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Clonal propagation, antioxidant activity and phenolic profiles of *Convolvulus galaticus* Rostan ex Choisy

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Abstract

Convolvulus galaticus Rostan ex Choisy (grizzle bindweed) is a medicinal plant in the family Convolvulaceae. The first objective of this study was to determine a highly efficient and rapid regeneration system for C. galaticus. Secondly, field-grown and in vitro-grown plants were compared in terms of antioxidant activities and phenolic constituents. C. galaticus leaves and stems were surface sterilized and three different explants (leaf, stem and node) were cultured. Regeneration was observed only with node explants. Best shoot proliferation was observed with 0.5 mg/l TDZ and 1.0 mg/l IAA, producing 15 shoots per explant at 84 % frequency. In vitro regenerated plants were also used as donor plants for explant source and best shoot formation was observed with 1.0 mg/l TDZ and 0.5 mg/l IBA with node explant, producing 23.7 shoots per explant at 60 % frequency. Regenerated shoots were transferred to rooting media and 1.0 mg/l IBA was the most effective for rooting. In the second part of this study, methanolic extract of field-grown and in vitro-grown C. galaticus were compared in terms of antioxidant activity and phenolic constituents. Field-grown plant showed higher antioxidant activities and phenolic content than in vitro-grown plant.

Keywords: antioxidant, *Convolvulus galaticus*, *in vitro* culture, LC-MS/MS, phenolics

1. Introduction

Convolvulus galaticus Rostan ex Choisy (Grizzle bindweed) is an endemic, prostrate, herbaceous, perennial herb in the family Convolvulaceae (DAVIS [1]). The natural habitat for *C. galaticus* is *Pinus* woods, open steppe, stony slopes, meadows, cultivated and fallow fields which are usually calcareous at 880-2000 m. It is found in the inner and rarely in the North part of Turkey (DAVIS [1]). According to some ethnobotanical studies, leaves of *C. galaticus* have been used as laxative, cholagogue, anthelminthic (BAYTOP [2]; YEŞIL [3]) animal fodder (ERTUG [4]) and cooked food among the people (YEŞIL [3]). Poultices obtained from the flowers (TUZLACI and DOGAN [5]) and mouthwashes obtained with infusion of *C. galaticus* (ALTUNDAG and OZTURK [6]) have been used in the treatment of toothache in folk medicine. Decoction obtained from the roots of *C. galaticus* is used because of the purgative effect (OZTURK and OLCUCU [7]). Threat category of the endemic *C. galaticus* is evaluated as LC (Least concern) (BOCUK & al. [8]; YILDIZTUGAY & al. [9]). Antibacterial (TURKER & al. [10]; TURKER and KOYLUOGLU [11]), antitumor (TURKER and KOYLUOGLU [11]) and anticancer (TOKGUN & al. [12]; KARAKAS & al. [13]) activities of *C. galaticus* have been reported. Antioxidant activities of some species of *Convolvulus* (*C. arvensis*, *C. microphyllus*, *C. hystrix* and *C. dorycnium*) were studied and it

was found that high phenolic content of these species led to strong antioxidant activities (AWAAD & al. [14]; NACEF & al. [15]; DONIA & al. [16]; JAIN & al. [17]).

Grizzle bindweed is a valuable medicinal herb, but there are no reports on an *in vitro* culture protocol of this species. The present work reports an *in vitro* culture procedure for rapid clonal propagation of *C. galaticus*. Antioxidant activities and phenolic contents of field-grown and *in vitro*-grown plant materials were also determined and compared by LC-MS/MS method for the first time.

