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In vitro propagation and phytochemical assessment of *Perovskia abrotanoides* Karel. (Lamiaceae) – A medicinally important source of phenolic compounds

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ABSTRACT

Perovskia abrotanoides Karel. (Lamiaceae) is an aromatic herb that is mainly grown in the mountains of Northeastern Iran across Northern Pakistan and Northwestern India. Here, a suitable system for regenerating *P. abrotanoides* via direct organogenesis was performed for the first time. *In vitro*-grown shoot tips were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations (1.0, 2.0, and 3.0 mg L^{-1}) of kinetin (KN) and 6-benzylaminopurine (BAP) alone or in combination with two concentrations (0.5 or 1.0 mg L^{-1}) of 2,4-dichlorophenoxyacetic (2,4-D), α-naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA). MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} IBA with shoot proliferation percentage (83.3%) and shoot-forming capacity (SFC) index (5.5) was the optimal medium for shoot induction in the plant. Rooting of elongated shoots was performed on half-strength MS medium fortified with different concentrations (0.1, 0.5, and 1.0 mg L^{-1}) of IBA, 2,4-D, and NAA. The highest rooting percentage (100%) with a mean root number (5.9 ± 1.35) was obtained on the free-hormone medium. Rooted plantlets were successfully acclimatized in the greenhouse (75%) and transferred to natural conditions. Several phenolic acids were quantitatively determined in the extract of both *in vitro* regenerated plant (IVRP) and wild plant (WP). Rosmarinic acid was the most prominent phenolic acid in the extract of both samples. Total phenolic content of IVRP (70.7 ± 9.1 mg/g DW) was higher than WP (54.9 ± 15.2 mg/g DW). Antioxidant activity (IC_{50}) of IVRP and WP extracts was 230.4 and 275.7 μg mL^{-1}, respectively.

1. Introduction

Perovskia Karel. is a small genus of Lamiaceae family comprises of aromatic shrubs which is growing in arid habitats of Central Asia. Some species such as Russian sage (*P. artiplicifolia* Benth.) are cultivated worldwide for ornamental purposes. The genus comprises seven species of which *P. atriplicifolia, P. artemisoides,* and *P. abrotanoides* are native to Iran (Mozaffarian, 1996). *P. abrotanoides* with the common Persian name of "*Brazambel*" is locally used for treating headache, typhoid, atherosclerosis, toothache, cough, liver fibrosis, and gonorrhea cardiovascular diseases (Tareen et al., 2010). Furthermore, pharmacological properties of the plant such as cytotoxicity, antiplasmodial, and anti-inflammatory effects have been reported (Beikmohammadi, 2012; Esmaeili et al., 2009; Sairafianpour et al., 2001). The plant roots contain a class of diterpene compound called tanshinone, which has shown various biological activities such as effects on heart function, antibacterial, antioxidant, and anti-cancer properties (Mozaffarian, 1996; Sairafianpour et al., 2001).

Propagation of *P. abrotanoides* is usually done by dividing the plant and sometimes by seeding. Since propagation of the plant is limited to seasonal variations and low seed germination rates, tissue culture technique is useful for multiplying and maintaining this species, which is difficult to regenerate with conventional methods and is becoming extinct. Moreover, *in vitro* plant regeneration via lateral bud's culture is a simple and economical method for gaining higher consistency and greater true-to-type plants during a short time (Shailja Kanwar et al., 2017). Developed *in vitro* multiplication techniques offer high-rate multiplication alternatives for horticultural plants with economic and medicinal importance (Deb and Pongener, 2012; Pati et al., 2006).

Nowadays, phenolic compounds are products of the shikimic acid, pentose phosphate, and phenylpropanoid pathways in plants which are currently considered as one of the best sources of natural antioxidant (Shahidi, 2000). They are mainly found in fruits, vegetables, cereals and beverages (Haung et al., 2005) and play a significant role in

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Abbreviation

BAP	6-Benzylaminopurine
CRD	Completely randomized design
2,4-D	2,4-Dichloro-phenoxyacetic
HPLC	High performance liquid chromatography
IBA	Indole-3-butyric acid
IVRP	*In vitro* regenerated plant
IVS	*In vitro* seedling
KN	Kinetin
LC-MS	Liquid chromatography-mass spectrometry
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
PGRs	Plant growth regulators
RA	Rosmarinic acid
RFC	Root-forming capacity
SFC	Shoot-forming capacity
WP	Wild plant
DPPH	2,2-diphenyl1-picrylhydrazyl

protecting tissues against the oxidizing effects of free oxygen radicals (Balasundram et al., 2006). Phenolic acids (PAs) are aromatic compounds derived from phenyl carboxylic or hydroxycinnamic acids which has been extensively reported in the different plant taxa (Arceusz et al., 2013). Lamiaceae, one of the most important families among the medicinal plants, has been recognized as a potential source of PAs (Zgórka and Glowniak, 2001). The chemical structure of some common PAs is shown in Fig. 1.

Total phenol and total flavonoid content, antioxidant, and antimicrobial activities as well as some pharmacological research on the extracts of *P. abrotanoides* have been already reported (Ghaffari et al., 2018; Tabefam et al., 2018; Ghafourian and Mazandarani, 2017), but as far as literature survey could ascertain, determination of phenolic acids has not yet been assessed in the plant. Also, *in vitro* micropropagation of *P. abrotanoides* has not previously been reported. Therefore, the objective of the present study was to provide a suitable and efficient protocol for *in vitro* propagation of *P. abrotanoides,* and identification and quantification of its PAs as well. Theses findings can be considered in further conservation, domestication, cloning, breeding, and biotechnological programs for the production of PAs-rich cultivars.

