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PLANT TISSUE CULTURE

Improvement of shoot proliferation and comparison of secondary metabolites in shoot and callus cultures of *Phlomis armeniaca* by LC-ESI-MS/MS analysis

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Abstract *Phlomis armeniaca* Willd. is a medicinal plant in the Lamiaceae family endemic to Turkey. The present study describes efficient plant regeneration and callus induction protocols for *P. armeniaca* and compares phenolic profiles, total phenol and flavonoid contents, and free radical scavenging activity of *in vitro*-derived tissues. Stem node explants from germinated seedlings were cultured on Murashige and Skoog medium (MS) supplemented with 75 plant growth regulator (PGR) combinations. The highest shoot number per explant, frequency of shoot proliferation, and frequency of highly proliferated, green, compact callus were obtained on MS medium containing 0.25 mg L^{-1} thidiazuron (TDZ) and 0.25 mg L^{-1} indole-3-acetic acid (IAA). The best root formation was on MS basal medium (control). Methanol extract of leaves obtained from regenerants contained higher total phenol and flavonoid contents than the callus extract. The callus extract showed stronger free radical scavenging activity than leaves with IC_{50} [concentration inhibiting 50% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical] values of 4.30 ± 0.08 and 2.21 ± 0.04 mg g^{-1} dry weight in leaves and callus, respectively. Apigenin, caffeic acid, *p*-coumaric acid, luteolin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, and chlorogenic acid were detected by liquid chromatography–electrospray ionization multistage tandem mass spectrometry (LC-ESI-MS/MS) analysis in *in vitro*-grown leaves and callus tissue. Rutin hydrate, *p*-coumaric acid, and vanillic acid were found at approximately tenfold higher levels in callus than in leaves. This new micropropagation protocol, the first for *P. armeniaca*, could be used in industrial production for new herbal tea and germplasm conservation.

Keywords Micropropagation · Plant growth regulators · Nodal explants · Antioxidant system · LC-ESI-MS/MS

Introduction

Different kinds of dried medicinal plant materials have appeared as herbal teas in the supermarkets and are used as natural antioxidants by the food industry. The antioxidant activity of some natural plant extracts is comparable to and sometimes higher than that of synthetic chemical antioxidants (Pokorny 1991). Many natural plant species that contain proteins, carotenoids, ascorbic acid, flavonoids, and other phenolic molecules might play a significant role as physiological and food antioxidants (Shahidi 2000). Natural antioxidants that are obtained from medicinal plants are known to show high levels of biological activities including anti-inflammatory, antiviral, antibacterial, antiallergic, antiaging, anticancer, neuroprotective, and cardioprotective effects (Cook and Samman 1996; Karakas *et al.* 2015).

Phlomis is a large genus in the Lamiaceae family that comprises about 100 species and encompasses species native to Turkey, North Africa, Europe, and Asia (Demirci *et al.* 2008). *Phlomis* species are used to remedy common diseases such as diabetes, gastric ulcers, hemorrhoids, inflammation, and wounds and to protect the liver, kidney, heart, veins, and bone from different pathologies (Limem-Ben Amor *et al.* 2009). These species have various uses in traditional medicine that differ from one country to another. For instance, *P. cephalotes* and *P. plukenetii* are used to treat fever, cough, and cold.

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P. bovei and *P. crinita* are utilized to heal burns, lesions, skin infections, and allergies (Limem-Ben Amor *et al.* 2009).

P. armeniaca Willd. is a medicinal plant in the Lamiaceae family endemic to Turkey. The aerial parts have been used as an herbal tea in traditional medicine for the treatment of inflammation, cold, diabetes, wounds, and gastrointestinal problems such as digestion problems, gastric ulcer, and stomachache (Baytop 1999; Uysal *et al.* 2016). Antimutagenic (DNA-protecting activity) and antioxidant activities (Yumrutas and Saygıdeger 2012; Sarikurkcu *et al.* 2015) as well as antibacterial and antitumor activities (Turker and Yıldırım 2013) have also been attributed to aerial parts obtained from natural *P. armeniaca*. This herbal tea is sometimes consumed daily instead of black tea (Gençay 2007; Dalar and Konczak 2014) in Turkey. The aerial parts of this plant are collected during early summer and used fresh or dried during autumn and winter. Some researchers have demonstrated that *P. armeniaca* includes glycosidic compounds such as betulalbuside (a monoterpene glycoside), ipolamiide (an iridoid glycoside), and teucrioside (a phenylpropanoid glycoside) (Saracoglu *et al.* 1995); phenolic compounds such as catechin, chlorogenic acid, caffeic acid, ferulic acid, rutin, rosmarinic acid, and apigenin (Sarikurkcu *et al.* 2015); and essential oils such as germacrene-D, beta-caryophyllene, caryophyllene oxide, (E)-beta-farnesene, and hexahydrofarnesyl acetone (Yasar *et al.* 2010).

Despite extensive uses of this endemic medicinal herb and daily consumption of its tea, the *in vitro* micropropagation of *P. armeniaca* and the phenolic composition of its tissue culture-derived plant materials have not previously been studied. *In vitro* micropropagation protocols are useful for plant breeding and commercial production of medicinal endemic plants. Therefore, the objective of this study was to establish an efficient *in vitro* regeneration and callus formation protocol and to determine the phenolic profiles and antioxidant capacities of *P. armeniaca* for the first time. Such research would illuminate the components of this plant as well as distribution of these components in various parts of its body and provide an efficient tool for researchers in both basic and applied science.

Materials and Methods

Plant material, surface sterilization of seeds, and culture conditions *P. armeniaca* seeds were collected from the natural habitat of Sünnet Lake, Bolu, Turkey, in September 2014. The plant was authenticated by Prof. Dr. Arzu Ucar Turker and an herbarium sample was deposited in the Department of Biology, Abant Izzet Baysal University, Bolu (accession number AUT-1954).

