

# The extracts from *Panax quinquefolium* shoots derived from somatic embryos accumulate ginsenosides and have the antioxidant properties

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Received: 12 April 2015 / Accepted: 15 October 2015 / Published online: 4 November 2015 / Editor: David Duncan  
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**Abstract** The present study was carried out to obtain somatic embryo-derived shoot cultures of *Panax quinquefolium* (American ginseng) and to examine the ginsenoside and total phenolic content (TPC) of these cultures. HPLC analysis revealed that the levels of six ginsenosides measured in shoot cultures totaled 9.76 mg g<sup>-1</sup> DW. TPC content was investigated using the Folin–Ciocalteu method and interpreted as gallic acid equivalents (GAE). The extracts from shoot cultures contained twice the level of TPC (12.75 mg GAE g<sup>-1</sup> of extract) found in aerial parts of field-cultivated *P. quinquefolium* (6.36 mg GAE g<sup>-1</sup> of extract). Antioxidant activity obtained from various plant materials (embryogenic callus, *in vitro*-grown shoots and aerial parts of field plants) was evaluated using two *in vitro* assays: ABTS (scavenging of free radicals) and ferric reducing antioxidant power (FRAP). The methanolic extracts from somatic embryo-derived shoots demonstrated 43% more antioxidant activity than did the extracts from the aerial parts of *P. quinquefolium* plants from field cultivation.

**Keywords** *Panax* · Ginsenosides · Antioxidant activity · Shoot cultures

## Introduction

*Panax quinquefolium* (American ginseng) is a dicotyledonous plant belonging to the Araliaceae family and is one of several known species of ginseng, the others being *P. ginseng*, *P. notoginseng*, and *P. vietnamensis*. These species are commonly cultivated and used for therapeutic purposes such as improving physical and mental efficiency, stimulating cell metabolism, and acting on the nervous, cardiovascular, and immune systems (Yuan *et al.* 2010). Ginseng has also demonstrated anticancer, antiobesity, and radioprotective properties (Xie *et al.* 2007; Lee *et al.* 2010).

The main active compounds of ginseng, ginsenosides, are responsible for most of its medicinal activity. Ginsenosides are triterpene saponins that can be divided into two major groups: 20(S)-protopanaxadiol derivatives (Rb group) and 20(S)-protopanaxatriol derivatives (Rg group). Metabolites Rb1, Rb2, Rc, and Rd belong to the Rb group; Rg1 and Rg2 belong to the Rg group. Ginsenosides are present not only in the roots, which are recorded as a source in many pharmacopoeias, but also in the aerial parts of the plant. For example, ginseng leaf appeared as a new crude drug in the 2005 version of China Pharmacopoeia (Pharmacopoeia of the People's Republic of China 2005) and is used as a tea or a component of dietary supplements in the Western market. Moreover, ginseng leaves are added into skin-care products and cosmetics due to their antioxidant properties (Zhang *et al.* 2014). However, obtaining ginseng from field cultivation is problematic because it takes 3–4 yr and is expensive. An alternative source of ginseng secondary metabolites may be *in vitro* shoot culture. Previous research on *in vitro* shoot cultures of *P. quinquefolium* focused on the multiplication and germination of somatic embryos and their further development (Tirajoh *et al.* 1998; Zhao and Brown 2006; Uchedu *et al.* 2011). Meanwhile, reports on the content of secondary metabolites in somatic embryo-derived

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plantlets of ginseng are limited, with most studies of antioxidant capacity and the phenolic content of ginseng confined to reports on field-grown plants. (Jung *et al.* 2005; Zhang *et al.* 2014). Therefore, the present study was intended not only to obtain somatic embryo cultures of *P. quinquefolium* that were capable of creating shoots but also to determine the levels of ginsenosides and polyphenolic compounds and their antioxidant activity in these cultures.