2. Material and methods

2.1. Plant materials

The seeds of *P. abrotanoides* were collected from wild growing plants (Fig. 2A) in the village of Qamsar (33° 45′ N, 51° 28′ E at the height of 1740 m), Kashan, Iran. A voucher specimen of the plant (MPH-2141) has been deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran. *P. abrotanoides* seeds were washed for 3 h under running water. Seeds were soaked in 70% ethanol for 60s and surface-sterilized with 2% (v/v) of commercial bleach for 10 min. Seeds were immediately irrigated three times in sterile distilled water. Sterilized seeds were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and then incubated in a growth room under a 16-h photoperiod and 25 ± 2 °C temperature. The germination rate was 35% from day 8 to day 19 of the establishment in culture. Shoot tips collected from *in vitro* seedling (IVS) were used for the next multiplication experiments.

2.2. Culture initiation and shoot multiplication

To study the effects of plant growth regulators (PGRs) on the proliferation of *P. abrotanoides* shoots, the shoot tips (2–3 cm) obtained from IVS (Fig. 2B), were initially cultured on MS medium supplemented with 1.0, 2.0, and 3.0 mg L^{-1} of both cytokinins 6-benzylaminopurine (BAP), kinetin (KN) and control (no PGRs). Shoot tips cultured in the baby food jars containing prepared agar-solidified media (40 mL) were incubated under a 16-h photoperiod provided for 30 days by cool-white fluorescent lamps (Philips, 58 W, Holland) at a photon flux density of 40 μ mol m^{-2} s^{-1} at 23 ± 2 °C. Each baby food jar was considered as an experimental unit and the experiment done with triplicates per treatment. Totally, five jars each containing three shoot tips were considered as experimental units in each replicate.

To investigate the effect of BAP and KN, and their concentrations on shoot proliferation percentage, both length, and number of obtained shoots were recorded after 35 days (Fig. 2C). Cytokinin type and its effective concentration were then tested in combination with two concentrations (0.5 or 1.0 mg L^{-1}) of different auxins α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) or 2,4-dichlorophenoxyacetic (2,4-D) to specify the best *in vitro* propagation condition. Both shoot length and number were recorded after 35 days (Fig. 2D and E).

2.3. In vitro rooting and acclimatization

Elongated shoots (about 2–3 cm) were transferred to ½ MS medium supplemented with 0.1, 0.5 or 1 mg L^{-1} NAA, IBA or 2, 4-D and control for rooting. The effect of each treatment was registered 35 days after inoculation and the percentage of rooted shoots, number, and length of the roots, were recorded. After 45 days, shoots with well-developed roots (Fig. 2F) were selected and gently washed under running tap water to remove the traces of medium. Thereafter, they were transferred to plastic cups (Fig. 2G, including peat and perlite (1:1)); the cups were covered with polythene bags to keep moisture. After four weeks, polyethylene bags were removed, and plantlets were transferred to clay pots including soil, animal manure, and sand (2:1:1) (Fig. 2H and I). Finally, they were transferred to fields after six weeks.

2.4. Extraction and determination of total phenolic content

Dried powder of the aerial parts (100 mg) of both *in vitro* regenerated plant (IVRP) and wild plant (WP) was extracted in 20 mL methanol then incubated for 20 min in an ultrasonic bath and centrifuged at 2000 rpm for 5 min. The methanolic extracts were vaporized

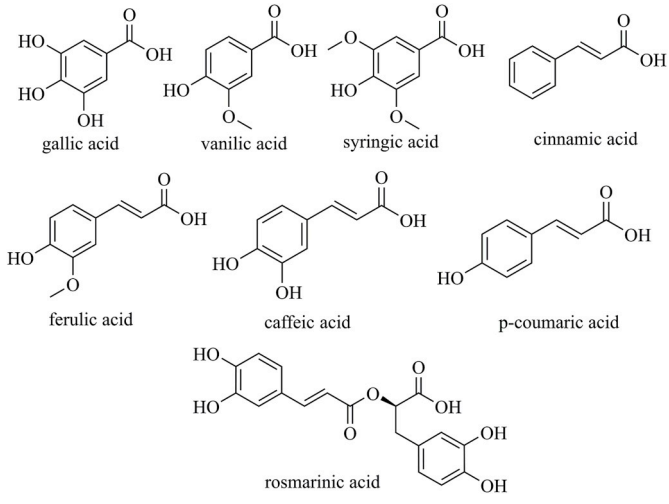

Fig. 1. The chemical structure of some common phenolic acids.

