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Growth of *Leonurus sibiricus* L. roots expressing the AtPAP1 transcriptional factor in closed bioreactor, production of bioactive phenolic compounds and evaluation of their biological activity

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Abstract

Fast-growing transgenic root cultures of *Leonurus sibiricus* transformed with the AtPAP1 transcriptional factor may be used as a base for potential large-scale phenolic acid production. The present study investigates the effect of different volumes of medium (300 mL, 1 L, 3 L, 5L flasks) and a 5 L bioreactor on biomass increase and phenolic acid production by transgenic roots of *L. sibiricus*. Of these cultures, those

from the 5 L bioreactor demonstrated the greatest increase in dry weight (20.83g/L) and highest yields of phenolic acids (chlorogenic acid 448 mg/L and caffeic acid 302 mg/L). Additionally, they also displayed a cytotoxic effect on melanoma cells across the range of tested concentrations, as well as antioxidant activity on human blood plasma. This approach may serve as an alternative to conventional field crops for enabling large-scale production of the active constituents of AtPAP1 root extract, which would be of great value for pharmaceutical production.

Keywords: Fresh and dry weight, bioreactor, AtPAP1transgenic roots of *Leonurus sibiricus*, phenylopropanoid pathway genes expression, antioxidant activity, cytotoxic effect

1. Introduction

Leonurus sibiricus L. belongs to the Lamiaceae family. It is an aromatic, herbaceous plant, which can be annual, bisannual or perennial. Most significantly from a pharmacological perspective, the species has been reported to have a wide range of anti-inflammatory, antioxidant, anti-diabetes, anti-bronchitis and anti-cancer effects (Sayed et al., 2016; Oliveira et al.,2017; Sitarek et al., 2017a; Sitarek et al., 2017b; Sitarek et al.,2016a). The present study describes a procedure to increase the production of secondary metabolites from the plants based on genetic manipulation. Previous studies describe the successful transformation of *L. sibiricus* roots by *Agrobacterium rhizogenes* infection (Sitarek et al., 2016b) and note that the hairy root cultures obtained have a different secondary metabolite content to untransformed roots (Sitarek et al., 2016b). Given the improved pharmacological content of hairy roots and the rich variety of beneficial compounds in *Leonurus sibiricus*, the species represents a good model for improving the production by refining biotechnology techniques (Sitarek et al., 2016b).

In the last two decades, genetic transformation and metabolic engineering have become powerful tools for transferring new genes into plants. This approach offers an attractive alternative to conventional breeding, because specific traits can be transferred into selected genotypes without adverse effects on desirable genetic backgrounds (Raghavendrarao et al.,2017; Rivera et al., 2012). Furthermore, they offer tremendous potential to modify, improve and enhance the production of various compounds. One such factor which can enhance the production of phenolic acids is AtPAP1 (*Arabidopsis thaliana* transcription factor) (Qui et al., 2014; Zhang et al., 2010). One effective approach to engineering high levels of bioactive compounds is by simulating the overexpression of the factors that regulate the transcription of the genes involved in the phenylpropanoid pathway (*PAL, 4CL, C4H*) (Anh Tuan et al ., 2014). The use of transgenic root cultures provides novel opportunities for the production of valuable phytochemicals synthesized in roots. They are genetically stable, they can accumulate biomass in a culture system and they are able to grow in hormone-free culture media (Srivastava and Srivastava, 2007). Moreover, they demonstrate rapid growth and

promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Bannerjee et al., 2012). Our earlier studies revealed that transgenic roots of *L. sibiricus* with AtPAP1 transcriptional factor produce higher levels of phenolic acids than roots without any such construct; in addition, they showed better biological properties Sitarek et al., 2018). However, despite these successes, no commercial process currently exists for the production of phenolic acids by transgenic roots. There remains a need to increase the rates of phenolic acid production for commercial exploitation.