## Materials and Methods

**Plant materials. Callus and regenerated shoot cultures.** Callus cultures of *P. quinquefolium* were initiated from the epicotyl of seedlings obtained from plants grown on a plantation of the University of Agriculture in Lublin (Poland). The seedlings were washed under running water with detergent, disinfected using 1% (v/v) sodium hypochlorite for 10 min, and cut into 1-cm pieces. Aseptic pieces of epicotyl were rinsed three times in sterile distilled water. The medium for callus initiation was composed of MS (Murashige and Skoog 1962) basal medium containing 3% (w/v) sucrose (Avantor™ Performance Materials, Gliwice, Poland), 0.7% (w/v) agar (Difco™ Bacto™ Agar; Sigma-Aldrich®, St. Louis, MO), and a combination of auxins (1 mg L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid [2,4-D] and 1 mg L<sup>-1</sup> 1-naphthaleneacetic acid [NAA]) and cytokinin (0.2 mg L<sup>-1</sup> 6-benzylaminopurine [BAP]) (all from Sigma-Aldrich®). Medium was sterilized for 25 min at 121°C after the medium pH was adjusted to 5.8.

The callus cultures were placed in plant culture tubes (19 × 2.5 cm, containing 25 mL of medium), at 26 ± 2°C, in the dark. The calluses were transferred into fresh medium every 5 wk. The developed embryoid structures were transferred to semi-solid half-strength MS medium supplemented with gibberellic acid (GA<sub>3</sub>; 0.5, 1, or 10 mg L<sup>-1</sup>) (Sigma-Aldrich®) and either BAP (0.5 or 1 mg L<sup>-1</sup>) or kinetin (kin; 1 mg L<sup>-1</sup>) (Sigma-Aldrich®). Shoot regeneration was observed after 4 wk of culture, and these shoots were rooted in semi-solid half-strength B5 medium (Gamborg *et al.* 1968) with 0.2 mg L<sup>-1</sup> 3-(benzo [b] selenyl) acetic acid (BSAA, Thermo SCI Acros Organics™, Geel, Belgium), 2 mg L<sup>-1</sup> indole-3-acetic acid (IAA) (Sigma-Aldrich®), and 50 g L<sup>-1</sup> sucrose. The cultivation of cultures during regeneration and the rooting of shoots took place in plant culture tubes under continuous light (40 μm m<sup>-2</sup> s<sup>-1</sup>) (white light, fluorescent lamp, Polamp, Pabianice, Poland), at 26 ± 2°C.

**Shoots from field cultivation.** The aerial parts of 4-yr-old plants from field cultivation were obtained from the plantation of the University of Agriculture in Lublin. Fresh plant material was dried on absorbent paper at room temperature and then processed (described below) to estimate antioxidant ability and ginsenoside content.

**Determination of ginsenosides and total phenolic content.** The procedures for extraction and quantitative analysis of ginsenosides were described in a previous report (Kochan *et al.* 2014). The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent according to Singleton and Rossi (1965). Briefly, a 400-μL extract of each sample was oxidized with 2 mL of Folin–Ciocalteu reagent (Avantor™ Performance Materials). This reagent had been previously diluted tenfold with distilled water. After 5 min at room temperature, the reaction was neutralized with 1.6 mL of 7.5% sodium carbonate. The mixture was then incubated for 30 min at room temperature. The absorbance of the resulting blue color was measured at 765 nm on a Rayleigh UV-1601 spectrophotometer (Beijing Reyleigh Corp., Beijing China) versus a blank sample. TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract. The calibration curve was obtained by preparing gallic acid solution at concentrations ranging from 1 to 400 mg L<sup>-1</sup>. The results are presented as means of triplicate analyses.