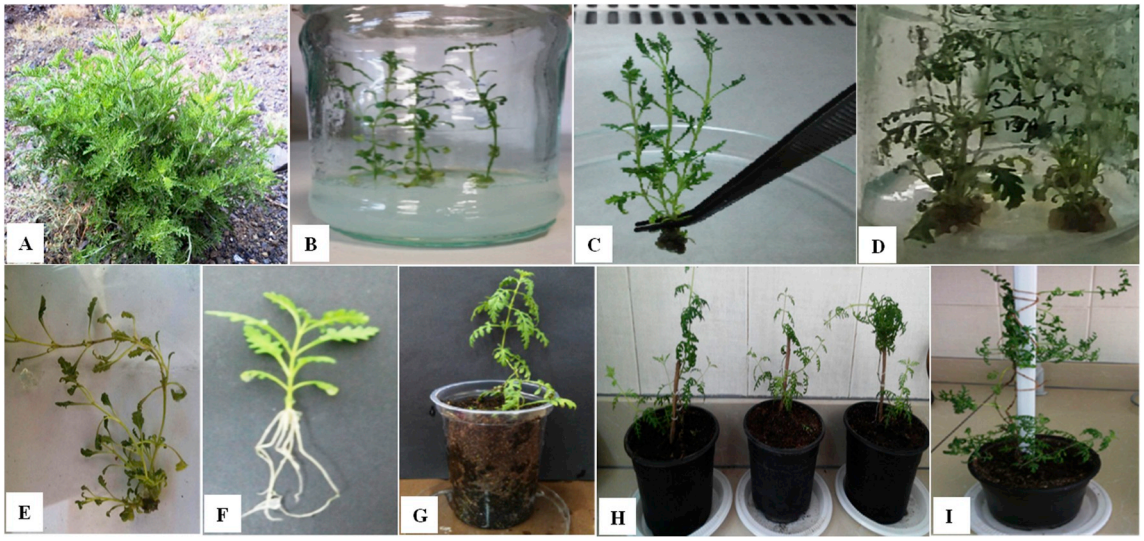

Fig. 2. *In vitro* propagation of *Perovskia abrotanoides*. (A) Wild plant that employed as seed and explant source, (B) sterile seedling establishment, (C) multiple shoot formation on MS medium supplemented with 3 mg L^{-1} BAP, (D) development of callus at the base of explants obtained on MS medium containing 1 mg L^{-1} IBA and 3 mg L^{-1} BAP, (E) multiple shoots obtained on MS medium supplemented with 0.5 mg L^{-1} IBA and 3 mg L^{-1} BAP, (F) formation of roots on 1/2 MS medium, (G, H & I) acclimatization of plantlets after 2, 4 and 6 weeks, respectively.

under reduced pressure (vacuum evaporator) at 35–40 °C. Total phenolic compounds were measured using the Folin-Ciocalteu method. 25 µL of extract solution was mixed with 125 µL of 10% Folin-Ciocalteu reagent. Following a 5 min incubation period, 100 µL sodium carbonate solution 7.5% (V/V) was added. The tubes were covered with aluminum foil and placed on a shaker at 80 rpm for 2 h. Then the absorbance was measured at a wavelength of 760 nm using a spectrophotometer (Model, Company, and Country). Gallic acid standard was used to perform a calibration curve as a positive control. The amount of total phenolic compounds was measured as mg gallic acid equivalent per gram of dry extract (mg GAE/100 g dry weigh) through the resulting equation from the standard curve (Kamtekar et al., 2014). Experiments were carried out with three replications, and the mean values were reported.

2.5. Determination of phenolic acids by HPLC-UV

The dried methanolic extracts as described above were dissolved in 10 mL high-performance liquid chromatography (HPLC) grade methanol. The segregation and designation of phenolic acids were carried out using HPLC (waters 2695 separations module), equipped with a C_{18} column (250 mm × 4.6 mm, 5 µm, Waters) and a UV detector (waters 2487). The mobile phase consisted of water (A) and methanol (B), respectively. Standard solutions for each phenolic acid were separately prepared in pure methanol and were diluted to plot the standard curve in the range of concentrations (6–100 ppm). Detection was carried out at two wavelengths of 254 and 320 nm with a flow rate of 1.0 mL min^{-1} and injection volume of 20 µL. Each sample injection was repeated three times.

2.6. LC-MS analysis

LC-MS analysis was performed on a Shimadzu Prominence HPLC system composed of a LC-20 AD binary pump, CTO-20AC column thermostat, SDP-M20A PDA detector, and CBM-20A system controller, coupled to LCMS-8030 Triple Quadrupole Mass spectrometer equipped with an electrospray ionization source (ESI). Conditions were as follows: capillary voltage, 4.5 V; desolvation line temperature, 250 °C; heat block temperature, 500 °C; drying gas (nitrogen) flow, 15 L min^{-1}; nebulizing gas (nitrogen) flow, 3 L min^{-1}. Data acquisition was performed in both positive and negative ionization modes. Full-scan (160–1500) was carried out with 6000 u/sec scan speed and 0.150 s per event time. LC separation was carried out on a C_{18} SunFire column (3.5 µm, 3 × 150 mm i.d., Waters) equipped with a guard column (3 × 20 mm i.d.). HPLC solvents both contained formic acid (0.1%, v/v), with the flow rate set to 0.4 mL min^{-1}. The following gradient was used: 5–100% B in 30 min; 100% B isocratic from 30–35 min. Data acquisition was performed with Lab Solutions software (Shimadzu). The mobile phase consisted of water (A) and acetonitrile (B). The injection volume was 10 µL.

2.7. Antioxidant capacity by DPPH assay

The radical scavenging capacity of both IVRP and WP extracts was determined by the method has been described previously (Nakajima et al., 2004). The test was performed using 50 µL of extract, which was added to 2000 µL of 6 × 10^{-5} mol. L^{-1} (free radical, 95%) in methanol. The room temperature incubation for 30 min; the absorbance of the mixture was measured (λ = 517 nm). The inhibition percentage of 2,2-diphenyl1-picrylhydrazyl (DPPH) radical was estimated through the following formula:

$$\text{DPPHsc } \% = \frac{(\text{Abs control})_{t=x\ \text{min}} - (\text{Abs sample})_{t=x\ \text{min}}}{(\text{Abs control})_{t=x\ \text{min}}} \times 100$$

Abs control: Absorbance of DPPH solution mixtures without extract.Abs sample: Absorbance of DPPH mixtures containing extract.