To break seed dormancy, seeds were kept at 4°C for 1 mo. Seeds were soaked in tap water containing 10% (*v*/*v*) antibacterial soap (Protex®, Denizli, Turkey) for 24 h and then rinsed with tap water until the foam cleared. To test the effects of bleach concentration on seed germination, surface sterilization was carried out with 10–40% (*v*/*v*) commercial bleach (Domestos®, Unilever, Istanbul, Turkey, containing 5% sodium hypochlorite) for 15 min followed by four to five washes with sterile distilled water. The sterilized seeds were placed in sterile Petri plates containing 4.43 g L^{-1} Murashige and Skoog (MS) medium (Duchefa Biochemie, Amsterdam, the Netherlands; Murashige and Skoog 1962) supplemented with 3% (*w*/*v*) sucrose (Merck®, Darmstadt, Germany) and 0.8% (*w*/*v*) Bacto agar (Difco®, Bordeaux, France). The medium was adjusted to pH 5.8 before being autoclaved for 20 min at 121°C and 105 kPa. All culture plates were maintained in a plant culture room at 22 ± 2°C with fluorescent light (cool-white light; 200 μmol m^{-2} s^{-1}, Polylux®, GE Lighting, Nagykanizsa, Hungary), 16-h photoperiod, and 60–65% relative humidity.

Shoot regeneration and callus induction Two weeks later, germinated seeds were placed into Magenta GA-7 vessels (Sigma-Aldrich® Chemical Co., St. Louis, MO) including MS medium supplemented with 0.5 mg L^{-1} gibberellic acid (GA_3) for shoot elongation for an additional 2 wk in the same culture conditions as described above.

Four different explant types (petiole, leaf, root, and stem node) were excised from 1-mo-old sterile seedlings for shoot regeneration and callus induction. The leaf lamina (5 × 5-mm pieces), petiole (4- to 5-mm fragments), node (single node with two axillary buds), and root (4- to 5-mm fragments) explants were transferred to sterile disposable Petri dishes (90 × 15 mm) containing MS medium supplemented with 75 different combinations and concentrations of plant growth regulators (PGRs): thidiazuron (TDZ; 0.1, 0.25, 0.5, 1.0, or 3.0 mg L^{-1}) and indole-3-butyric acid (IBA; 0, 0.5, 1.0, or 3.0 mg L^{-1}), TDZ (0.1, 0.25, 0.5, 1.0, or 3.0 mg L^{-1}) and indole-3-acetic acid (IAA; 0, 0.25, 0.5, or 1.0 mg L^{-1}), benzyl adenine (BA; 0.25, 0.5, 1.0, 2.0, or 3.0 mg L^{-1}) and naphthalene acetic acid (NAA; 0, 0.25, 0.5, or 1.0 mg L^{-1}), or BA (0.1, 0.5, 1.0, 3.0, 5.0, or 10.0 mg L^{-1}) and IAA (0.1, 0.25, 0.5, 1.0, or 3.0 mg L^{-1}). Filter-sterilized GA_3 was added to cooled MS medium after autoclaving. All other used PGRs were added before pH adjustment and then being autoclaved. Shoot regeneration and callus development experiments were repeated three times; each replicate for every treatment contained 3 Petri plates with 6 explants (i.e., each replicate used 18 explants and a total of 54 explants per treatment for the whole experiment). The number of shoots per shooted explant, shoot frequency, and callus formation were recorded 8 wk after culture initiation. A scale of one to four plus (+) signs was used to identify callus formation size [−, no callus development; +, callus development only on edges of explant (Fig. 1*f*); ++, callus development 0.5–1.0 cm in diameter (Fig. 1*g*); +++, callus development 1.0–1.5 cm in diameter

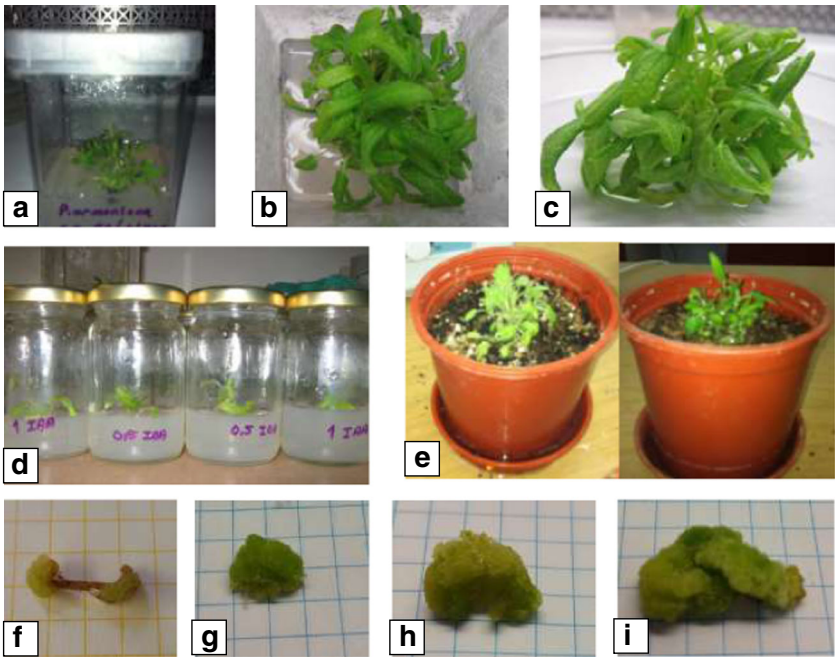

Figure 1. (*a*) Shoot regeneration from stem node explants of *P. armeniaca* seedling. (*b*) Shoot elongation on MS medium including 0.5 mg L^{-1} gibberellic acid (GA_3). (*c*) Shoots of *P. armeniaca* before being separated for rooting. (*d*) Rooting media. (*e*) Plantlets maintained under plant growth room conditions. (*f–i*) Illustrations of callus size scale. *f* +: callus development only on edges of explant. *g* ++: callus development is 0.5–1.0 cm in diameter. *h* +++: callus development is 1.0–1.5 cm in diameter. *i* ++++: callus development is 1.5–2.0 cm in diameter. Each edge of the *squares* in (*f–i*) is 0.5 cm

(Fig. 1*h*); ++++, callus development 1.5–2.0 cm in diameter (Fig. 1*i*)] .