2.Materials and Methods

2.1 Clonal propagation

In vitro regeneration of *C. galaticus* was attempted by using two different explant sources (field-grown plants and *in vitro* regenerated seedlings). Field-grown plant parts (leaves and stems) were collected from AIBU Campus, Bolu, Turkey. Identification of the species was made by using "Flora of Turkey and The East Aegean Island" (DAVIS [1]) and voucher specimens (AUT-2026) were deposited at Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Field-grown plant parts were washed 2 hours under running water and then kept in sterile distilled water containing tween 20 (10 drops in 100 ml water) for 15 minutes. Surface sterilization was achieved in 0.1 % mercuric chloride ($HgCl_2$) for 15 min and then 70 % ethanol (EtOH) for 2 minutes. Plant parts were rinsed with sterile distilled water 5 times. After surface sterilization of the stems and leaves, leaf, stem internode and stem node explants were excised and placed in sterile disposable petri plates (80 x 15 mm) containing 15 ml of Murashige and Skoog medium (4.43 g/l, MS, Sigma Chemical Co., St. Louis, MO, USA) (MURASHIGE and SKOOG [18]), 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 min at 121°C and 105 kPa) with different combinations and concentrations of plant growth regulators. In the second part of the experiment, *in vitro* regenerated plantlets from stem node explant were used as donor plants. Three different explants (leaf, stem internode and stem node) were excised and placed on MS medium with different combinations and concentrations of plant growth regulators. All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$). After 6-8 weeks, regenerated explants were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MS medium with 1 mg/l gibberellin (GA_3) for shoot elongation for an additional two weeks. The shoot number and percentage of explants producing shoots were recorded after 10 weeks for all explants. Tests had 10 replications for each explant and the experiment was repeated three times.

Shoots were then separated individually and placed in rooting medium containing MS including 1 mg/l GA_3 and varying concentrations of different auxins. After 6 weeks, the number of roots and percentage of explants producing roots were recorded. There were 10 replications and experiment was replicated three times. Rooted explants were transferred to vermiculate (Agrekal®) in Magenta containers for acclimatization and after 3-4 weeks they were transferred to plastic pots containing potting soil.

All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA).

2.2 Plant material and extraction

Two different sources of the plant (field-grown and *in vitro*-grown) were used for extractions. All plant materials were dried in a room avoiding sun light and then ground into

a powder. Field-grown aerial parts and *in vitro* grown seedlings of *C. galaticus* were extracted with methanol. In methanol extraction, 20 g of each plant sample were soxhlet extracted with 300 ml of methanol at 65 ºC for 12 hours and then filtered. Filtrates were evaporated under vacuum using rotary evaporator to give the crude extracts.

2.3 Antioxidant assay

2.3.1Free radical scavenging activity

Free radical scavenging activity of methanolic extracts of *C. galaticus* was determined spectrophotometrically by monitoring the disappearance of 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma-Aldrich Chemie, Steinheim, Germany) at 517 nm, according to the method described by BRAND-WILLIAMS & al. [19].

2.3.2 Determination of total phenolics content

The phenolic contents in methanolic extracts of *C. galaticus* were determined according to the procedure described by SLINKARD and SINGLETON [20] with the slight modification of using a Folin-Ciocalteu phenolic reagent.

2.3.3 Determination of total flavonoid

The amount of total flavonoids in methanolic extracts was measured by aluminum chloride ($AlCl_3$) colorimetric assay according to the procedure described by MARINOVA & al. [21].

2.4 LC-ESI-MS/MS analysis of the selected phenolics

Analysis was performed on a Triple Quadrupole LC-MS system with an Agilent 1290 Infinity LC (Agilent) in Central Laboratory, METU, Ankara, Turkey. Triple Quadrupole LC-MS system is equipped with a Jet Stream ESI source. Compounds were separated on an Zorbax SB-C18 column (Agilent, 50 mm x 2.1 mm; 1,8µm particle size). The mobile phase consisted of: (A) water containing 0.05 % formic acid + 5 mM ammonium formate (v/v) and (B) methanol. A stepwise gradient from 5 % to 95 % solvent B for 13 min was applied to run the separation. 5 µl of each sample extract was injected and flow rate was 0.3 ml/min. The column temperature was maintained at 35 °C.