2.8. Statistical analysis

All experiments were factorial based and conducted using a completely randomized design (CRD) with three replicates. Data of *in vitro* multiplication include shoot proliferation percentage, the mean number of shoots, mean shoot length and shoot-forming capacity (SFC) index after 35 days of culture (Lambardi et al., 1993). Rooting data were evaluated 35 days after transferring the elongated shoots onto the rooting medium and consisted of rooting percentage, mean root number, mean root length and the root-forming capacity (RFC) index. SFC and RFC indices were calculated in accordance with the following formula:

SFC/RFC = (mean number of shoots or mean number of roots per regenerating explant) × (percentage of regenerating explant)/ 100

Data were analyzed using SAS software ver. 9.4. Means were assessed using Duncan's multiple range tests at $p < 0.05$.

3. Results

3.1. Effects of BAP and KN on shoot multiplication

The mean comparison results showed no statistical difference between BAP and KN in terms of overall average results (showed bold in Table 1). However, different concentrations of BAP and KN had significant effects on the induction of multiple shoots. The shoot tips on control (PGR free) medium did not give any shoot multiplication. The highest shoot number (3.4 ± 0.4) with an average length (3.8 ± 0.6 cm), shoot proliferation (75%), and SFC index (2.6) were obtained on MS medium supplemented with 3 mg L^{-1} BAP. The highest length of 4.1 ± 0.8 cm was obtained in the treatment of 2 mg L^{-1} BAP which revealed no significant difference with 3 mg L^{-1} BAP treatment. In contrast, medium containing 1 mg L^{-1} BAP reduced shoot number (2 ± 0.3), shoot proliferation (41.7%), and SFC index (0.8). Increase in KN concentration had inhibiting effects on *in vitro* proliferation of *P. abrotanoides*. The lowest SFC index (0.7) was obtained at a concentration of 3 mg L^{-1} KN. Overall, BAP in comparison with KN was more effective on the formation of multiple healthy shoots. 3 mg L^{-1} BAP was the best concentration for shoot proliferation of *P. abrotanoides* (Fig. 2C).

3.2. Effect of auxins on shoot proliferation

The results showed that the interaction of 3 mg L^{-1} BAP and auxin was more effective on measured parameters including shoot proliferation, number of shoots, and shoot elongation compared to cytokinins alone. Means comparison showed no statistical difference between auxin types in terms of shoot proliferation (Table 2). However, the highest shoot proliferation was obtained in BAP hormonal treatment combined with IBA. Adding IBA to MS medium containing BAP increased the shoot proliferation, the average of shoot number and SFC by 1.5, 2.2, and 2.7 folds, respectively, in comparison with BAP treatment alone. While their combination reduced the average length of 1.1 folds compared to BAP alone (2.2 ± 0.4 cm versus 3.8 ± 0.6 cm).

The highest shoot length with an average of 4.6 ± 1.7 cm was obtained from 3 mg L^{-1} IBA and 1 mg L^{-1} IAA. The average number of the shoot in this treatment was less than 3. In terms of measured parameters, there is no significant difference between 0.5 and 1 mg L^{-1} IBA concentrations, whereas medium supplemented with 1 mg L^{-1} IBA and 3 mg L^{-1} BAP resulted in the formation of callus at the base of explants (Fig. 2D). In terms of regeneration rate, treatment of 3 mg L^{-1} BA and 1 mg L^{-1} NAA had a positive impact on shoot formation, leading to the development of callus at the base of explants. The combination of BAP and auxin on shoot formation had an adverse effect on regeneration rate which was shown in medium supplemented with 2, 4-D. The application of both concentrations of this hormone (0.5 and 1 mg L^{-1}) with 3 mg L^{-1} BAP reduced regeneration rate compared to BAP alone (58.3 vs. 75).

3.3. In vitro rooting and acclimatization

Strong and healthy shoots obtained at the end of the proliferation stage (2–3 cm) were transferred to full-strength MS medium containing 3% (w/v) sucrose and 10 g L^{-1} agar. After 3 weeks, the roots were formed in this medium and rooting percentage was less than 30% (data not shown). In order to increase rooting percentage with the suitable number and length, shoots were transferred to half-strength MS medium (1/2 MS) containing 7 g L^{-1} agar and 1.5% (w/v) sucrose without PGRs or with different concentrations (0.1, 0.5, 1 mg L^{-1}) of auxins (2,4- D, IBA, NAA). Shoots in these media presented different responses in terms of root number per shoot and root length (Table 3). In ½ MS medium without any PGRs, rooting was observed on shoots. In this medium, the highest RFC index (5.9), the highest rooting rate (100%), and average root number (5.9 ± 1.4) with an average length (3 ± 0.5 cm) were obtained (Fig. 2F). Also, the highest root length (7.5 ± 1.6 cm) was recorded on the medium containing 0.5 mg L^{-1} IBA. Root length was decreased at a concentration of 1 mg L^{-1} IBA (1.7 ± 0.5). Rooted explants on ½ MS medium without PGRs were transferred to 250 mL pots and were then successfully acclimatize (75%) after 2 weeks (Fig. 2G). In vitro seedlings adapted to soil after 6 weeks (Fig. 2I).