Rooting For *in vitro* root induction, the regenerants (5 to 8 cm long) obtained from node explants (Fig. 1*b*) were transferred to MS medium supplemented with IAA (0.5, 1.0, and 3.0 mg L^{-1}), IBA (0.5, 1.0, and 3.0 mg L^{-1}), NAA (0.5, 1.0, and 3.0 mg L^{-1}), or 2,4-D (0.05, 0.5, and 1.0 mg L^{-1}), or to MS control medium (without PGRs). All used auxins were added to MS medium before pH adjustment and then being autoclaved. After 5 wk, the number of roots and percentage of the regenerated shoots producing roots were recorded. The rooted shoots were cleaned of medium and transferred to Magenta containers including autoclaved vermiculite (Agrekal®, Antalya, Turkey) and sterile distilled water and maintained in a plant culture room (22 ± 2°C with fluorescent light, 16-h photoperiod, and 60–65% relative humidity). Two weeks later, surviving plantlets were transferred to plastic pots containing commercial soil (Emin Torf, Bolu, Turkey) and vermiculite (3:1 *w*/*w*) for acclimatization and growth under growth room conditions (16-h photoperiod, 22 ± 2°C, and 25–35% humidity).

Plant extraction *In vitro*-grown leaves and callus of *P. armeniaca* were collected from 2-mo-old *in vitro*-cultured plant materials on MS medium containing 0.25 mg L^{-1} TDZ and 0.25 mg L^{-1} IAA. The tissues were freeze-dried using a lyophilizer (Christ®, Osterode, Germany) at −55°C and then powdered with a grinder. One gram of the powdered plant material was transferred to a glass test tube containing 10 mL of 80% (*v*/*v*) methanol and incubated for 18 h at 35°C in an agitated hot-water bath for extraction. After incubation, the test tubes were centrifuged at 5000 rpm (2795×*g*) for 10 min. The supernatant was filtered with a Whatman syringe filter (pore size 0.45 μm) and transferred to a new glass test tube. The extracts were stored in a deep-freeze (−80°C) until required for analysis.

Total phenolic determination The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent according to the procedure reported by Dewanto *et al.* (2002) with some modifications. Gallic acid (Sigma-Aldrich®) and tannic acid (Sigma-Aldrich®) are accepted standards used as reference phenols. A 125-μL sample from each calibration solution, sample, or blank was placed into a separate cuvette. Then, 500-μL distilled water and 125 μL Folin–Ciocalteu reagent (Merck®, Darmstadt, Germany) were added to each and mixed well. After 5 min, 125 μL of 10% (*w*/*v*) sodium carbonate (Na_2CO_3) solution was added and the final solution was adjusted to 3 mL with distilled water. Then, the solution was shaken very well by vortexing. The solutions were incubated at 22 ± 2°C for 90 min, and the absorbance of each solution was measured at 760 nm using a spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200 V, Tokyo, Japan). The TPC values of methanol extracts obtained from *in vitro*-grown leaves and callus were calculated as mg gallic acid equivalent (GAE) and mg tannic acid equivalent (TAE) g^{-1} dry weight (dw) of plant material according to the calibration curves. The gallic acid and tannic acid calibration curve ranges was 12.5–250 mg L^{-1} (R^2 = 0.9992 and R^2 = 0.9962, respectively). Experiments were repeated three times for each tested extract with three replicates.

Total flavonoid determination The total flavonoid content (TFC) of methanol extracts from *in vitro*-grown leaves and callus of *P. armeniaca* was determined and measured by an aluminum chloride ($AlCl_3$) colorimetric assay (Chang *et al.* 2002). Catechol (Sigma-Aldrich®) and quercetin (Sigma-Aldrich®) were used as reference flavonoids. In order to obtain calibration curves of catechol and quercetin solutions at 12.5–250 mg L^{-1}, known concentrations were prepared in 80% methanol. A total of 500 µL of extract solution or standard solution of catechol or quercetin was added to a 10-mL test tube containing 2-mL distilled water. At time 0, 150 µL of 5% (*w/v*) sodium nitrate ($NaNO_2$) was added to the test tube. After 5 min, 150 µL of 10% (*w/v*) $AlCl_3$ was added. At 6 min, 1000 µL of 1 M sodium hydroxide (NaOH) was added to the mixture. Immediately, the reaction tube was diluted to a volume of 5 mL with the addition of 1200-µL distilled water and thoroughly mixed. Absorbance of the mixture (three replicates for each extract), pink in color, was determined at 510 nm versus a blank (Chang *et al.* 2002). The TFC of methanol extracts of *in vitro*-grown leaves and callus from *P. armeniaca* was expressed as mg catechol equivalents (CE) and mg quercetin equivalents (QE) g^{-1} dw of plant material according to the calibration curves (R^2 = 0.9996 and R^2 = 0.9906, respectively). Experiments were repeated three times.

Determination of free radical scavenging activity by DPPH assay The free radical scavenging activity of methanol extracts from *in vitro*-grown leaves and callus of *P. armeniaca* was determined spectrophotometrically against a stable DPPH· (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich®). The free radical scavenging activity of extracts was measured by slight modifications of the method of Brand-Williams *et al.* (1995), as described below. The extract solutions were diluted with 80% methanol (to 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg mL^{-1}) from the stock extract solutions. The solution of DPPH· in 80% (*v/v*) methanol (1.5×10^{-5} M) was prepared daily. For each sample to be analyzed, 0.5 mL of this solution was mixed with 1.5 mL of extract solution in a 10-cm path-length glass test tube (final mass ratio of extract to DPPH· was approximately 3:1). The samples were kept in the dark for 30 min at 22 ± 2 °C and then the decrease in absorption was measured. Absorption of a blank sample (positive control) containing the same amount of 80% (*v/v*) methanol and DPPH· solution was measured. The experiment was carried out in triplicate. Quercetin and ascorbic acid (Sigma-Aldrich®) were used as the reference samples. Radical scavenging activity (% inhibition) of *in vitro*-grown leaves and callus of *P. armeniaca* was calculated by the formula of Gülçin *et al.* (2003): %Inhibition = [(AB–AA)/AB] × 100, where AB is the absorption of the blank sample (control) and AA is the absorption of the tested extract solution. The results were also expressed as IC_{50} (µg mL^{-1}), the amount of sample necessary to decrease the absorbance of DPPH by 50%.