3. Results and Discussion

Although *C. galaticus* is a very valuable medicinal plant, there is no any study about *in vitro* propagation of this plant. We therefore aimed to develop an *in vitro* culture protocol for high frequency regeneration of grizzle bindweed. Two different donor plants (field-grown plants and *in vitro* regenerated seedlings) were used as an explant source. In the first part of the experiment, field-grown plant parts (leaves and stems) were collected, surface sterilized and three different explants (leaf, stem internode and stem node) were taken. Explants were cultured on MS medium containing TDZ in combination with IAA or IBA (Table 1); BA in combination with IAA or NAA (Table 2); KIN in combination with IAA (data not provided). Among used explants only stem node explant was successful for shoot regeneration. Best shoot regeneration was obtained with stem node explant on medium containing 0.5 mg/l TDZ and 1.0 mg/l IAA (15.0 shoots per explant; 84 % explants formed shoots). Although 1 mg/TDZ and 1 mg/l IBA combination was effective in terms of mean number of shoots (12 shoots), shoot frequency was very low (20 %) (Table 1). The regeneration efficiency was not found to be high with BA and IAA combinations and better shoot proliferation was observed when BA was used alone (1, 3 and 5 mg/l) with regard to the mean number of shoots. But, percentage of explants forming shoots was not efficient with these concentrations (53 %, 60 % and 40 %, respectively) (Table 2). KIN and IAA combinations were not effective for shoot regeneration (2.3-3.3 shoots per shooting explant) (data not provided). In the second part of

the experiment, *in vitro* grown seedlings were used as donor plant. Three different explants (leaf, stem internode and stem node) were excised and cultured on medium containing TDZ in combination with IAA or IBA; BA in combination with NAA (Table 3).

Table 1. Effects of TDZ in combination with IAA or IBA on shoot regeneration from field- grown plants as an explant source. Means with the same letter within columns are not significantly different at P>0.05.

Plant Growth Regulators		EX PLANTS					
		Leaf		Stem internode		Stem node	
		Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots
Control		- -	-	- -	-	- -	-
TDZ (mg/l)	**IAA (mg/l)**						
0.05	0.0	- -	-	- -	-	4.3 ± 0.3^{cde}	20
0.05	0.1	- -	-	- -	-	- -	-
0.05	0.5	- -	-	- -	-	2.7 ± 0.3^{def}	50
0.05	1.0	- -	-	- -	-	3.0 ± 0.0^{def}	50
0.1	0.0	- -	-	- -	-	5.3 ± 0.3^{bcde}	20
0.1	0.1	- -	-	- -	-	2.0 ± 0.6^{ef}	25
0.1	0.5	- -	-	- -	-	3.3 ± 0.9^{def}	33
0.1	1.0	- -	-	- -	-	3.7 ± 0.6^{def}	67
0.5	0.0	- -	-	- -	-	- -	-
0.5	0.1	- -	-	- -	-	5.7 ± 0.9^{bcde}	33
0.5	0.5	- -	-	- -	-	- -	-
0.5	1.0	- -	-	- -	-	15.0 ± 3.1^{a}	84
1.0	0.0	- -	-	- -	-	2.3 ± 0.3^{def}	25
1.0	0.1	- -	-	- -	-	6.0 ± 1.5^{bcd}	33
1.0	0.5	- -	-	- -	-	7.7 ± 0.9^{bc}	33
1.0	1.0	- -	-	- -	-	8.0 ± 2.2^{b}	50
TDZ (mg/l)	**IBA (mg/l)**						
0.5	0.1	- -	-	- -	-	5.3 ± 2.0^{bcde}	33
0.5	0.5	- -	-	- -	-	- -	-
0.5	1.0	- -	-	- -	-	8.7 ± 0.9^{b}	25
1.0	0.1	- -	-	- -	-	- -	-
1.0	0.5	- -	-	- -	-	- -	-
1.0	1.0	- -	-	- -	-	12.0 ± 1.2^{a}	20
3.0	0.1	- -	-	- -	-	- -	-
3.0	0.5	- -	-	- -	-	- -	-
3.0	1.0	- -	-	- -	-	- -	-
5.0	0.1	- -	-	- -	-	- -	-
5.0	0.5	- -	-	- -	-	- -	-
5.0	1.0	- -	-	- -	-	- -	-