3.4. Phenolic content and antioxidant activity

Total phenolic content of IVRP (70.7 ± 9.1 mg/g DW) was higher than that of the WP (54.9 ± 15.2 mg/g DW). Quantitative and qualitative analysis indicated the presence of several phenolic acids in the extract of both IVRP and WP of *P. abrotanoides* (Fig. 3). Phenolic acids content of the extract from the air-dried aerial parts of WP and IVRP are presented in Table 4. Rosmarinic acid (RA) was the most prominent phenolic acid in the extract of both IVRP and WP. The presence of RA *in vitro* regenerated plant extract was confirmed by HPLC-PDA-ESI mass analysis; the typical UV spectrum of RA is λ_{max} at 328 nm (Fig. 4A). The presence of RA was confirmed in the ESI-MS spectra by *m/z* 359 [M-H]- and *m/z* 719 [2M-H] (Fig. 4B). The amount of RA and *p*-coumaric acid in IVRP was 3, 3.8, and 3 times higher than WP, respectively. While the highest amounts of syringic acid and ferulic acid were observed in WP. Radical-scavenging activity of the extracts of WP and IVRP of *P. abrotanoides* was assessed by DPPH radical assay. The concentrations that led to 50% inhibition (IC_{50}) are presented in Table 4. The IC_{50} of the methanolic extract of WP and IVRP were found 275.7 ± 10.2 and 230.4 ± 8.6 μg/mL, respectively.

4. Discussion

Shoot-tip culture results in a rapid propagation (due to pre-existing meristems) and progeny's genetic stability compared to the mother plant (Vincent et al., 1992). Cytokinins have important roles in various aspects of growth, metabolism, and plant development of which overcoming apical dominance and lateral buds growth are the most important (Benková et al., 1999; Sánchez-Gras and del Carmen Calvo, 1996). In the current study, cytokinins were used to overcome the apical dominance of main shoots cultured for proliferation and to increase shoot proliferation in petiole angle. Cytokinins used in this study can be divided into two groups; (1) BAP group with weaker physiological effects and more effective in shoot induction, and (2) kinetin group with stronger physiological activity in elongation and cell division (Chiwocha et al., 2005). The results showed that BAP treatments at

Table 1
Effects of BAP and KIN on multiple shoot formation of *P. abrotanoides*.

Cytokinin concentration (mg L^{-1})	Shoot proliferation (%)	MSN [a] (no. ± SE[d])	MSL[b] (cm ± SE)	SFC[c] index
Control	0.0	0.0	0.0	0.0
BAP	**58.3 A**	**2.6 ± 0.6 A**	**2.7 ± 1.3 A**	**1.6**
1	41.7 a	2.0 ± 0.3d e	2.0 ± 0.2 cd	0.8
2	66.7 a	2.6 ± 0.1 b c	4.1 ± 0.8 a	1.7
3	75.0 a	3.4 ± 0.4 a	3.8 ± 0.6 ab	2.6
KIN	**52.8 A**	**2.4 ± 0.7 A**	**2.5 ± 0.5 A**	**1.5**
1	58.3 a	3.0 ± 0.3 ab	2.4 ± 0.3 c	2.1
2	50.0 a	2.4 ± 0.4 cd	3.2 ± 0.1 b	1.4
3	41.7 a	1.7 ± 0.4 e	1.4 ± 0.1 d	0.7

[a]Mean shoot number; [b]Mean shoot length; [c] Shoot-forming capacity; Different capital letters indicate significant differences between cytokinin types. Lower case letters indicate significant differences ($P \leq 0.05$) between the different concentrations of each cytokinin; [d] standard error.

Table 2
Effect of BAP (3 mg L^{-1}) and auxin combinations on multiple shoot formation of *P. abrotanoides*.

Auxin concentration (mg L^{-1})	Shoot proliferation (%)	MSN [a] (no. ± SE[d])	MSL[b] (cm ± SE)	SFC[c] index
Control	0.0	0.0	0.0	0.0
NAA	**70.8 A**	**3.5 ± 0.6 B**	**2.0 ± 0.3 A**	**2.5**
0.5	58.3 a	3.3 ± 0.6 c	2.0 ± 0.2 b	2.0
1	83.3 a	3.6 ± 0.5 bc	2.2 ± 0.6 b	3.0
IBA	**87.5 A**	**5.9 ± 1.2 A**	**2.2 ± 0.4 A**	**4.5**
0.5	83.3 a	5.5 ± 1.1 ab	2.3 ± 0.2 b	4.6
1	91.7 a	6.3 ± 1.4 a	2.2 ± 0.7 b	5.8
IAA	**54.2 A**	**2.4 ± 1.2 B**	**3.2 ± 1.3 A**	**1.5**
0.5	50.0 a	2.0 ± 1.7 c	2.7 ± 1.4 b	1.5
1	58.3 a	2.9 ± 0.4 c	4.6 ± 1.7 a	1.6
2,4-D	**58.3 A**	**3.4 ± 1.2 B**	**3.3 ± 0.9 A**	**2.8**
0.5	41.7 b	3.2 ± 1 c	3.0 ± 1.4 ab	1.3
1	75.0 a	3.7 ± 1.5 bc	2.0 ± 0.2 b	2.8

[a] Mean shoot number; [b] Mean shoot length; [c] Shoot-forming capacity; Different capital letters indicate significant differences between auxins. Lower case letters indicate significant differences between the different concentrations of each auxin ($P \leq 0.05$); [d] Standard error; [e] Callus induction at the basal end of the explants.

Table 3
Effects of type and different concentrations of auxins on *in vitro* rooting of *P. abrotanoides*.