Determination of selected phenolic compounds by LC-ESI-MS/MS analysis The amounts of 20 selected phenolic compounds, apigenin, caffeic acid, *p*-coumaric acid, gallic acid, genistein (BioChemica®, AppliChem, Darmstadt, Germany), kaempferol, luteolin, myricetin, procyanidin-*C1*, quercetin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, chlorogenic acid, hesperidin, naringenin, rosmarinic acid, and isorhamnetin, in methanol extracts of leaves obtained from plantlets and callus of *P. armeniaca*, were detected using the liquid chromatography–electrospray ionization multistage tandem mass spectrometry (LC-ESI-MS/MS). All 20 phenolic compounds, except genistein, were obtained from Sigma-Aldrich®. Analysis was performed by METU Central Laboratory, Molecular Biology–Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey, with an Agilent 6460 Triple Quadrupole System (ESI + Agilent Jet Stream) coupled with an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA). A mobile phase including a mixture of 5 mM ammonium formate +0.05% (*v/v*) formic acid (solvent A) and methanol (MS grade, Merck) (solvent B) was delivered at a flow rate of 0.3 mL min^{-1} for 13 min (run time). All analysis conditions were used according to Karakas and Turker (2013).

Statistical analysis The statistical analysis of the experimental data was performed using SPSS Version 22.0 (SPSS Inc., Chicago, IL, USA). The Duncan's multiple range test was used to show statistical differences among means at $p < 0.05$ (ANOVA).

Results and Discussion

Seed germination Seed germination (100%) of *P. armeniaca* was accomplished by using a high concentration of commercial bleach (40%) for the seed surface sterilization in 7 d. In the experiment, different percentages of bleach from low to high doses were used in the seed germination process. Low doses of bleach were not effective to break the seed coat, and seeds were not germinated after 2 mo. On the other hand, the seed coats were separated easily from the seeds treated with a high concentration of bleach (40%), and then all seeds germinated successfully (100%) in MS medium after 5–7 d.

Shoot proliferation and callus induction systems The number of shoots per shooted explant, shoot frequency, and callus development were recorded 8 wk after shoot culture initiation (Tables 1 and 2).

Table 1. The effect of different auxin/cytokinin (TDZ/IBA and TDZ/IAA) combinations on shoot regeneration from stem node explants and callus regeneration from petiole, leaf, root, and node explants of *P. armeniaca* on MS medium after 8 wk of culture

Treatment (mg L^{-1})		Stem node explant		% of explants showing callus development, callus diameter, and callus color[z]			
		Mean number of shoots per shooted explants (±SD)	Explants forming shoots (%)	Petiole	Leaf	Root	Node
MS control		–	–	–	–	–	–
TDZ	IBA						
0.1	0	–	–	–	–	–	–
0.1	0.5	1.93 ± 0.88bcd	93.34	–	–	100, +, G	100, +, G
0.1	1.0	2.53 ± 0.83b	100	–	–	100, +, LG	100, +, G
0.1	3.0	1.33 ± 0.61cdef	100	100, +, G	–	100, ++, G	100, ++, G
0.25	0.0	–	–	–	–	–	–
0.25	0.5	1.53 ± 0.63cde	100	–	–	–	100, ++, G
0.25	1.0	2.46 ± 0.74b	100	–	–	100, +, B	100, ++, G
0.25	3.0	1.26 ± 0.70cdef	100	–	–	93.33, +, B	100, +, G-B
0.5	0.0	–	–	–	–	–	–
0.5	0.5	1.26 ± 0.59def	86.67	86.66, +, B	–	100, ++, G	100, ++, G-B
0.5	1.0	0.53 ± 0.51f	46.66	100, +, G	33.33, +, G	100, +, G	100, +, G-B
0.5	3.0	–	–	26.66, +, G	–	100, ++, G	100, ++, G
1.0	0.0	–	–	–	–	–	–
1.0	0.5	2.13 ± 0.83 bc	100	100, +, G	–	100, +, G	100, +, G
1.0	1.0	1.13 ± 0.74def	73.34	100, +, G	100, +, B	100, ++, G	100, ++, G
1.0	3.0	0.86 ± 0.63ef	66.67	66.66, ++, G	–	100, ++, G	100, +++, G
3.0	0.0	–	–	–	–	–	–
3.0	0.5	–	–	–	–	–	–
3.0	1.0	–	–	–	–	–	–
TDZ	IAA						
0.1	0.0	–	–	–	–	–	–
0.1	0.25	–	–	–	–	–	–
0.1	0.5	–	–	–	–	–	–
0.1	1.0	–	–	–	–	–	–
0.25	0.0	–	–	–	–	–	–
0.25	0.25	25.86 ± 4.17a	100	46.6 +, G	–	93.33 ++, G	100, ++++, G
0.25	0.5	2.46 ± 1.24b	100	73.33, ++, G	–	33.33, +, G	100, +++, G
0.25	1.0	1.33 ± 0.61cdef	86.67	100, ++, G	100, +, B	100, ++, G	100, ++, G
0.5	0.0	–	–	–	–	–	–
0.5	0.25	1.26 ± 0.59cdef	100	100, ++, G	–	90, +, G	100, ++, G
0.5	0.5	1.46 ± 0.83cdef	93.34	100, +, G	100, +, G-B	80, +, G	100, +, G
0.5	1.0	1.13 ± 0.63def	93.34	100, +, G	100, +, G-B	100, +, G	100, +, G
1.0	0.0	–	–	–	–	–	–
1.0	0.25	–	–	–	–	–	–
1.0	0.5	–	–	100, +, G	100, +, B	100, +, G	100, ++, G
1.0	1.0	–	–	100, +, G	100, +, B	100, ++, G	100, ++, G-B
3.0	0.0	–	–	–	–	–	–
3.0	0.5	–	–	–	–	–	–
3.0	1.0	–	–	–	–	–	–

Mean values within a *column* followed by the same *letter* are not significantly different at the 0.05 probability level of Duncan's multiple range test. A *dash* (−) indicates that there was no formation of the indicated tissue (shoot or callus)

TDZ thidiazuron, *IBA* indole-3-butyric acid, *IAA* indole-3-acetic acid, *B* brown, *G-B* greenish brown, *LG* light green, *G* green

[z] Callus diameter is indicated on a scale of + (callus development only on edges of explant) to ++++ (callus development 1.5–2.0 cm in diameter)