Best shoot proliferation was obtained with node explant with 1 mg/l TDZ and 0.5 mg/l IBA (23.7 shoots per explant; 60 % explants formed shoots) (Table 3, Figure 1A). But, twofold increase of IBA concentration (from 0.5 to 1 mg/l) decreased the shoot number twice (from 23.7 to 12.3 shoots) (Table 3). Regarding the shoot frequency, best combination was 0.5 mg/l TDZ and 0.5 mg/l IBA with 87 % explants forming shoots (11.6 shoots) (Table 3). Shoot regeneration was not obtained with leaf and stem internode explants (Table 3). Control treatments involving no plant growth regulators produced no shoots in all 3 explants for all experiments. (Table 1, 2 and 3). Indirect organogenesis was observed for all experiments because of callus formation before shoot development.

Table 2.Effects of BA in combination with IAA or NAA on shoot regeneration from field- grown plants as an explant source. Means with the same letter within columns are not significantly different at P>0.05.

Plant Growth Regulators		EXPLANTS					
		Leaf		Stem internode		Stem node	
		Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots
Control		- -	-	- -	-	- -	-
BA (mg/l)	**IAA (mg/l)**						
0.5	0.0	- -	-	- -	-	3.3 ± 0.3^{def}	58
0.5	0.1	- -	-	- -	-	5.0 ± 0.6^{def}	33
0.5	0.5	- -	-	- -	-	6.3 ± 0.3^{bcde}	33
0.5	1.0	- -	-	- -	-	5.3 ± 0.7^{def}	33
1.0	0.0	- -	-	- -	-	13.3 ± 3.1^{a}	53
1.0	0.1	- -	-	- -	-	5.7 ± 0.3^{cdef}	67
1.0	0.5	- -	-	- -	-	3.7 ± 1.5^{def}	75
1.0	1.0	- -	-	- -	-	7.3 ± 2.3^{bcde}	75
3.0	0.0	- -	-	- -	-	9.3 ± 0.9^{abcd}	60
3.0	0.1	- -	-	- -	-	- -	-
3.0	0.5	- -	-	- -	-	11.3 ± 0.3^{abc}	75
3.0	1.0	- -	-	- -	-	5.7 ± 0.3^{cdef}	67
5.0	0.0	- -	-	- -	-	12.0 ± 0.6^{ab}	40
5.0	0.1	- -	-	- -	-	4.3 ± 0.9^{def}	40
5.0	0.5	- -	-	- -	-	- -	-
5.0	1.0	- -	-	- -	-	- -	-
BA (mg/l)	**NAA (mg/l)**						
0.5	0.1	- -	-	- -	-	- -	-
0.5	0.5	- -	-	- -	-	- -	-
0.5	1.0	- -	-	- -	-	- -	-
0.5	3.0	- -	-	- -	-	- -	-
1.0	0.1	- -	-	- -	-	5.0 ± 0.7^{def}	75
1.0	0.5	- -	-	- -	-	3.0 ± 0.6^{ef}	71
1.0	1.0	- -	-	- -	-	2.0 ± 0.0^{ef}	67
1.0	3.0	- -	-	- -	-	4.0 ± 0.6^{def}	33
3.0	0.1	- -	-	- -	-	7.5 ± 1.1^{bcde}	67
3.0	0.5	- -	-	- -	-	- -	-
3.0	1.0	- -	-	- -	-	- -	-
3.0	3.0	- -	-	- -	-	- -	-
5.0	0.1	- -	-	- -	-	- -	-
5.0	0.5	- -	-	- -	-	- -	-
5.0	1.0	- -	-	- -	-	- -	-
5.0	3.0	- -	-	- -	-	- -	-