Auxin concentration (mg L^{-1})	Rooting rate (%)	MRN[a] (no. ± SE[d])	MRL[b] (cm ± SE)	RFC[c] index
Auxin-free (1/2MS)	**100 A**	**5.9 ± 1.4 A**	**3 ± 0.5 B**	**5.9**
NAA	**55.6 B**	**4.1 ± 1.3 B**	**1.5 ± 1 B**	**2.5**
0.1	41.7 b	3.2 ± 1.2 abc	2.2 ± 1.05 b	1.3
0.5	58.3 ab	4.0 ± 1.08 abc	1.1 ± 1.04b	2.6
1	66.7 ab	5.2 ± 1.05 ab	1.3 ± 0.9 b	3.5
IBA	**52.7 B**	**3.8 ± 1.6 B**	**3.8 ± 2.2 A**	**2.1**
0.1	41.7 b	3.0 ± 1.1c	2.2 ± 0.07 b	1.2
0.5	50.0 ab	5.2 ± 1.9 ab	7.5 ± 1.6 a	2.9
1	66.7 ab	3.1 ± 0.9 abc	1.7 ± 0.5 b	2.2
2,4-D	**47.2 B**	**2.4 ± 0.8 C**	**3.4 ± 3.7 A**	**1.1**
0.1	75.0 ab	1.9 ± 0.4 c	2.2 ± 0.6 b	1.4
0.5	41.7 ab	3.2 ± 0.8 abc	7.0 ± 2.4 b	1.3
1	33.3 ab	2.0 ± 0.6 c	0.83 ± 0.3 c	0.6

[a] Root number mean; [b] root length mean; [c] Root-forming capacity; Different capital letters indicate significant differences between auxins, while lower case letters indicate significant differences between the different concentrations of each auxin at $P \leq 0.05$; [d] Standard error.

a concentration of 3 mg L^{-1} were more effective than KN in multiple shoot formation. In contrast, favorable effect of KN compared with the other cytokinins (BAP and 2-isopentenyl adenine in promoting *in vitro* regeneration of *Thymus vulgaris* (Furmanowa and Olszowska, 1992) has been reported. These data correlate with a well-known physiological fact that cytokinins usually block ethylene action and promote shoot multiplication (Ozudogru et al., 2011).

Other studies have reported stronger effects of BAP than KN on the induction of multiple shoots in the Lamiaceae family (Chishti et al., 2006; Dias et al., 2002). Such a desirable effect of BAP on shoot formation from germinated seeds of *P. abrotanoides* has been reported in *in vitro* condition (Saboora and Shokri, 2013), although their optimum concentration reported was lower than the optimum concentration determined in the present study. In addition, other studies have shown that an average of 52–91 percent of *Melissa officinalis* explants cultured in media containing 3 mg L^{-1} BAP formed about 3–4 new shoots which are in consistent with the results (Meftahizade et al., 2010).

In general, a combination of two or more PGRs is needed for a successful *in vitro* propagation from the shoot (Bakhtiar et al., 2014; Caraballo et al., 2010). In the study on Lamiaceae family, it has been shown that shoot induction on medium containing auxin and cytokinin is preferred to medium containing cytokinin alone (Castillo and Jordan, 1997). Adding auxin will not accelerate stem proliferation. However, if used in small quantities, it will neutralize inhibitory effects of large amounts of cytokinin on the growth of lateral shoots and restore the stem growth process to normal mode (Caraballo et al., 2010; Robert et al., 1992). Furthermore, low concentrations of auxin are used to stop the production of callus (Verma et al., 2016). Therefore, in the second stage of shoot induction, the optimized concentration of BAP (3 mg L^{-1}) was used in combination with four different auxins (IBA, IAA, 2, 4-D and NAA) at two levels (0.5 and 1 mg L^{-1}) to assess shoots formation. Overall results showed that in the presence of BAP combined with auxin, the main shoots of *P. abrotanoides* had a better response. In this study, IBA had a more effective impact on the induction of shoot formation of *P. abrotanoides* compared to the other tested auxins. Adding concentrations of IBA (0.5 and 1 mg L^{-1}) to MS medium containing 3 mg L^{-1} BAP increases shoot formation, average shoot number, and SFC index. Overall, there was no significant statistical difference between both IBA concentrations (0.5 and 1 mg L^{-1}) on the assessed traits. The highest number of shoots (6.3 ± 1.4) and shoot induction percentage (91.66%) were obtained from 3 mg L^{-1} BAP in combination with 1 mg L^{-1} IBA. This combination leads to unwanted callus formation at the place of lateral bud formation. The callus formation was insignificant at the base of explants placed in the hormonal combination of 3 mg L^{-1} BAP and 0.5 mg L^{-1} IBA. Explants having callus formation were not used for all subsequent experiments because of the possible genetic diversity (Kaeppler et al., 2000). The highest shoot length mean (4.6 ± 1.7 cm) was observed in plants treated with 3 mg L^{-1} BAP and 1 mg L^{-1} IAA, but the new shoots obtained from these treatments were very weak with a lower number of leaves in bright green. Thus, MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} IBA is the optimal medium for shoot induction in *P. abrotanoides*. Ozudogru et al. (2011) reported that the highest shoot proliferation of *Thymus vulgare* was obtained from MS medium containing BAP combined with IBA (Ozudogru et al., 2011). Moreover, in a study conducted on *in vitro* proliferation of *Ocimum gratissimum* through direct regeneration, the highest number of shoots in desirable length was obtained in a medium containing 0.5 mg L^{-1} IAA and 0.25 mg L^{-1} BAP (Gopi et al., 2006). In the present study, medium supplemented with 2, 4-D had the lowest shoot number and shoot formation percentage. The same negative effect of 2,4-D on shoot formation percentage and SFC index has been reported for *Thymus persicus* (Bakhtiar et al., 2014).