Table 2. The effect of different auxin/cytokinin (BA/NAA; BA/IAA) combinations on shoot regeneration from stem node explants and callus regeneration from petiole, leaf, root, and node explants of *P. armeniaca* on MS medium after 8 wk of culture

Treatments (mg L^{-1})		Stem node explant		% of explants showing callus development, callus diameter, and callus color[z]			
		Mean number of shoots per shooted explant (±SD)	Explants forming shoots (%)	Petiole	Leaf	Root	Node
MS control		–	–	–	–	–	–
BA	NAA						
0.25	0.0	–	–	–	–	–	–
0.25	0.25	1.73 ± 0.79fghij	93.34	40, +, G-B	–	80, +, G	100, ++, G
0.25	0.5	0.86 ± 0.51ij	80	86.66, +, LG	–	100, +, LG	100, ++, G
0.25	1.0	–	–	100, +, LG	–	–	100, ++, G
0.5	0.0	–	–	–	–	–	–
0.5	0.25	1.26 ± 0.48ghij	66.67	–	–	–	100, +, B
0.5	0.5	0.67 ± 0.45hij	100	100, ++, G	100, +, B	100, +, G-B	100, ++, G
0.5	1.0	–	–	100, ++, G	–	100, ++, G-B	100, +, G-B
1.0	0.0	–	–	–	–	–	–
1.0	0.25	2.06 ± 0.70fgh	100	73.33, +, G	–	100, ++, G	100, ++, G
1.0	0.5	1.93 ± 0.88fghi	100	100, +, G-B	100, +, B	66.66, ++, B	100, +, B
1.0	1.0	1.06 ± 0.45hij	93.34	60, +, G	100, ++, B	86.66, ++, B	100, +++, B
2.0	0.0	–	–	–	–	–	–
2.0	0.25	4.26 ± 1.83de	100	33.33, +, G	–	100, ++, G	100, ++, G
2.0	0.5	1.33 ± 0.48ghij	100	83.33, ++, G	–	60, ++, G	100, +++, G
2.0	1.0	1.20 ± 0.67ghij	86.67	26.66, ++, G	–	100, ++, G	100, ++, G
3.0	0.0	–	–	–	–	–	–
3.0	0.25	–	–	26.66, ++, G	–	100, +++, G	100, ++, G
3.0	0.5	–	–	100, ++, G	100, ++, B	73.33, ++, B	100, ++, G
3.0	1.0	–	–	100, ++, G	80, ++, B	100, ++, B	100, ++, G
BA	IAA						
0.1	0.5	–	–	–	–	–	–
0.5	0.1	7.13 ± 1.88b	100	–	–	100, ++, G	100, ++, G
0.5	0.25	3.66 ± 1.11e	100	–	–	100, +, G	100, ++, G
1.0	0.5	2.06 ± 0.79fgh	100	–	–	–	100, +, G
1.0	1.0	2.00 ± 0.92fgh	93.34	33.33, +, G	–	66.66, ++, G	100, ++, G
1.0	3.0	1.86 ± 1.12fghi	73.34	93.34, ++, G	–	100, ++, G	100, ++, G
3.0	0.5	1.13 ± 0.51hij	100	33.33, ++, G	–	100, ++, G	100, +, G
3.0	1.0	–	–	–	–	100, ++, G	100, ++, G
3.0	3.0	–	–	–	–	100, +++, G	100, ++, G
5.0	0.1	8.06 ± 2.12a	100	–	–	–	100, ++, G
5.0	0.5	5.13 ± 1.72d	100	–	–	100, +, G	100, ++, G
5.0	1.0	2.26 ± 1.33fg	93.34	–	–	100, ++, G	100, +, G
5.0	3.0	1.20 ± 0.56ghij	86.67	–	–	100, +, G	100, +, G
10.0	0.5	6.20 ± 2.88c	100	–	–	–	100, +, G
10.0	1.0	2.73 ± 1.98f	93.34	–	–	–	100, +, G
10.0	3.0	1.06 ± 0.88hij	73.34	–	–	86.66, ++, G	100, ++, G

Mean values within a *column* followed by the same *letter* are not significantly different at the 0.05 probability level of Duncan's multiple range test. A *dash* (−) indicates that there was no formation of the indicated tissue (shoot or callus)

BA benzyl adenine, *NAA* naphthalene acetic acid, *IAA* indole-3-acetic acid, *B* brown, *G-B* greenish brown, *LG* light green, *G* green

[z] Callus diameter is indicated on a scale of + (callus development only on edges of explant) to ++++ (callus development 1.5–2.0 cm in diameter)

The success of *in vitro* propagation depends on the concentration and combination of endogenous plant hormones and exogenous PGRs, composition of the culture medium, culture conditions, genotype, physiological properties of the donor plant, and suitable choice of explant type (Gonçalves and Romano 2013). In the present study, there were significant differences in the effect of different concentrations and combinations of PGRs and the effect of explant type on shoot number and frequency of *P. armeniaca* (Tables 1 and 2 and Fig. 1*a*). The explant type plays an important role in the efficiency of regeneration (Karakas and Turker 2013). In the present study, when petiole, leaf, root, and stem node explants were tested, only stem node explants of *P. armeniaca* were capable of shoot proliferation (Tables 1 and 2). With stem node explants, the highest number of shoots per explant was obtained on MS medium containing 0.25 mg L^{-1} TDZ and 0.25 mg L^{-1} IAA (25.86 ± 4.17 shoots with 100% shoot formation frequency; Table 1 and Fig. 1*b*, *c*). Likewise, the nodal explants of some medicinal plant species were identified as the most suitable explant for axillary shoot multiplication in *Galega officinalis* (Pehlivan Karakas *et al.* 2016), *Swertia corymbosa* (Mahendran and Narmatha Bai 2014), *Lavandula* spp. (Gonçalves and Romano 2013), and *Scutellaria alpina* (Grzegorczyk-Karolak *et al.* 2015).

In the present study, a combination of TDZ and IAA was the most effective for shoot regeneration (Table 1). Huetteman and Preece (1993) and De Gyves *et al.* (2001) pointed out the success of TDZ in the regulation of adventitious shoot formation, especially in combination with endogenous and some exogenous auxins. Similarly, an induction effect of TDZ in combination with IAA has been shown in many medicinal plant species such as *Bellis perennis* L. (Karakas and Turker 2013), *Cichorium intybus* L. (Yucesan *et al.* 2007), and *Achillea millefolium* L. (Turker *et al.* 2009).

