different varieties of *Cosmos bipinnatus* and hairy roots of *C. bipinnatus* 'Dazzler', were extracted with High-Performance Liquid Chromatography (HPLC) grade ethanol (purity ≥99.9%). A powdered sample (1 g each) was placed in falcon (12 mL), and ethanol (10 mL) was added to the tube. Each sample was sonicated for 2 min, centrifuged at 15000g for 5 min, and the supernatant collected and filtered through a 0.2 µm filter. We employed HPLC to measure the yield of apigenin.

HPLC analysis of apigenin content

The HPLC analysis was performed using an HPLC system (WATER, USA) equipped with a UV detector (WATER, model Breeze, USA). The separation of apigenin was performed using a Fortis C18 column a C8 analytical column (250 mm×4.6 mm, particle size 5 µm; Perfectsill, MZ-Analysentechnik, Germany) equipped by a guard column of the same packing. The mobile phase was composed of water-acetonitrile (55:45 v/v), 340 nm for detection wavelength, an injection volume of 20 µL by a loop injector (Rheodyne ®7725i, Cotati, CA, USA), and the flow rate of 1 mL/min. The outputs were recorded and analyzed using software (Breeze, USA). The method accuracy and sensitivity were evaluated according to our previous study [15]. Stock solutions of apigenin were prepared in HPLC grade ethanol at concentrations of 1 mg/mL. Dilutions of working solutions were prepared daily in HPLC grade ethanol in the appropriate series of standard solutions of 0.05, 0.0125, 0.005, 0.001, 0.0005, 0.0001, 0.00005, and 0.00001 mg/mL.

Establishment of hairy root cultures of *C. bipinnatus* 'Dazzler'

Seed sterilization and germination

C. bipinnatus 'Dazzler' seeds were obtained from the Institute of Pakan Bazr, Isfahan, Iran. After sterilization with 70% v/v ethanol for 30 seconds, followed by 10% (w/v) sodium hypochlorite for 15 minutes and rinsing with sterile distilled water for 3–4 times, the seeds were germinated on Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.7% agar at 25°C under a 16-h photoperiod regime. Seed germination was started within 5-6 days, and 1-month-old in vitro grown seedlings were applied for hairy root induction.

Hairy root induction

Hairy root induction occurred through the infection of stem explants of *C. bipinnatus* 'Dazzler' variety with *A. rhizogenes* MSU 440 strain according to Jaberi et al. study [16]. After 1 week of incubation, the hairy roots emerged from inoculation sites, were excised and transferred to solid half-strength MS medium containing 400 mg/L cefotaxime, and cultured for three weeks at 28°C. To establish the liquid culture of hairy roots, thirty 1-cm root tips were inoculated into 250 mL flasks containing 50 mL of half-strength MS medium and maintained on an orbital shaker at 90 rpm, at 28°C, and in continuous darkness. After one month, the hairy roots were harvested to examine the transgenic nature and their apigenin content.

Extraction of genomic DNA and Polymerase Chain Reaction (PCR)

Genomic DNA of the transformed root of *C. bipinnatus* 'Dazzler' was extracted using the modified cetyltrimethylammonium bromide method [17]. The forward and reverse primers for the fragments of Root Loci B (*rol*B) gene were 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3', respectively [18]. The PCR conditions for amplification of the rolB gene were as follows: Initial denaturation at 94°C for 5 min, followed by 30 cycles of amplification in 3 steps (94°C for 1 minute, primer annealing at 58°C for 1 minute, and primer elongation at 72°C for 1 minute and 72°C for 10 minutes for the final extension). The purity of PCR amplified DNA was checked by electrophoresis on 1% agarose gel stained with DNA safe stain (FluoroVue™) and visualized by a UV transilluminator.

Ferric Reducing Antioxidant Power (FRAP) assay

The ferric reducing ability of hairy root extract was assessed according to the method of Benzie and Strain [19]. A standard curve of $FeSO_4$ was prepared. About 200 mg of hairy root and wild root materials were powdered and extracted with 1 mL of 100% ethanol three times for 8 hours and then shaken thoroughly. Then, they were sonicated for 10 min, centrifuged for 15 min at 5000× g, and the supernatant was collected for analysis. About 50 µL of filter-sterilized hairy root and wild root extracts (1 mg/mL) were added to premixed freshly prepared 1.5 mL of FRAP reagent (0.3 M, pH 3.6 acetate buffer: 20 mM $FeCl_3$: 10 mM TPTZ = 10:1:1). Then, the sample absorbance was recorded at 593 nm after 30 min incubation at 37°C. Ferric Reducing Antioxidant Power (FRAP) activity was calculated and expressed in equivalents of µM Fe^{2+}/mg DW.