**Determination of antioxidant activities. ABTS (radical scavenging assay).** The antioxidant activity was determined using the ABTS radical cation decolorization test according to Re *et al.* (1999). An ABTS stock solution contained 7 mM 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 2.45 mM potassium persulfate (both from Sigma-Aldrich®). This mixture was kept in the dark at room temperature for 12–16 h. Then, 2 mL of ABTS solution was mixed with 2 mL of methanol and the absorbance was measured as a negative control (determined to be 0.7 ± 0.05 at 734 nm). The assay was performed by adding 2 mL of extract at concentrations of 2, 20, 100, 200, and 500 μg mL<sup>-1</sup> to 2 mL of ABTS solution. The absorbance at 734 nm was measured after 10 min. The results were expressed as EC<sub>50</sub> (μg mL<sup>-1</sup>): the concentration of the sample at which 50% maximum scavenging activity was recorded. Additionally, the results were calculated as (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma-Aldrich®) micromolar equivalents per gram of dry extract using a calibration curve obtained by measuring a Trolox standard at concentrations from 1 to 20 μM.

**Ferric reducing antioxidant power (FRAP) assay.** The FRAP assay is a method of measuring the ability of reductants (antioxidants) to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup>. The formation of blue Fe<sup>2+</sup>-TPTZ complex (Fe<sup>2+</sup>-2,4,6-tripyridyl-s-triazine) increases absorbance at 593 nm. The method of Pulido *et al.* (2000) was used with some modifications. The FRAP reagent was freshly prepared by mixing 2.5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl), 2.5 mL of FeCl<sub>3</sub>·6H<sub>2</sub>O solution (20 mM), and 45 mL of acetate buffer (300 mM at pH 3.6). The FRAP reagent (3 mL) was added to 300 μL redistilled water and 100 μL of the sample methanol extract.

The reaction mixture was incubated at 37°C for 15 min. After this time, the absorbance was measured at 593 nm relative to a blank: a sample containing methanol instead of extract. The antioxidant potential of the sample was determined against a standard  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  value calculated from a calibration curve plotted with concentrations from 0 to 2000  $\mu\text{M}$ .

**Statistical analysis.** All treatments were performed in triplicate. Data were analyzed using the Kruskal–Wallis test. Differences between values were considered significant at  $p \leq 0.05$ . Statistica Version 10 software was used for all statistical analyses (StatSoft [www.statsoft.com/Products/STATISTICA-Features/Version-10](http://www.statsoft.com/Products/STATISTICA-Features/Version-10)).

## Results and Discussion

**Development of embryoids and regenerated shoots.** The pieces of sterile epicotyl derived from seedlings placed in MS medium with the addition of 1  $\text{mg L}^{-1}$  2,4-D, 1  $\text{mg L}^{-1}$  NAA, and 0.2  $\text{mg L}^{-1}$  BAP were covered with callus tissue after 2 wk of culture. Initially, the callus tissue was opaque, light-yellowish, and friable, without any structures visible under macroscopic observation. After three subcultures, spherical structures developed spontaneously on the surface of the callus. Spontaneous development of embryoid structures was also observed by Tang (2000) and Choi *et al.* (1997) for other species of ginseng. These authors suggest that the presence of 2,4-D in the medium induces the formation of high-quality somatic embryos of *P. ginseng* (Tang 2000) and *Acanthopanax koreanum* (Choi *et al.* 1997). In the present study, the embryoid structures of *P. quinquefolium* initially appeared as small yellowish-white globular masses (Fig. 1). The frequency of spherical structure formation was 65%, and the competence for structure formation increased with the age of the culture, whereas Tirajoh *et al.* (1998) showed lower frequencies (40, 30, and 15.6%) of somatic embryo formation on calluses derived respectively from roots, mature leaves, and seedling-leaf explants of American ginseng. Similarly, Punja *et al.* (2004) observed that the type of explant influenced callus development and formation of embryonic structures. These authors stated that 11 lines of American ginseng calluses, which derived from 11 seed sources from different geographic regions and were incubated in MS medium containing 10  $\mu\text{M}$  NAA and 9  $\mu\text{M}$  2,4-D, significantly differed in their formation of embryonic structures.