Regenerated shoots were cultured on shoot elongation medium containing 1 mg/l GA_3 for additional 2 weeks (Fig. 1B). After 2 weeks, regenerated shoots were separated individually and cultured on MS medium including 1 mg/l GA_3 and IAA, IBA, 2.4-D or NAA. Root induction was not occurred on the control treatment to which no auxin was added to the media. Of the different auxins investigated for rooting, 1 mg/l IBA was more efficient in terms of mean number of roots (5.5 roots). Regarding the root frequency, 5 mg/l IBA was superior with the greatest percentage of root formation (100 %) (Table 4, Figure 1 C). Root development was observed in 6 weeks. Medium containing IAA concentrations were also effective for root formation. Although 1 mg/l NAA caused root formation (4 roots), increasing concentrations of NAA (3, 5 and 7 mg/l) severely inhibited shoot development. 2,4-D concentrations were not effective in root formation (Table 4). The rooted plants were transferred to Magenta containers including vermiculate for acclimatization (Fig. 1D). After 3-4 weeks, they were transferred to soil and kept under growth room conditions (Fig. 1E and F).

Table 3. Effects of TDZ in combination with IAA or IBA and BA in combination with NAA on shoot regeneration from *in vitro* regenerated seedlings as an explant source. Means with the same letter within columns are not significantly different at P>0.05.

Plant Growth Regulators		EX PLANTS					
		Leaf		Stem internode		Stem node	
		Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots	Mean number of shoots per shooting explant (±SE)	% explants forming shoots
Control		- -	-	- -	-	- -	-
TDZ (mg/l)	**IAA (mg/l)**						
0.5	0.0	- -	-	- -	-	- -	-
0.5	0.5	- -	-	- -	-	6.7 ± 0.3^{ef}	67
0.5	1.0	- -	-	- -	-	9.3 ± 0.3^{bcde}	67
1.0	0.0	- -	-	- -	-	- -	-
1.0	0.5	- -	-	- -	-	- -	-
1.0	1.0	- -	-	- -	-	5.3 ± 0.3^{f}	75
TDZ (mg/l)	**IBA (mg/l)**						
0.5	0.5	- -	-	- -	-	10.8 ± 1.6^{bcd}	87
0.5	1.0	- -	-	- -	-	10.7 ± 0.3^{bcd}	25
1.0	0.5	- -	-	- -	-	23.7 ± 0.3^{a}	60
1.0	1.0	- -	-	- -	-	12.3 ± 1.2^{b}	57
BA (mg/l)	**NAA (mg/l)**						
1.0	0.5	- -	-	- -	-	6.3 1.3^{ef}	87
1.0	1.0	- -	-	- -	-	- -	-
3.0	0.5	- -	-	- -	-	11.6 0.8^{bc}	87
3.0	1.0	- -	-	- -	-	- -	-
5.0	0.5	- -	-	- -	-	4.7 ± 0.3^{f}	50
5.0	1.0	- -	-	- -	-	- -	-
7.0	0.5	- -	-	- -	-	8.3 ± 0.7^{cdef}	63
7.0	1.0	- -	-	- -	-	7.7 ± 0.3^{def}	50
10.0	0.5	- -	-	- -	-	6.3 ± 0.9^{ef}	50
10.0	1.0	- -	-	- -	-	6.3 ± 0.3^{ef}	45

Table 4. Effects of the tested auxins on root formation from regenerated shoots. Means with the same letter within columns are not significantly different at P>0.05.

Treatments	Mean number of roots per explant (±SE)	% explants forming roots
Control	- -	-
IAA (mg/l)		
1.0	1.7 ± 0.3 ^{bc}	40
3.0	2.0 ± 0.4 ^{bc}	60
5.0	1.7 ± 0.3 ^{bc}	75
7.0	1.3 ± 0.3 ^{bc}	75
10.0	4.0 ± 0.0 ^{ab}	75
IBA (mg/l)		
1.0	5.5 ± 1.6 ^{a}	80
3.0	3.8 ± 0.9 ^{ab}	83
5.0	3.2 ± 0.9 ^{ab}	100
7.0	2.3 ± 0.9 ^{bc}	75
10.0	3.5 ± 1.6 ^{ab}	80
NAA (mg/l)		
1.0	4.0 ± 0.0 ^{ab}	33
3.0	- -	-
5.0	- -	-
7.0		-
2,4-D (mg/l)		
0.5	- -	-
1.0	- -	-