Statistical analysis

The experiments were performed in Completely Randomized Design (CRD) with three replicates. The statistics tests of ANOVA, Tukey post hoc test, Student t-test, linearity studies, and calculation of SE were done in SPSS v. 22. Excel 2010 software was applied for drawing charts.

Results

Quantification of apigenin content

The method produced linearity over the apigenin concentration range of 0.05-0.00005 mg/mL with a correlation coefficient (R^2) of 0.999, which is satisfactory for intended purposes. A typical linear calibration curve of the method was y= 1E + 07x + 2533.1, with x and y representing apigenin concentration (mg/mL) and peak area, respectively. The limit of quantification and limit of detection for apigenin were found to be 0.00005 and 0.00001 mg/mL, respectively. Chromatographic profiles of the standard and the hairy root sample were presented in Figure 1.

Analysis of the apigenin content of the genetically transformed root culture of *C. bipinnatus* 'Dazzler' and root, stem, leaf, and flowers of wild *C. bipinnatus* varieties, including *C. bipinnatus* 'Dazzler', 'Xanthos', and 'Sensation Pinkie', resulted in identification and quantification of apigenin (Figure 2). We found that the apigenin content of *C. bipinnatus* 'Dazzler' was comparatively higher than that of *C. bipinnatus* 'Xanthos' and *C. bipinnatus* 'Sensation Pinkie' varieties (Figure 2).

Table 1. The extraction recovery determined for apigenin from *C. bipinnatus* varieties

Plant	Tissue	Amount of Extracted Apigenin as Mean±SD (mg/100 mg)	Recovery (%)
C. bipinnatus 'Dazzler'	Hairy root	0.7971±0.0328	100
	Root	0.42±0.0166	100
	Stem	0.315±0.0158	100
	Leaf	0.1781±0.0071	100
	Flower	0.7996±0.0217	100
C. bipinnatus 'Xanthos'	Root	0.165±0.0054	100
	Stem	0.1561±0.0066	100
	Leaf	0.101±0.0047	100
	Flower	0.2446±.0116	100
C. bipinnatus 'Sensation Pinkie'	Root	0.2371±0.0098	100
	Stem	0.1867±0.0087	100
	Leaf	0.0885±0.0022	100
	Flower	0.1108±0.00515	100

In wild plants, the highest content of apigenin was found in the flowers (0.7996±0.0217 mg/100 mg), followed by the root explants (0.42±0.0166 mg/100 mg) of *C. bipinnatus* 'Dazzler' (Figure 2). As the amounts of apigenin in *C. bipinnatus* 'Xanthos' (0.2446±0.0116 mg/100 mg, $P<0.001$) and *C. bipinnatus* 'Sensation Pinkie' (0.1108±0.00515 mg/100 mg, $P<0.001$) flowers were significantly lower than that of *C. bipinnatus* 'Dazzler' (Figure 2). *C. bipinnatus* 'Dazzler' was selected for hairy root induction and antioxidant activity studies based on its higher content of apigenin. The extraction recovery determined for apigenin was shown in Table 1.

Establishment of hairy root cultures

The genetically transformed root cultures of *C. bipinnatus* 'Dazzler' were established using *A. rhizogenes* MSU 440 strain, half-strength MS medium as co-cultivation, and stem explants (Figure 3). The first hairy roots were observed at wounded sites, 5-6 days after inoculation (Figure 3). No hairy roots were observed in non-infected stem explants.

PCR confirmed the integration of the *rol*B gene in *C. bipinnatus* 'Dazzler' transgenic hairy root lines, and a fragment with 425 bp was amplified from each selected line (Figure 4). Among 4 different selected hairy root lines, hairy root line 1 exhibited a vigorous growth with a tendency to produce more branches as compared to other lines. Therefore this line was selected for HPLC analysis of apigenin content and antioxidant activity.

The transgenic hairy roots of *C. bipinnatus* 'Dazzler' displayed a significantly higher apigenin accumulation (0.7971±0.0328 mg/100 mg) than that of non-transformed root (0.42±0.0166 mg/100 mg, $P<0.001$), as judged from HPLC analyses. However, the aforementioned factor remained insignificant while compared to the amounts of apigenin in flowers of *C. bipinnatus* 'Dazzler' (0.7996±0.0217 mg/100 mg, $P>0.05$) (Figure 2).