Most (91%) of the spherical structures of *P. quinquefolium* cultivated in MS medium with the addition of 1  $\text{mg L}^{-1}$   $\text{GA}_3$  and 0.5  $\text{mg L}^{-1}$  BAP regenerated into multiple or single shoots, frequently without roots. Some of the shoots displayed an abnormal morphology: they were slender and elongated, sometimes hyperhydric and with an abnormal shape to the lamina. The shoots with normal morphology were rooted; of

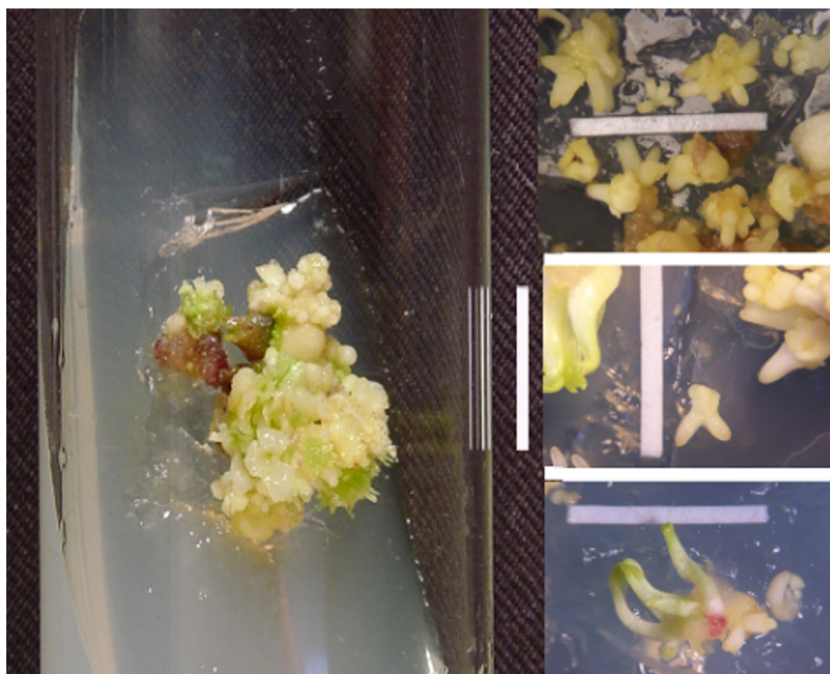
these, only 11% (17 shoots) developed roots. Numerous previous reports indicated that the development of embryoids was sometimes abnormal and that plantlets regenerated from such embryoids lacked roots or had a poorly developed root system (Wang 1990; Choi *et al.* 1998; Tirajoh *et al.* 1998). Choi *et al.* (1998) suggested that the inability of regenerated plants to form roots might be related to embryonic structural abnormalities within morphologically abnormal embryoids (*e.g.*, multicotyledonary or multiple embryoids). In contrast, Zhao and Brown (2006) reported that about 85% of germinated embryos of *P. quinquefolium* converted into plants with well-developed taproot systems and appeared normal when transplanted to growth chambers and field plots. According to that study, such results were possible through the use of higher concentrations of  $\text{GA}_3$  (10–20  $\text{mg L}^{-1}$ ) in the germination medium and further development of shoot cultures in half-strength SH basal medium supplemented with 0.5% charcoal.

**Ginsenoside content.** Saponin production was studied in somatic embryo-derived shoots after 4 wk of cultivation as well as in the aerial parts of 4-yr-old *P. quinquefolium* plants cultivated in the field. The somatic embryo-derived shoots contained a total of nearly 9.8  $\text{mg g}^{-1}$  DW of the six measured saponins (Table 1 and Fig. 2). Wang *et al.* (1999a) reported a lower level (6.6  $\text{mg g}^{-1}$  DW) of total ginseng saponins from embryo-derived plantlets of *P. quinquefolium*, whereas Asaka *et al.* (1994) reported higher levels (18.78–21.35  $\text{mg g}^{-1}$  DW) from such plantlets of *P. ginseng*. The present study indicated that the aerial parts of field-grown *P. quinquefolium* contained about 13  $\text{mg g}^{-1}$  DW of the six measured saponins. Kim *et al.* (2014b) presented comparable levels (10.92  $\text{mg g}^{-1}$  DW) of ginsenosides in the leaves of 3-yr-old plants of *P. ginseng* from hydroponic cultures. By comparison, ginsenoside accumulation in callus was significantly lower (3.26  $\text{mg g}^{-1}$  DW; Fig. 2).