Only one investigation has been carried out on *in vitro* regeneration of members of the genus *Convolvulus*. ABBAS & al. [22] reported the *in vitro* culture protocol of *Convolvulus scindicus* Stocks. They obtained best shoot proliferation with 2.5 mg/l BA along with 0.5 mg/l KIN and 0.5 mg/l NAA. Similar to our results, they used nodal segments for establishing *in vitro* cultures. They observed maximum number of roots (1.5) per explant and maximum rooting frequency of 67 % with MS medium containing 2 mg/l IAA (ABBAS & al. [22]). On the other hand, best root formation was obtained with 1, 3 and 5 mg/l IBA in our study with rooting frequencies of 80, 83 and 100, respectively (Table 4).

Leaf and stem internode explant of *C. galaticus* were unsuccessful to develop adventitious shoots. Shoot multiplication was obtained with stem node explant including meristematic cells for this plant. Micropropagation of many medicinal plant species has been achieved through different tissue culture techniques. In many cases, actively growing shoot-tips or axillary buds, both of which already contain *de nova* primordia, were used as a starting material (ROUT & al. [23]). This method remains the most widely used method in commercial micropropagation and produces the most true-to-type plantlets (BROWN and THORPE [24]; KANE [25]). It seems that the proliferative potential of meristematic cells of the stem node explant including axillary bud in our study is readily stimulated by exogenously added growth regulators, resulting in multiple shoot formation (KANE [25]). Our findings indicated that TDZ was the most critical plant growth regulator for multiple shoot formation from stem node segments when used in combination with IAA or IBA. A possible synergism between TDZ and auxins, both endogenous and some of the exogenous may lead to multiple shoot formation. The promoting effect of TDZ on *in vitro* development has been lately reported for many species (HUETTEMAN and PREECE [26]; LU [27]; YILDIRIM and TURKER [28]). MURCH & al. [29] showed that the occurrence of regenerants in TDZ-treated plants may be an adaptive reproductive mechanism to overcome the imposed stress. MURTHY & al. [30] hypothesized that under the influence of TDZ, a relatively high level of accumulation of minerals or other metabolites occurs in the tissues, and this causes a stress in plants (explants). To overcome this physiological stress, the plant tissue modifies its metabolic processes, resulting in the production and accumulation of various metabolites and culminating in the formation of regenerants.

The scavenging activity of DPPH radical caused by antioxidants was determined by measuring the decrease in its absorbance at 517 nm. Ascorbic acid was used as the antioxidant standard in this experiment. In the present study, antioxidant activity of methanolic extract of field-grown and *in vitro*-grown plants was assessed. The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteau) and total flavonoid content (aluminum chloride colorimetric) were used in this assessment. Methanolic extract of field-grown *C. galaticus* showed better free radical scavenging activity than *in vitro*-grown plants. Although over 50 % inhibition of DPPH was obtained at 50 µg/ml concentration with field-grown *C. galaticus*, 100 µg/ml concentration was required for *in vitro*-grown *C. galaticus*. The free radical scavenge tendency of both extracts increased when their concentrations increased (Table 5). The best DPPH scavenging activity of field-grown and *in vitro*-grown plants was obtained at 200 µg/ml concentrations (90.12 % and 92.75 %, respectively) that they showed high active radical scavenge capability as much as ascorbic acid (99.53 %) (Table 5).

Figure 1. *In vitro* regeneration of *C. galaticus*. (A**)** Shoot regeneration from stem node explant on medium containing 1 mg/l TDZ and 0.5 mg/l IBA, (B) Shoot elongation of regenerated shoots on medium containing 1 mg/l GA_3, (C) Rooting of the regenerated shoots on medium containing 3 and 5 mg/l IBA, (D) Regenerated plant in magenta container including vermiculate for acclimatization, (E) Regenerated plants transferred to cups containing sterile potting soil under high humidity conditions, (F) Regenerated plants transferred to cups containing sterile potting soil under growth room conditions.

Table 5. % inhibition of DPPH by *C. galaticus* extracts.