When stem node explants were cultured on MS media including BA in combination with NAA or IAA, the best shoot formation was obtained with 5.0 mg L^{-1} BA in combination with 0.1 mg L^{-1} IAA (8.06 ± 2.12 shoots with 100% shoot formation) (Table 2). It was clear that the shoot regeneration capacity and frequency of *P. armeniaca* were related to the levels of IAA. For instance, increasing the level of IAA from 0.1 to 3.0 mg L^{-1} decreased the shoot number from 8.06 to 1.86 shoots per explant. Likewise, the shoot frequency decreased from 100 to 73.34% (Table 2). Shoot induction was not obtained with petiole, leaf, or root explants on any of the tested media. Shoot and callus development were not observed with nodal explants of *P. armeniaca* when cytokinins were used alone (Tables 1 and 2). On the other hand, when TDZ or BA was combined with IAA, NAA, or IBA, shoot regeneration was achieved (Tables 1 and 2). Other studies have shown that the presence of auxins is essential for the induction of shoot development, because auxins can regulate/organize cytokinin biosynthesis in plant species (Nordström *et al.* 2004; Karakas and Turker 2013). However, a critical equilibrium between PGRs provided to the culture medium and endogenous concentration of plant hormones in the explant part is needed (Mallon *et al.* 2011). The MS control treatment (without PGRs) did not produce any shoots or callus on petiole, leaf, root, or stem node explants (Tables 1 and 2).

Callus tissue formation was observed on all tested explant types of sterile seedlings of *P. armeniaca*. The efficiency of callus development was evaluated by measuring % callus development, diameter, and color (Tables 1 and 2). The callus had different colors such as green (G), light green (LG), brown (B), and greenish brown (G-B). The best callus development was observed from stem node explants, with 100% callus development frequency, green color, and 2.0-cm diameter on MS medium containing 0.25 mg L^{-1} TDZ and 0.25 mg L^{-1} IAA (Table 2 and Fig. 1*i*). This callus induction combination was used to produce callus for measurement of antioxidant activity and analysis of the phenolic profile.

Rooting and acclimatization After 8 wk of culture, 5 to 7 cm long shoots of *P. armeniaca* were counted, separated, and transferred to rooting MS medium containing various types and levels of auxins (Fig. 1*d*). After 5 wk, 90% of the plants survived and rooted only in MS control medium (without PGRs). The plantlets showed moderate survival rates (over 55%; data not shown) (Fig. 1*e*).

The present study, being the first report to define an *in vitro* plant production protocol for *P. armeniaca*, can provide a method for mass production of pesticide-, herbicide-, and disease-free plants for industrial and pharmaceutical uses.

Estimation of phenolic content of *in vitro*-propagated leaf and callus obtained from *P. armeniaca* The amounts of 20 different phenolic compounds in *in vitro*-grown leaf and callus methanol extracts obtained from *P. armeniaca* were calculated by using LC-ESI-MS/MS analysis. The total content of these 20 phenolic compounds in methanol extract of *in vitro*-propagated leaves (134.4769 μg g^{-1} dw) was five times higher than *in vitro*-grown callus (26.5926 μg g^{-1} dw), both grown on MS medium containing 0.25 mg L^{-1} TDZ and 0.25 mg L^{-1} IAA (Table 3). Apigenin, caffeic acid, *p*-coumaric acid, luteolin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, and chlorogenic acid were detected in both extracts. The amounts of *p*-coumaric acid (Fig. 2), rutin hydrate, and vanillic acid were approximately tenfold higher in callus than in leaf (Table 3). Kaempferol, myricetin, naringenin, rosmarinic acid, quercetin, and isorhamnetin were only detected in methanol extract of *in vitro*-grown leaves (Table 3). The predominant phenolic secondary metabolite in both extracts was chlorogenic acid (121.74 μg g^{-1} dw in leaf and 22.385 μg g^{-1} dw in callus; Table 3 and Fig. 3). Sixteen of the 20 phenolic compounds were present at or above the limit

Table 3. Names of studied phenolic compounds, their retention times, and amount of phenolic compounds in methanol extract of *in vitro*-grown leaves (PAL) and *in vitro*-grown callus (PAC) of *P. armeniaca*

Phenolic compound	Retention time (min)	Amount of phenolics of extracts (μg g^{-1} of dry weight)	
		PAL	PAC
Apigenin	6.998	1.4669 ± 0.1563	0.2019 ± 0.0012
Caffeic acid	3.150	0.2893 ± 0.0084	0.5230 ± 0.0106
p-coumaric acid	3.959	0.0624 ± 0.0006	0.5711 ± 0.0062
Gallic acid	0.943	Nd	Nd
Genistein	6.493	Nd	Nd
Kaempferol	6.864	0.050 ± 0.001	Nd
Luteolin	6.489	3.8269 ± 0.0993	0.2179 ± 0.0030
Myricetin	5.347	0.4240 ± 0.0156	Nd
Procyanidin-*C1*	3.181	Nd	Nd
Quercetin	6.135	1.3937 ± 0.0037	Nd
Rutin hydrate	4.985	0.0622 ± 0.0074	0.6725 ± 0.0319
Vanillic acid	3.120	0.1448 ± 0.0033	1.0292 ± 0.0308
Ferulic acid	4.271	0.1223 ± 0.0018	0.5711 ± 0.0062
Salicylic acid	3.933	0.1350 ± 0.0009	0.2680 ± 0.0362
Sinapic acid	4.377	0.0580 ± 0.0016	0.1529 ± 0.0008
Chlorogenic acid	2.807	121.74 ± 0.0163	22.385 ± 0.0062
Hesperidin	5.169	Nd	Nd
Naringenin	6.235	0.1462 ± 0.0073	Nd
Rosmarinic acid	4.949	1.0759 ± 0.1980	Nd
Isorhamnetin	7.003	3.4793 ± 0.0082	Nd
Total phenolics		134.4769	26.5926