The ratio of protopanaxadiol derivatives to protopanaxatriol derivatives (Rb group/Rg group ratio) is used as an indicator for qualitative evaluation of the ginseng material. In methanolic extracts obtained from *in vitro* cultures, the ratios of Rb group to Rg group ginsenosides were 1.67 and 3.44, respectively, for somatic embryo-derived shoots and callus cultures. Rb group saponins constituted about 66.3% of the total amount of examined ginsenosides from those cultures. In contrast to these results, the ratio of Rb group to Rg group saponins in the aerial parts of field-cultivated plants was 0.78. Protopanaxatriol derivatives (Rg group) quantitatively predominated in these extracts (Fig. 2). Several reports (Asaka *et al.* 1994; Wang *et al.* 1999a; Kim *et al.* 2014b) showed that the Rb group/Rg group ratio in extracts from plantlets derived from somatic embryos as well as in leaves of field-grown plants was  $< 1$ .

The contents of six individual saponins (Rb1, Rb2, Rc, Rd, Re, and Rg1) were also compared (Table 1). Their relative

**Fig. 1** The development of somatic embryos of *P. quinquefolium*.



levels differed in *in vitro* shoot cultures and the aerial parts of field-grown *P. quinquefolium*. Ginsenosides Rb1 and Re predominated quantitatively in shoots regenerated from embryoids, and their levels were similar, each about  $3 \text{ mg g}^{-1} \text{ DW}$ . The amount of metabolite Re in regenerated shoots was about ninefold that of Rg1 saponin, another ginsenoside belonging to the Rg group. Wang *et al.* (1999a) noted that metabolite Rd dominated quantitatively among Rb group derivatives in somatic embryo-derived plantlets, and Asaka *et al.* (1994) reported that the three ginsenosides they examined, Rb2, Rc, and Rd, were present in larger quantities in plantlets regenerated from somatic embryoids of *P. ginseng* than were callus and embryoid cultures. These reports (Asaka *et al.* 1994; Wang *et al.* 1999a) confirm that Re ginsenoside is the main component of the derivatives in the Rg group.

Metabolite Rb2 was found to be the most plentiful saponin of the Rb group in the aerial parts of field plants (Table 1). The levels of Rb1, Rc, and Rd ginsenosides were significantly lower than in the shoots obtained from *in vitro* cultures. Metabolite Re predominated quantitatively and represented almost 50% of the total examined saponins,

and the Rg1 content in the aerial parts was higher than that observed in *in vitro* cultures. Searels *et al.* (2013) and Li *et al.* (2012) reported Rd, Re, and Rg1 to be the most prevalent saponins in Korean ginseng leaves regardless of the age of the plants.

A comparative analysis of data described in the literature and presented in this paper reveals significant differences in the contents of total and individual saponins in shoots or plantlets regenerated from somatic embryoids as well as in the leaves or the aerial parts of ginseng plants cultivated in the field. This may be due to several factors, such as the species of ginseng, growing conditions (field cultivation or *in vitro* culture), and the type of explant from which the callus or somatic embryoids are derived.