	% Inhibition of DPPH				
	Concentrations				
Treatments	**12.5 µg/ml**	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**
Ascorbic acid	95.59	95.73	96.2	96.14	99.53
Field-grown *C. galaticus*	24.13	42.71	76.33	88.34	90.12
In vitro* -grown *C. galaticus	14.97	27.79	46.05	71.89	92.75

Methanol extract of field-grown plant contained higher phenolic (84.689 mg gallic acid equivalent/g dried extract) and flavonoid (48.760 mg catechol acid equivalent/g dried extract) content than *in vitro*-grown plant (43.573 mg gallic acid equivalent/g dried extract, 30.110 mg catechol acid equivalent/g dried extract, respectively) (Table 6). Hydroxyl groups on phenolic compounds have scavenging ability so they are very important plant constituents. A number of studies reported a significant relationship between the phenolic contents of plant extracts and their antioxidant properties (GULCIN & al. [31]). Field-grown leaves of *C. galaticus* had higher phenolic and flavonoid content thereby having higher DPPH scavenging activity (Table 5 and 6). Numerous studies have revealed that environmental stress often raise the accumulation of the phenolics, which are believed that to play a regulatory role in some metabolic process (DIXON and PAIVA [32]; SOLECKA [33]; JANAS & al. [34]). THAKRAL & al. [35] reported the antioxidant activity of aerial parts of *Convolvulus arvensis* by DPPH method. Similar to our results, *C. arvensis* extracts showed dose dependent free radical scavenging property. THAKRAL & al. [35] showed that 50 % DPPH inhibition was observed at 131.03 µg/ml concentration with methanolic extract of *C. arvensis*. On the other hand, 50 % DPPH inhibition was obtained at 25-50 µg/ml concentrations with field-grown *C. galaticus* and at 50-100 µg/ml concentrations with *in vitro*-grown *C. galaticus* in our study (Table 5). JAIN & al. [17] reported that methanol extract of *Convolvulus microphyllus* showed 50 % DPPH inhibition at 75 µg/ml concentration with DPPH method. Ethyl acetate and n-butanol fractions of *Convolvulus dorycnium* leaves showed 50 % DPPH inhibition at 3.2 µg/ml and 6.9 µg/ml, respectively (NACEF et al. [15]).

Table 6.Total phenolic and flavonoid content of *C. galaticus* extract.

Treatments	**Total Phenolics in mg GA/g dry extract**	**Total Flavonoids mg CE/g dry extract**
Field-grown *C. galaticus*	84.689 ± 0.000	48.760 ± 0.001
In vitro* -grown *C. galaticus	43.573 ± 0.000	30.110 ± 0.001

Methanolic extracts of field-grown and *in vitro*-grown *C. galaticus* were subjected to Liquid Chromotography-Tandem Mass Spectrometry analyses and results were summarized in Table 7. The chromatogram of phenolic standards (each standard, 5 ppm in mixture) was obtained via gradient methanol flow. The phenolic content of the methanolic extracts was compared with their standard chromatograms and identified with mass spectrometer (MS). Finally, quantity of each phenolic compound in the extract was determined (Table 7).

According to LC-ESI-MS/MS results, the amounts of studied phenolic compounds in field-grown plant were higher than those in *in vitro*-grown plant (Table 7). Concentrations of phenolic compounds in field-grown plant extract were at least 3 times higher than the other extract. Caffeic acid was found in field-grown plant nearly 10 times higher (157.432 µg/g) than *in vitro*-grown plant (16.464 µg/g). Similarly, rutin was found 28 times higher (286.9 µg/g) than *in vitro* grown plant (10.45 µg/g). Although rutin was dominant compound in methanol extracts of field-grown leaves, caffeic acid was dominant in *in vitro*-grown leaves. Methanol extracts of field-grown plant contained from the highest to lowest amount rutin

(286.9 µg/g), caffeic acid (157.432 µg/g), coumarin (15.382 µg/g), kaempferol (14.832 µg/g), vanillic acid (6.264 µg/g), coumaric acid (4.01 µg/g) and epigallocatechin (0.094 µg/g). Methanol extracts of *in vitro*-grown plant contained from highest to lowest amount caffeic acid (16.464 µg/g), rutin (10.45 µg/g), vanillic acid (2.37 µg/g) and coumaric acid (0.204 µg/g). The reason of high phenolic content of field-grown plant may be due to the stress conditions in the natural environment (MICHALAK & al. [36]). When plants are exposed to different types of stress, such as drought, heat, ultraviolet light, air pollution, and pathogen attack, the synthesis of some phenolic compounds is induced adapting to these stresses (RIVERO& al. [37]).