Nd not detected

of detection (LOD) in at least one of the extract types. The levels of gallic acid, genistein, procyanidin-*C1*, and hesperidin were lower than the LOD in both tested extracts, and the levels of kaempferol, myricetin, naringenin, rosmarinic acid, quercetin, and isorhamnetin were lower than the LOD in the methanol extract of *in vitro*-grown callus but could be detected in

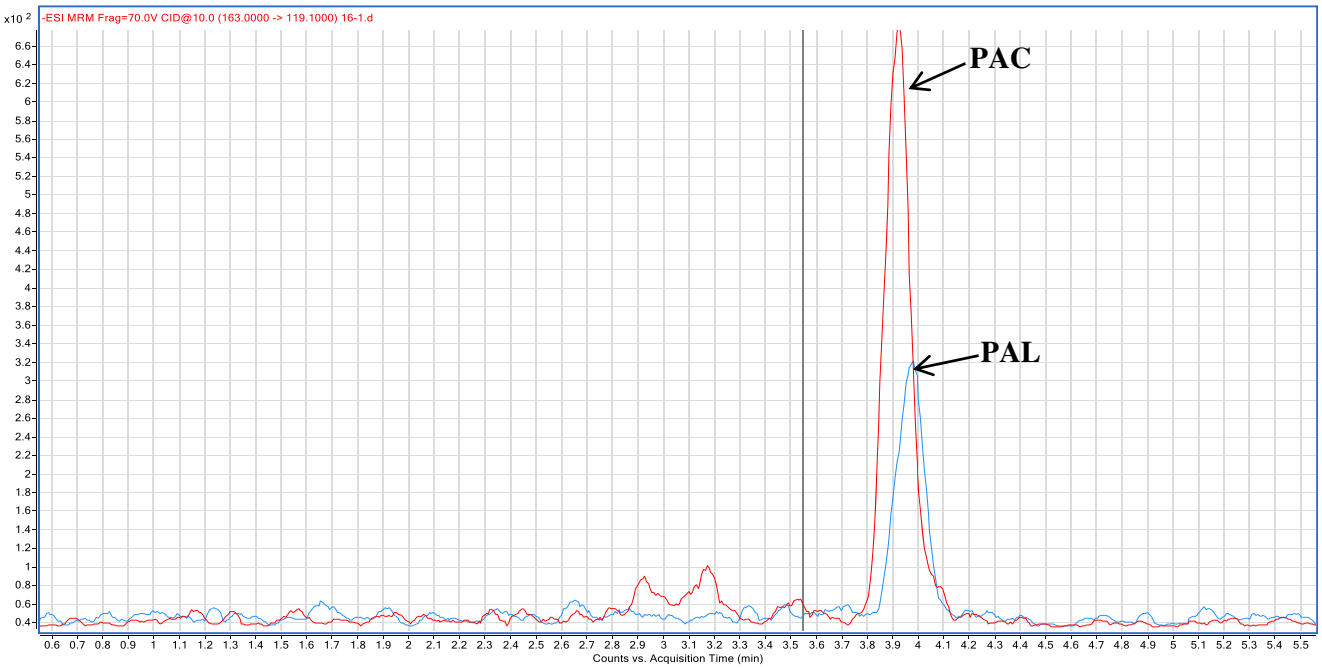

Figure 2. Chromatogram of *p*-coumaric acid obtained from leaves (PAL) and callus (PAC) of *Phlomis armeniaca* by LC-ESI-MS/MS analysis

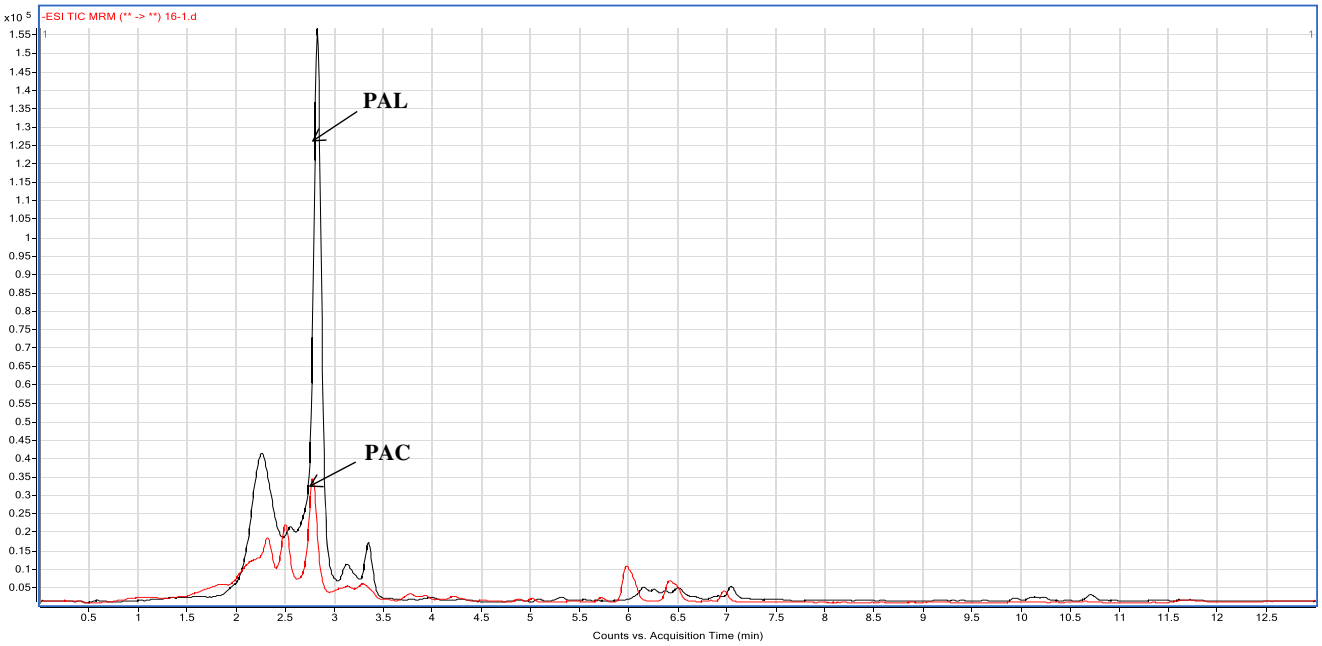

Figure 3. Chromatogram of chlorogenic acid obtained from leaves (PAL) and callus (PAC) of *Phlomis armeniaca* by LC-ESI-MS/MS analysis