One of the methods for increasing production is by the use of a bioreactor (Sivakumar 2006). In a biochemical context, bioreactors are defined as self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems. The use of bioreactors may be an effective way for increasing the production of transformed roots and their secondary metabolites to an industrial scale: many studies have discussed such upscaling (Wu et al. 2007; Kusakari et al., 2012). However, their use can be complicated by the continuous growth of the transformed roots. The bioreactor must have a special configuration to compensate for the heterogeneous, structured and entangled nature of fibrous roots (Sivakumar 2006).

The current study presents the first analysis of the phenolic acid content of the transgenic roots of *Leonurus sibiricus,* and their productivity, following transformation with a AtPAP1 transcription factor, while examining the effects of using various media volumes and bioreactor sizes that may be suitable for commercial-scale production. It also evaluates the expression of some of the genes involved in the phenylpropanoid pathway and determines the antiproliferative activity of the transgenic root extract in human melanoma cells and its antioxidant effect on human plasma.

2. Materials and methods

2.1 Establishment and confirmation of L. sibiricus transgenic root culture with transcriptional factor AtPAP1

The AtPAP1 root culture was established as described previously (Sitarek et al., 2018). The confirmation of the AtPAP1 root transgenic itself (Sitarek et al., 2018).

2.2 Plant material and extract preparation

Lyophilized and powdered AtPAP1 root was used for analysis (10 g d.w.). The material was subjected to extraction in an ultrasonic bath for 15 minutes with 80% (v/v) aqueous methanol (500 mL) at 35°C, followed by two further 15-minutes extractions with 300 mL 80% (v/v) aqueous methanol. The root extract was filtered, combined and evaporated under reduced pressure and then lyophilized. It was kept dry and stored in the dark until further use. The yield (w/w) was 50.25% for the AtPAP1 extract (Sitarek et al., 2018).

2.3 Determination of phenolic acids by HPLC analysis

LC-MS/MS was used to identify the phenolic acids. The content of the phenolic acids in the AtPAP1 root extract was determined by HPLC according to our earlier studies (Sitarek et al., 2018).

2.4 Growth of AtPAP1 transgenic roots of L. sibiricus culture in various volumes of medium

To determine the effect of growth volume, 6 g/L f.w. of *L. sibiricus* AtPAP1 transgenic roots were cultured in full strength SH medium in four Erlenmeyer flasks of different volume: a 300 mL flask containing 80 ml of medium, a 1L flask with 500 mL medium, a 3L flask with 1.5L medium and a 5L flask with 2.5 L medium. No phytohormone was added to any of the cultures. The AtPAP1 transgenic root cultures were kept on a rotary shaker at 75 rpm and incubated at 25 ± 2ºC. The fresh and dry weights of AtPAP1 transgenic roots were measured after 30 days of culture. The data was recorded as mean mass of three transgenic root cultures after each incubation period.

2.5 Scaling up the AtPAP1 transgenic root culture in the bioreactor

An attempt was made to scale up the transformed *L. sibiricus* roots to grow in a glass and stainless steel 5L bioreactor consisting of two containers: a main container (volume 5 L) in which AtPAP1 root growth took place, and an auxiliary container (volume 1.5 l) serving as a reservoir for the nutrient medium [liquid SH medium supplemented with 3% (w/v) sucrose)]. The medium (1L of SH medium) was supplied through with a polypropylene spray nozzle using a peristaltic pump which operated in bursts of 40s, supplying 75 ml of medium, separated by 1.5 minute breaks. The nozzle was situated at the bottom of the growth chamber; from here it provided the nutrient medium to the AtPAP1 roots, which were supported on a stainless steel wire mesh (with 10 mm pore size), situated 18 cm above the bottom. A more detailed description of the bioreactor configuration and operation is given by Chmiel et al,. 2001. During the experiment, an autoclavable nylon mesh was tightened just beneath the medium surface to prevent the roots from sinking to the bottom or becoming submerged in the medium during the growth phase. The entire vessel was divided into lower and upper compartments by a mesh septum. The sterile culture vessel was inoculated under aseptic conditions. The vessel was inoculated with 0.6 g/L of transgenic AtPAP1 root. The experiment duration was 30 days and cultures were incubated at 25 ± 2ºC. Growth was measured as fresh and dry weight.