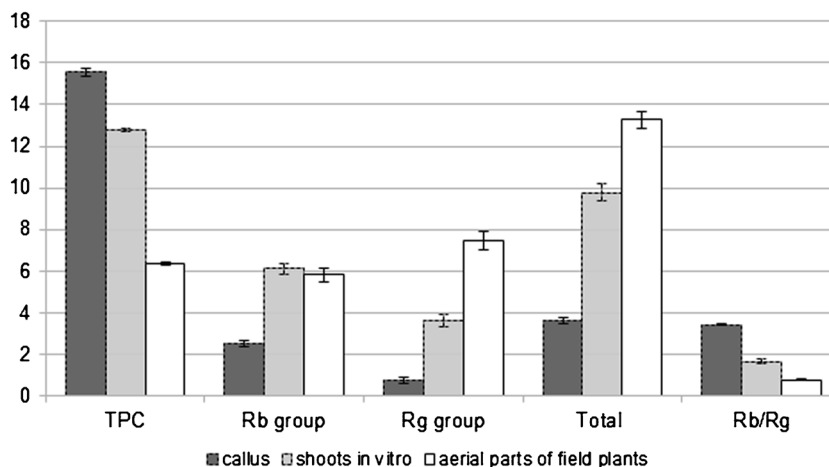
**Total content of phenolics.** TPC was measured in the callus, the shoots regenerated from somatic embryo and the aerial parts of field-cultivated plants by using the Folin–Ciocalteu method. As shown in Fig. 2, TPC for shoot cultures was  $12.75 \text{ mg GAE g}^{-1}$  of extract, whereas the value for the aerial

**Table 1** Ginsenoside in *P. quinquefolium* derived from *in vitro* culture and field cultivation

	Ginsenosides [ $\text{mg g}^{-1} \text{ DW}$ ] $\pm$ SD					
	Rb1	Rb2	Rc	Rd	Re	Rg1
Embryogenic callus	$1.1 \pm 0.352a$	$1.007 \pm 0.097a$	$0.226 \pm 0.06a$	$0.193 \pm 0.029a$	$0.565 \pm 0.076a$	$0.169 \pm 0.019a$
Shoots	$3.065 \pm 0.950b$	$0.941 \pm 0.164a$	$0.787 \pm 0.248b$	$1.316 \pm 0.260b$	$3.276 \pm 0.497b$	$0.373 \pm 0.018b$
Aerial part field plant	$1.776 \pm 0.655a$	$3.165 \pm 0.346b$	$0.432 \pm 0.162c$	$0.458 \pm 0.035a$	$6.311 \pm 0.932c$	$1.148 \pm 0.09c$

The values are means $\pm$ SE. The means with the same *letter* do not differ significantly according to the Kruskal–Wallis test ( $p \leq 0.05$ )





**Fig. 2** Ginsenoside and total phenolic content (TPC) levels in *in vitro*-derived and field-grown *Panax quinquefolium*. *Rb group* contains protopanaxadiol derivatives (Rb1, Rb2, Rc and Rd). *Rg group* contains protopanaxatriol derivatives (Rg1 and Re). *Total* contains all six

ginsenosides. *Rb/Rg* is the ratio of *Rb group* contents to *Rg group* contents. Data are presented as milligrams per gram of DW, except for TPC (mg GAE g<sup>-1</sup>) and *Rb/Rg* (dimensionless). Bars indicate mean ± SD.

parts of field-cultivated *P. quinquefolium* (6.36 mg GAE g<sup>-1</sup> of extract) was half that amount. These TPC levels were, respectively, four and two times the levels in ginseng root reported by Hwang *et al.* (2010).

A comparative analysis of TPC and total saponin contents showed that their levels were inversely related. Higher TPC levels were associated with lower total ginsenoside levels; conversely, lower TPC levels corresponded to higher total ginsenoside levels (Fig. 2). Similarly, protopanaxatriol derivative (*Rg group*) content was inversely related to both *Rb group*/*Rg group* ratio and TPC content of the studied extracts (Fig. 2).