½ MS without PGRs with 7 g L^{-1} agar is reported as the optimal medium for root induction and obtaining healthy root from propagated shoots of *P. abrotanoides*. It has been reported that in the rooting stage, the concentrations of macro and micro nutrients are usually reduced to half of the standard concentration. The favorable effect of reducing the elements might be related to less nitrogen requirement of explants. Culture conditions may also affect *in vitro* rooting of plants. Due to the lack of ventilation in medium with high amounts of agar, no hairy roots are formed on roots grown in these conditions (Hammerschlag et al., 1987). In many species, including *Lavandula vera*, the fastest root formation is obtained in a low strength MS medium (Andrade et al., 1999). ½ MS without PGRs with 1.5% (W/V) sucrose has been reported as the optimal medium for rooting of proliferated shoots of *Coleus forskohlii* (Krishna et al., 2010). In the present study, the maximum length of roots was observed in ½ MS supplemented with 0.5 mg L^{-1} IBA. In this medium, a high amount of callus developed at the base of the explants. Callus development as a result of auxin in the rooting medium causes abnormal vascular connections between the stem and adventitious roots (Ziv, 1986; Zuzarte et al., 2010). In many Lamiaceae family plants, IBA at low concentrations has a positive impact on the formation and elongation of roots. For example, *Ocimum basilicum* shoots cultured on the medium containing 0.2 mg L^{-1} IBA showed the highest root

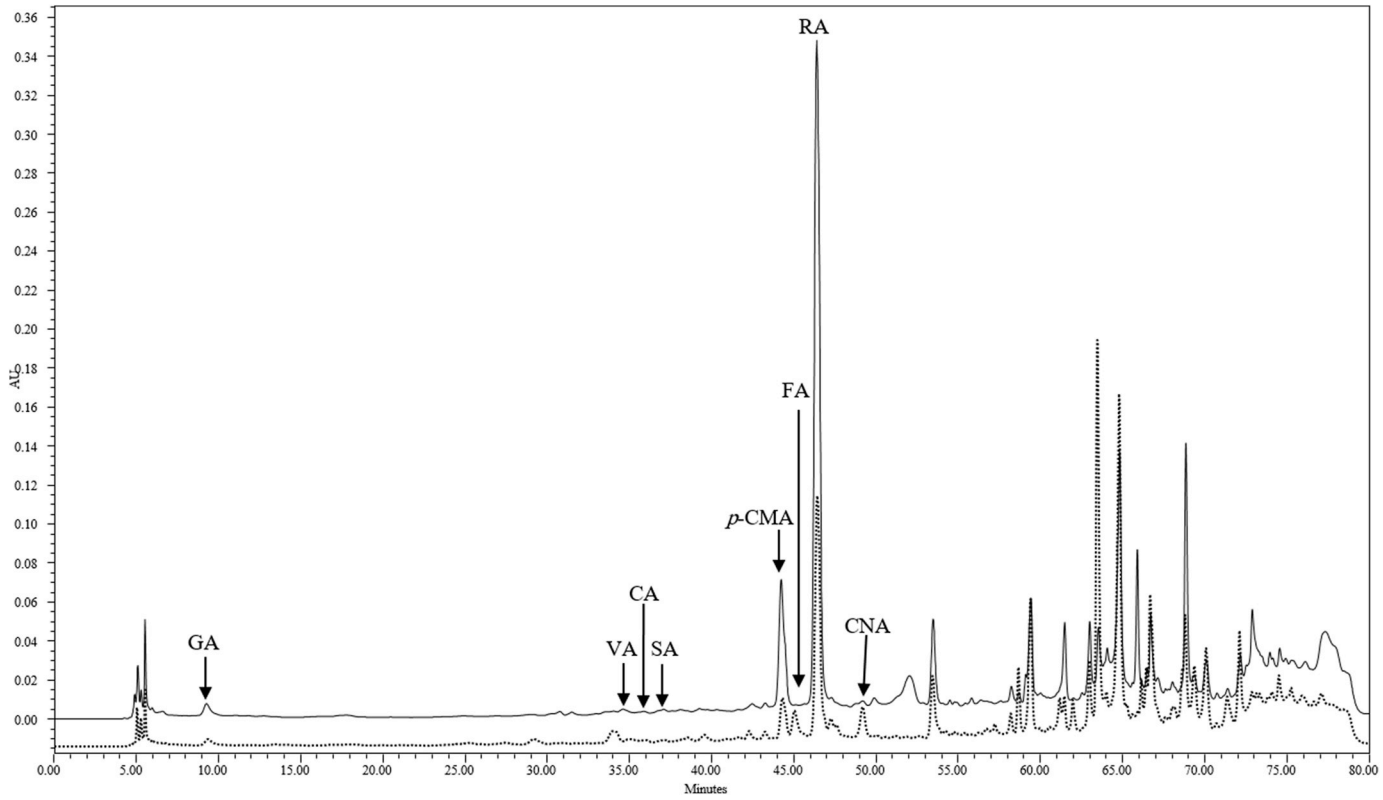

Fig. 3. The HPLC-DAD profiling of phenolic acids i.e. gallic acid (GA), vanilic acid (VA), caffeic acid (CA), syringic acid (SA), *p*-coumaric acid (*p*-CMA), ferulic acid (FA), rosmarinic acid (RA), cinnamic acid (CAN) for *in vitro* regenerated plant (bold line) and wild plant (dotted line) of *P. abrotanoides*. The condition is explained in the experimental section.

Table 4
Total phenol, phenolic acids content (mg/100 g DW ± SD) and antioxidant activity of *in vitro* regenerated plant (IVRP) and wild plant (WP) of *P. abrotanoides*.