Antioxidant activity

In the present study, the antioxidant activity of *C. bipinnatus* 'Dazzler' hairy root extract was evaluated using FRAP assay. Our results revealed that FRAP radical scavenging activity of *C. bipinnatus* 'Dazzler' transgenic hairy roots (668.1±64.33 µM Fe^{2+}/g) was significantly higher than non-transformed roots (426.23±47.29 µM Fe^{2+}/g) ($P<0.01$) (Figure 5).

Discussion

Apigenin (4′,5,7-trihydroxyflavone) is a natural flavonoid widely distributed in several dietary plant foods such as vegetables, herbal spices, and fruits [20]. It is

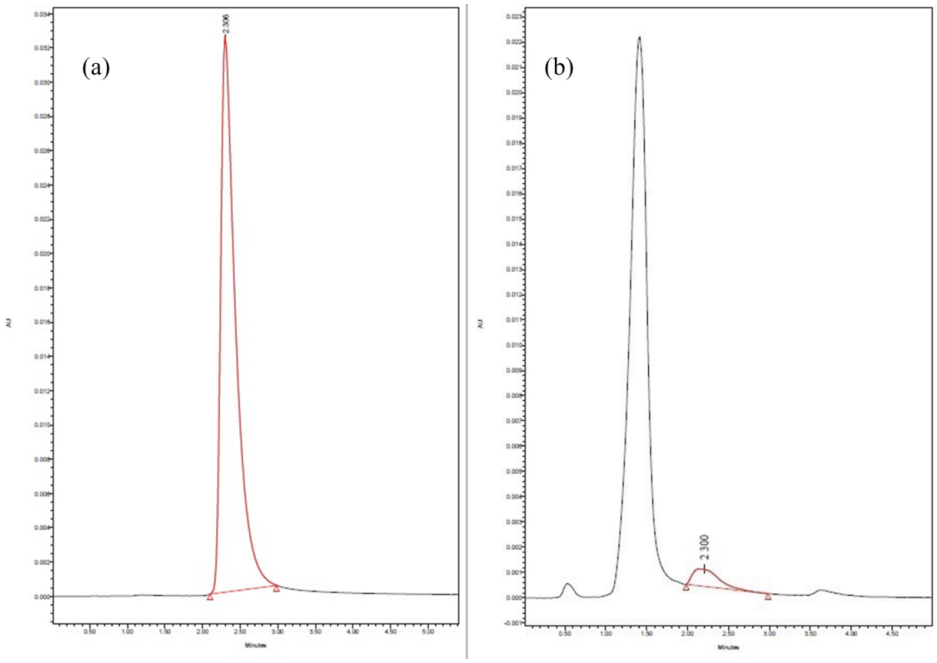

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Figure 1. Representing High-Performance Liquid Chromatography (HPLC) chromatograms of the standard (a) and *C. bipinnatus* 'Dazzler' hairy root extract (B)

also one of the most known and well-studied phenolics. Apigenin, as a biologically active flavonoid, has potential anti-inflammatory [21], anticancer [22], and antioxidant properties [23]. In the current study, there was a wide range of apigenin content among different studied varieties of *C. bipinnatus*, from 0.08 mg/100 mg (*C. bipinnatus* 'Sensation Pinkie' leaf's) to 0.8 mg/100 mg (*C. bipinnatus* 'Dazzler' flower's) (Figure 2). Since naturally occurring apigenin abundance is very low, in this work, *C. bipinnatus* 'Dazzler' genetically transformed root cultures were established as an alternative approach to enhance the production of apigenin.

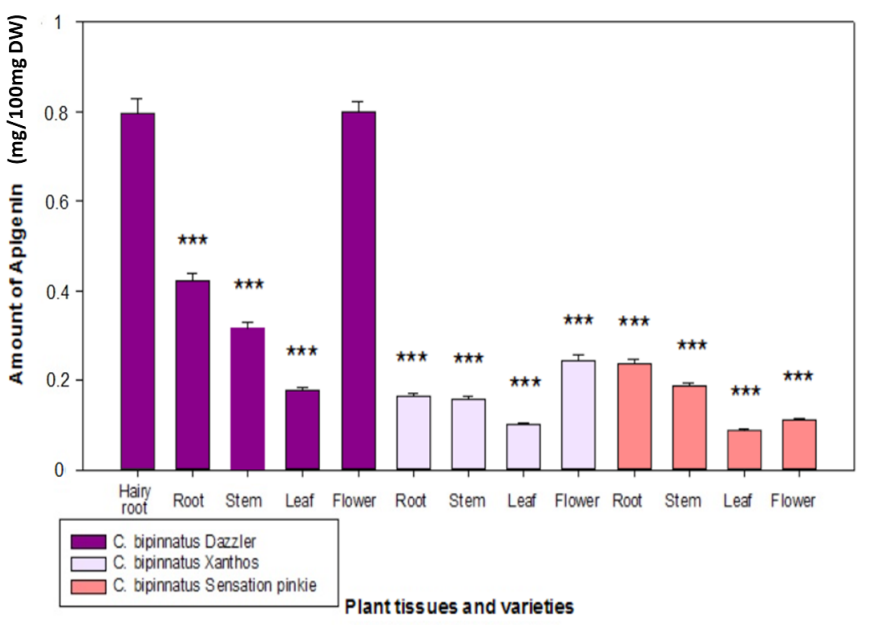

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Figure 2. The apigenin content in three varieties of *C. bipinnatus*