AL-RIFAI & al. [38] determined the flavonoids (kaempferol and quercetin) in methanolic extract of *Convolvulus pilosellifolius* Desr and kaempferol was more abundant than quercetin in *C. pilosellifolius*. Quantity of quercetin and kaempferol was found as 4.27 µg/ml extract and 6.14 µg/ml extract, respectively. Similarly, kaempferol was found more than quercetin in our study. On the other hand, quantity of kaempferol was found as 14.832 µg/g and quercetin was lower than 0.01 µg/g in our study (Table 7).

Table 7**.** Identified phenolic compounds and their amounts in the methanolic extracts of field-grown plant and *in vitro*-grown plant of *C. galaticus*. "[a]" indicates peaks for standards having the same-close retention times.

STANDART COMPOUNDS	**Peak number**	**Retention time (min)**	**EXTRACTS (µg/g of dry extract)**	
			Field-grown *C. galaticus*	***In vitro*-grown C. galaticus**
Gallic acid monohydrate	1	0.92	≤ 0.01	≤ 0.01
Pyrocatechol	2*	1.902	≤ 0.01	≤ 0.01
Procyanidin B1		2.225	≤ 0.05	≤ 0.05
(-) epigallocatechin	3*	2.497	0.094 ± 0.006	≤ 0.01
(+) catechin		2.539	≤ 0.01	≤ 0.01
Procyanidin B2	4	2.886	≤ 0.05	≤ 0.05
Vanillic acid	5*	3.063	6.264 ± 0.26	2.37 ± 0.112
Caffeic acid		3.092	157.432 ± 0.998	16.464 ± 0.166
Procyanidin C1	6*	3.216	≤ 0.5	≤ 0.5
(-) epicatechin		3.32	≤ 0.01	≤ 0.01
p-coumaric acid	7	3.884	4.01 ± 0.168	0.204 ± 0.004
(±) Taxifolin hydrate	8	4.07	≤ 0.01	≤ 0.01
Coumarin	9*	4.716	15.382 ± 0.442	≤ 0.025
Luteolin-7-O-β-D glucoside		4.809	≤ 0.025	≤ 0.025
Rutin hydrate		4.898	286.9 ± 2.222	10.45 ± 0.46
Resveratrol		4.956	≤ 0.01	≤ 0.01
Myricetin	10	5.27	≤ 0.01	≤ 0.01
Kaempferol	11	5.42	14.832 ± 0.124	≤ 0.01
Daidzein	12	5.829	≤ 0.01	≤ 0.01
Quercetin	13	6.073	≤ 0.01	≤ 0.01
Genistein	14	6.425	≤ 0.01	≤ 0.01
Apigenin	15	6.945	≤ 0.01	≤ 0.01

4. Conclusion

This paper, as being the first report, described an efficient and rapid regeneration system for *C. galaticus*, an endemic plant. Plant tissue culture is an alternative method of commercial propagation and is being widely used for the commercial propagation of a large number of plant species, including many medicinal plants (ROUT & al. [23]). It is believed that this protocol will have an important contribution for *in vitro* conservation and mass propagation of this endemic plant. Furthermore, phenolic constituents and antioxidant activity of this plant was revealed for the first time with this study. Comparison between *in vitro*-grown and field-grown plants in terms of their phenolic constituents and antioxidant activities was performed revealing the quality of *in vitro*-grown plants. *C. galaticus* contained the considerable amounts of phenolic compounds, such as rutin and caffeic acid. Considering the strong biological activity of phenolic compounds, future studies should be focused to increase the amount of phenolics in *in vitro*-grown plant parts by applying different stress conditions.

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