**Antioxidant activities.** The antioxidant potentials of methanolic extracts of *P. quinquefolium* shoots regenerated from somatic embryos were compared with those of the aerial parts of field-cultivated plants. Two methods based on different reaction mechanisms, the FRAP and ABTS assays, were used to measure antioxidant activity (Table 2). In the FRAP assay, the extracts of callus tissue demonstrated the strongest antioxidant capacity (181 μmol Fe (II)g<sup>-1</sup> DW of extract). Furthermore, the shoot cultures had 43% more Fe-reducing ability than the aerial parts had. The ABTS assay (Table 2) also showed the methanolic extract of shoots regenerated from somatic embryoids to be a more potent scavenger than that from the aerial parts of field-grown plants. However, it should be noted that the effectiveness of the extracts as antioxidants was relatively low in comparison to those of well-known products such as blueberries or blackberries (respectively, 257 and 344 μmol Fe (II)g<sup>-1</sup> DW; Henríquez *et al.* 2011) or cranberry powder (242–319 μmol Fe (II)g<sup>-1</sup> DW; Kim *et al.* 2014a). On the other hand, the antioxidant properties of the extracts described in this paper were stronger than those of other

medicinal plants such as *Aloe vera* (for fresh leaf extract, 37 μmol Fe (II)g<sup>-1</sup> DW) or *Coriandrum sativum* (for fruit extract, 23 μmol Fe (II)g<sup>-1</sup> DW) (Guleria *et al.* 2013).

The results reported here demonstrated a relationship between antioxidant activity and total phenolic level: the extracts found to have higher antioxidant activity, as confirmed by both FRAP and ABTS assays, were those with a higher TPC (Table 2 and Fig. 2). This observation was not surprising and has been described for other species (Henríquez *et al.* 2011; Guleria *et al.* 2013; Kim *et al.* 2014a). On the other hand, an inverse correlation was found between antioxidant capacity and the amount of total saponins. These results differed from those of Zhang *et al.* (2014), who showed a strong correlation between antioxidant capacities and the contents of ginsenosides in leaf extracts from 1- to 13-yr-old ginseng samples.

**Table 2** Antioxidant abilities of methanolic extracts of *P. quinquefolium* derived from *in vitro* culture and field cultivation

	FRAP <sup>a</sup>	ABTS	
		EC <sub>50</sub> <sup>b</sup>	TEAC <sup>c</sup>
Embryogenic callus	180.58±0.90 <sup>a</sup>	178.26±2.34 <sup>a</sup>	59.05
Shoots <i>in vitro</i>	108.28±1.27 <sup>b</sup>	182.36±1.83 <sup>a</sup>	59.16
Aerial part of field plant	75.77±2.18 <sup>c</sup>	220.26±1.99 <sup>b</sup>	49.05

The values are given as means ± SE. The means with the same letter do not differ significantly according to the Kruskal-Wallis test ( $p \leq 0.05$ )

<sup>a</sup> Ferric reducing antioxidant power expressed in μmol Fe (II)g<sup>-1</sup> extract DW, 15 min of incubation

<sup>b</sup> EC<sub>50</sub> the concentration of sample [mg ml<sup>-1</sup>] showing 50% of maximal radical scavenging activity

<sup>c</sup> Micromolar trolox (TEAC) equivalent antioxidant capacity expressed in micromolar standard equivalents per gram of extract DW

## Conclusions

Embryogenic callus and somatic embryo-derived shoot cultures were obtained from *P. quinquefolium*. In the studied extracts, higher total saponin levels, higher protopanaxatriol (Rg group) derivative content, and Rb group/Rg group ratio corresponded to lower TPC levels. Stronger antioxidant activities (according to both FRAP and ABTS) were observed in extracts with a higher total phenol content. On the other hand, saponin content was inversely related to the free-radical scavenging capacity of the studied extracts.

**Acknowledgments** The project was financed by the Medical University of Lodz from research grant no. 502-13-754 and by the State Committee for Scientific Research grant no. 3PO5F01523.

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