Values are expressed as the Mean±SD and were analyzed using 1-way ANOVA followed by Tukey post hoc test.

***P<0.001 compared with the amount of apigenin in the hairy root (n=8-10).

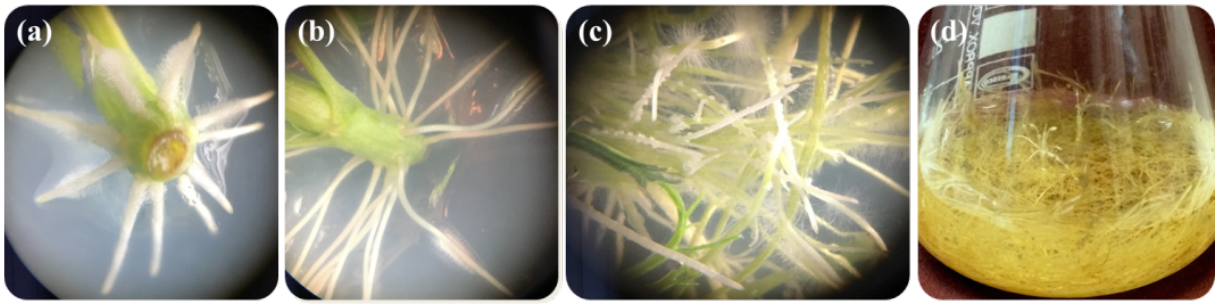

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Figure 3. Hairy root induction and growth

A: Hairy root induction on stem explants of *C. bipinnatus* 'Dazzler' after 2 weeks; B: After 3 weeks; C: After 4 weeks using A. rhizogenes strain MSU 440; D: Hairy root growth in liquid ½ Murashige and Skoog (MS) medium.

According to the results, the maximum hairy roots induction was achieved using examined factors, including *A. rhizogenes* MSU 440 strain, stem explant, and half-strength MS medium as co-cultivation media (Figure 3). HPLC analysis of *C. bipinnatus* 'Dazzler' hairy roots ethanolic extract revealed the high content of apigenin in transformed roots (0.8 mg/100 mg) that is approximately a 1.9-fold increase in apigenin content as compared with wild roots (Figure 2). Hairy root cultures as a promising alternative source of high-value secondary metabolites have been attracted more attention. The hairy root is typically formed when the T-DNA of the root-inducing (Ri) plasmid of *A. rhizogenes* is integrated into the genome [24]. Root Loci (*rol*) genes (*rol*ABC) harbored by the Ri plasmid of this bacterium causes the hairy root disease by incorporating the host plant genome in wounded sites of higher plants. Of the *rol* genes, *rol*B is effective for the induction of hairy roots and the most potent stimulating gene for secondary metabolites production [25]. The high-*rol*B-expressing cultures of *Vitis amurensis* and *Rubia cordifolia* were positively correlated with enhanced biosynthesis of resveratrol and anthraquinone up to 100 and 15-fold, respectively [26, 27]. Previously, several studies have focused on the production of flavonoids in hairy root cultures of medicinal plants [28-30]. Production of apigenin in hairy root cultures of several plants has been reported [31, 32], but its production was not reported in hairy root cultures of *C. bipinnatus* 'Dazzler'. The enhanced production of apigenin was reported in *Saussurea involucrata* hairy roots overexpressing the chalcone isomerase (*chi*) gene [32]. The *chi* gene was introduced into the genome of *S. involucrate* using *A. rhizogenes*-mediated transformation and overexpressed, which elevated the accumulation of apigenin up to 12 times than control hairy roots [32]. Additionally, the