Phenolic compounds	WP	IVRP
Gallic acid	26.2 ± 0.04	34.0 ± 0.5
Vanillic acid	14.0 ± 0.01	14.1 ± 0.02
Caffeic acid	15.4 ± 0.1	16.3 ± 0.2
Syringic acid	17.9 ± 0.1	15.0 ± 0.06
p-Coumaric acid	72.5 ± 0.3	212.0 ± 0.4
Ferulic acid	17.0 ± 0.3	2.0 ± 0.03
Rosmarinic acid	341.1 ± 7.2	1017.6 ± 1.5
Cinnamic acid	65.4 ± 0.3	13.2 ± 0.03
TPC (mgGAE/100 g)	54.9 ± 15.2	70.7 ± 9.1
Antioxidant activity (μg/ml)	275.7 ± 10.2	230.4 ± 8.6

SD, Standard deviation; TPC, total phenolic content.

length and number mean (Siddique and Anis, 2008). Given that the number of roots and RFC rate are essential parameters for successful adaptation stage (Ozudogru et al., 2011). IVRPs rooted on free-PGRs ½ MS medium were taken to the adaptation stage. After transferring to pots, 75% of the explants survived and adapted to the *in vivo* conditions.

In recent years, the global market value of herbal medicines including medicinal plants and their by-products has significantly increased. For instance, using plant tissue and organ cultures as well as biotechnological techniques to enhance the quantity and quality of medicinal plants are very important. Tissue culture provides a rapid proliferation tool to produce a large number of uniform plants while maintaining their genotype. In addition to the lack of secondary metabolite production constraints like climate and planting season, the rapid production of metabolites due to the rapid growth in tissue culture is a slew of other benefits. Development of an efficient micropropagation protocol and improvement of secondary metabolites performance in *in vitro*-grown plants have been reported for the other member of Lamiaceae (Bakhtiar et al., 2014; Karam et al., 2003). In this study, the conditions of direct micropropagation of *P. abrotanoides* were optimized. Quantification of several valuable PAs in *in vitro* regenerates compared with wild growing plant were also reported for the first time. RA (1017.6 ± 1.5 mg 100 g dw^{-1}) was identified as the major phenolic acid in the extract of the plant. Our results show that IVRPs were more effective than WPs in producing these compounds. The higher levels of phenolic compounds in IVRPs, compared to those grown at natural habitats (WP), have been attributed to the higher concentrations of nutrients in culture medium. Phenolic compounds are strongly influenced by ecological and edaphological conditions (Casagrande et al., 2018; Rawat et al., 2017), so the difference in the content of these compounds is also justified for studied WP and IVRPs of *P. abrotanoides*. It has been claimed that UV irradiation is able to destroy phenolic compounds, which would subsequently affect the total phenol content of the plant extracts (Swanson et al., 1996). Ghaffari et al. (2018) have recently been reported the variability of total phenol content (19.80–66.86 mg GAE/g DW) among the wild populations of *P. abrotanoides*.

In the present study, the highest antioxidant activity was obtained from the extract of IVRPs which can be attributed to their higher content of phenolic compounds. Antioxidant activity of *P. abrotanoides* populations has recently been reported by Ghaffari et al. (2018). The authors also confirmed the contribution of phenolic compounds to the antioxidant activity. These results are in agreement with those of Orsavová et al. (2019) and Turumtay et al. (2014).

5. Conclusion

We achieved an excellent reproducible protocol for *in vitro* cloning of *P. abrotanoides* as a natural source of potent antioxidant phenolic compounds. This protocol can also be used for mass production and

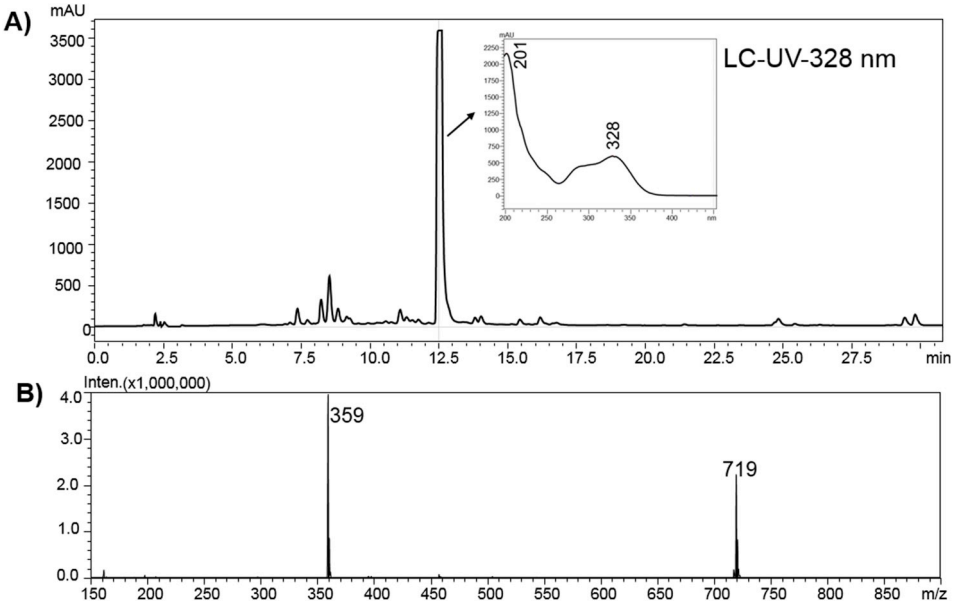

Fig. 4. A) HPLC-UV chromatograms (328 nm) corresponding to the MeOH extract of *in vitro* regenerated of *Perovskia abrotanoides* B) ESI mass spectrum of rosmarinic acid in negative mode, *m/z* 359 shows $[M-H]^-$ and *m/z* 719 reveals $[2M-H]^-$.

germplasm conservation of the plant. This research also contributes to the secure implementation of research to enhance the antioxidant compounds production including RA through various biotechnological strategies such as cell suspension culture and large scale culture and production in bioreactors.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101113.

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