leaves (Table 3). In the present investigation, the levels of apigenin, luteolin, and chlorogenic acid were higher in the leaves derived from regenerants than in callus. On the other hand, levels of caffeic acid, *p*-coumaric acid (Fig. 2), rutin hydrate, vanillic acid, ferulic acid, salicylic acid, and sinapic acid were higher in callus than in leaf (Table 3). The presence and accumulation of secondary metabolites is affected by the level of cellular differentiation and cellular organization of the tissue (Verpoorte and Memelink 2002; Mahendran and Narmatha Bai 2014). For that reason, the amount and type of phenolic compounds may vary in tissue-cultured leaf and callus of *P. armeniaca*. Some researchers have shown that flavonoids such as apigenin, luteolin, naringenin, kaempferol, eriodictyol, and chryseriol are the dominant secondary metabolites isolated from *Phlomis* species (Limem-Ben Amor *et al.* 2009). Verbascoside, a phenylethanoid glycoside, was also found in some *Phlomis* species such as *P. crinita* (Kabouche *et al.* 2005), *P. carica* (Yalcin *et al.* 2003), *P. sintenisii* (Calis *et al.* 2002), *P. tuberosa* (Ersoz *et al.* 2001), and *P. armeniaca* (Saracoglu *et al.* 1995).

The existence of some phenolic compounds in *in vitro*-grown plant parts of *P. armeniaca* can be beneficial as a basis for future studies with the goal of raising the amount of phenolics in *in vitro*-grown plant parts through the optimization of culture conditions or by applying different stress treatments.

Antioxidant activities of *in vitro*-cultured leaves and callus of *P. armeniaca* The antioxidant activity, TPC, and TFC of *in vitro*-grown leaves and callus of *P. armeniaca* are shown in Table 4. These results demonstrated approximately a twofold higher TPC level in methanolic extract of *in vitro*-grown leaves (66.36 ± 3.01 mg GAE g^{-1} dw and 76.90 ± 7.02 mg TAE g^{-1} dw) than in *in vitro*-grown callus (35.92 ± 2.98 mg GAE g^{-1} dw and 41.39 ± 3.48 mg TAE g^{-1} dw) (Table 4). When TFC values were compared, the methanol extract of *in vitro*-grown leaves contained higher TFC (102.50 ± 3.07 mg CE g^{-1} dw and

Table 4. DPPH radical scavenging activity, total phenolic content (TPC), and total flavonoid content (TFC) of *in vitro*-grown leaves (PAL) and callus (PAC) methanol extracts obtained from *P. armeniaca*

Treatment	IC_{50} DPPH (μg mL^{-1})	TPC (mg g^{-1} dw)^{z}	TPC (mg g^{-1} dw)^{y}	TFC (mg g^{-1} dw)^{x}	TFC (mg g^{-1} dw)^{w}
PAL	4.30 ± 0.08 ^{c}	66.36 ± 3.01	76.90 ± 7.02	102.50 ± 3.07	119.44 ± 3.69
PAC	2.21 ± 0.04 ^{b}	35.92 ± 2.98	41.39 ± 3.48	90.76 ± 1.83	105.35 ± 2.20
Ascorbic acid	2.01 ± 0.04 ^{b}	–	–	–	–
Quercetin	1.10 ± 0.24 ^{a}	–	–	–	–

Values are means ($n = 3$) ± standard deviation. Mean values within a *column* followed by the same *letter* are not significantly different ($P < 0.05$). *Dashes* (–) indicate that TPC and TFC analyses were not performed for the positive controls (ascorbic acid and quercetin). *dw* dry weight

^{z} *GAE* gallic acid equivalents, ^{y} *TAE* tannic acid equivalents, ^{x} *CE* catechol equivalents, ^{w} *QE* quercetin equivalents

119.44 ± 3.69 mg QE g^{-1} dw) than of *in vitro*-grown callus (90.76 ± 1.83 mg CE g^{-1} dw and 105.35 ± 2.20 mg QE g^{-1} dw) (Table 4). *In vitro*-grown leaves of *P. armeniaca* contained higher TPC and TFC than callus, probably due to a tissue structure associated with their growth and developmental period.

Although the methanol extract of callus had lower TPC and TFC than that of the leaves, it had better antioxidant activity in the DPPH assay (IC_{50} = 2.21 ± 0.04 μg mL^{-1}) comparable to the positive reference ascorbic acid (IC_{50} = 2.01 ± 0.04 μg mL^{-1}) (Table 4). The methanol extract of *in vitro*-grown leaves showed a higher IC_{50} value (IC_{50} = 4.30 ± 0.08 μg mL^{-1}) (lower antioxidant activity) than that of callus in the DPPH assay, which means that high total phenolic and/or flavonoid content is not the main reason for the antioxidant activity of the callus methanol extract of *P. armeniaca*. There was no apparent correlation between TPC, TFC, and antioxidant activity of the methanol extract of *in vitro*-grown leaves and callus in the present study. Moreover, Babbar *et al.* (2011) similarly demonstrated that phenolic molecules alone are not completely responsible for the antioxidant activity of plants. Other secondary metabolites such as tocopherols, terpenes, ascorbates, carotenoids, and pigments as well as the synergistic effect among them could possibly contribute to the total antioxidant activity (Fernandes de Oliveira *et al.* 2012).

Conclusions

The present study generated the first micropropagation protocol for the endemic medicinal plant *P. armeniaca*, which is consumed as an herbal tea in traditional medicine. A significant effect of 0.25 mg L^{-1} TDZ + 0.25 mg L^{-1} IAA was observed in shoot and callus proliferation. This protocol can be beneficial for the conservation of this endemic medicinal plant and for future biochemical, pharmacological, and molecular studies in *P. armeniaca*. *In vitro*-propagated plant materials (leaves and callus) of *P. armeniaca* have a high content of certain pharmaceutical molecules and a high antioxidant activity similar to the positive control ascorbic acid. Therefore, future studies are required to define which phenolic compounds are responsible for the free radical scavenger activity of *P. armeniaca*, and to evaluate the way in which the phenolic compounds promote high antioxidant activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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