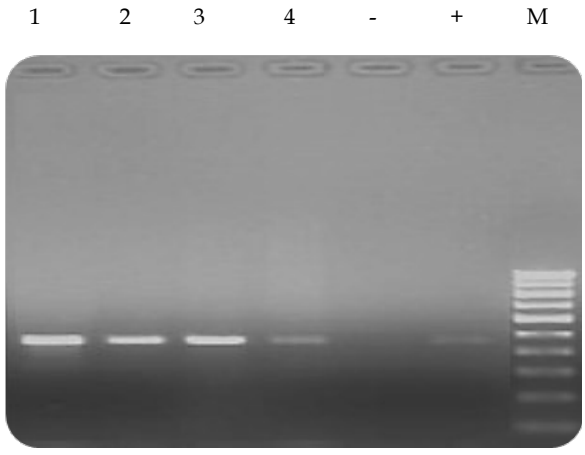

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Figure 4. PCR-amplified products of the rolB gene (425 bp) in hairy root lines of *C. bipinnatus* Lanes 1–4 show the presence of the rolB gene in the 4 hairy root lines; Lanes 5 and 6 refers to plasmid DNA and wild root DNA, respectively; Lane M refers to the 100 bp molecular size marker.

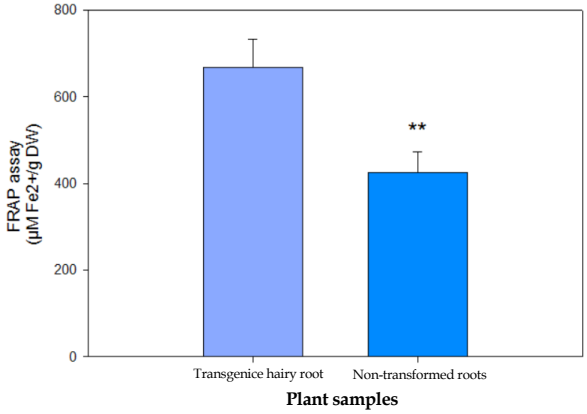

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Figure 5. Assessment of antioxidant activity in ethanolic extracts of the hairy roots and non-transformed roots of *C. bipinnatus* 'Dazzler' using Ferric Reducing Antioxidant Power (FRAP) assay

Values are expressed as the Mean±SD and were analyzed using t-test.

**P<0.01 compared with transgenic hairy root (n=8-10).

content of apigenin was increased more than 6-fold in methyl jasmonate-induced hairy root cultures of *Erigeron breviscapus* compared with control [33].

FRAP assay measures the amount of antioxidants based on their ability to reduce Fe^{3+} to Fe^{2+}. In the current study, the antioxidant molecules present in the ethanolic extract of *C. bipinnatus* 'Dazzler' hairy roots could reduce the TPTZ-Fe^{2+} to TPTZ-Fe^{3+}. Hairy root extract exhibited significantly higher antioxidant potential when compared to the wild root (control) (Figure 5). The high production of secondary metabolites in the genetically transformed roots could increase FRAP scavenging potential [34, 35]. These results are similar to those recorded by Muthusamy and Shanmugam [36] and Kudale et al. [37].

As stated before, excessive ROS gives rise to a phenomenon known as oxidative stress. This is a potentially harmful process that can damage cells, proteins, and DNA. This event can contribute to several diseases such as cancer, cardiovascular, neurological, respiratory, rheumatoid arthritis diseases, and delayed sexual maturation [38]. Several studies proved that herbal medicines like plant extracts are a rich source of polyphenols, flavones, and phenolic acids, which are the most important natural antioxidants [8, 39, 40]. It has been observed that flavonoids such as curcumin possess protective activity against aluminum chloride-induced oxidative stress and hepatotoxicity in rats [41]. Apigenin, as one of the flavonoids isolated from the cosmos genus and *Cosmos bipinnatus* 'Dazzler' hairy roots, exhibits antioxidant properties, protecting DNA against free radicals generated by H2O2 or Fe^{2+} and is considered to possess several biological activities such as anti-inflammatory and anticancer [42]. Therefore the high content of apigenin in transformed root cultures of *Cosmos bipinnatus* 'Dazzler' could be assumed as a new and alternative source of apigenin for future pharmaceutical applications.

Conclusion

As we observed, there is a noticeable difference in the amount of apigenin between varieties of *Cosmos bipinnatus*. The Dazzler variety had the highest amount of apigenin, and the best part for extraction of apigenin would be the flowers of the Dazzler variety. In addition, it has been proved that hairy roots of *C. bipinnatus* 'Dazzler' are a potential source of apigenin, and ethanolic extract of *C. bipinnatus* 'Dazzler' hairy root exhibits excellent scavenging activity against free radicals.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Investigation, Data collection (laboratory): Soroush Bijani, Zahra Gharari, Alireza Ahmadnia; Methodology: Ali Sharafi and Hossein Danafar; Supervision, writing – original draft, data analysis: Ali Sharafi.

Conflict of interest

All authors declared no conflict of interest.

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