2.6 RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA from the transgenic roots was isolated with Syngen Plant RNA MINI Kit reagent First strand cDNA synthesis was performed using a TranScriba kit (A&A Biotechnology, Poland) in a 20 µl reaction mix according to the manufacturer's protocol. Quantitative real-time PCR analysis was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using a RT-PCR Mix SYBR® (A&A Biotechnology, Poland). The following Primer sequences were used : *PAL* F-5'ACC TAC CTC GTC GCC CTA TGC3' and R-5'CCA CGC GGA TCA AGT CCT TCT3'; *C4H* F-5'CCA GGA GTA CCA TTG ACA GAG CC3' and R-5'CAG CCA CCA AGC GTT CAC CAA3'; *4CL* F-5' CTT TGC CAA AGA

GCC ATT CGA G3' and R-3'CTT TCT GTG GCC TCT GGA TCA T. Briefly, each reaction was performed in a 25 µL mix containing RT-PCR SYBR® Master Mix (10µL), 0.5 µL of each primer (10 µM), 1 µL of cDNA and water. The elongation factor 1α (*EF-1α*) gene was chosen as an internal control for normalization (Primers F-5'-TGAGATGCACCACGAAGCTC- 3'and R- 5'-CCAACATTGTCACCAGGAAGTG -3').

The potential amino acid sequences for *PAL*, *4CL* and *C4H* were analysed for homology by BLAST searches of the NCBI GenBank database (http://www.ncbi.nlmnih.gov/BLAST). The PCR procedure was performed under the following conditions: five minutes at 95˚C, followed by 35 cycles of 95˚C at 30 s, 60-65 ˚C for each gene at 30 s and 72˚C at 60 s. Each sample was analysed in triplicate. In the melting curve analysis, the levels of the genes were normalized to that of the elongation factor 1α (*EF-1α*) gene used to test the specificity of amplification. The expression of the genes was calculated by the comparative C_t method (Schmittgen and Livak 2008).

2.7 Antiproliferative activity of AtPAP1 roots extracts in human melanoma cells

The antiproliferative activity of AtPAP1 root extract on melanoma cells was studied in the A2058 (ATCC® CRL-11147) cell line. A2058 are highly invasive human epithelial adherent melanoma cells, derived from lymph node metastatic cells obtained from a 43-year-old male patient. They are tumorigenic at 100% frequency in nude mice, and considered as very resistant to anticancer drugs. All cell culture experiments were performed at 37 °C. Cells were grown to confluence in 75 cm² flasks in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Dominique Dutscher, France), in a 5% CO_2 humidified atmosphere. Extracts were solubilized in DMSO at 5 $mg.mL^{-1}$ and diluted to concentrations of 5 to 80 µg mL^{-1} in cell culture medium. The confluent cells were trypsinized and centrifuged in FCS at 1500 *g* for five minutes. The supernatant containing trypsin was discarded and the cell pellet was suspended in cell culture medium to obtain a 4.10^4 cell mL^{-1} suspension. Following this, 50 µL of the extracts were deposited in a 96-well flat-bottom microplate, and 50 µL of cell suspension was added. The 2000 cells were then grown for 72 hours in cell culture medium containing 2.5 to 40 $\mu g.mL^{-1}$ of transgenic AtPAP1 root extract (containing 0.05 to 0.8% DMSO). After 72 hours, 30µL of a 5 gL^{-1} MTT solution were added to each well of the microplate and left for four hours, during which time, the living cells containing a functional mitochondrial succinate dehydrogenase were able to metabolize MTT to the corresponding blue formazan salt. The cell culture medium was removed using an Eppendorf epMotion 5070 pipetting robot (Eppendorf, France) and formazan crystals were dissolved in 200 µL DMSO. The microplates were placed at 37 °C for five minutes to solubilize the formazan crystals and absorbance was read at 550 nm using a VERSA max microplate reader (Molecular devices, France). The percentage of growth inhibition was calculated as GI (%) = 100- ((A550 nm sample – A550 nm BG) / (A550 nm control – A550 nm BG)) x 100 with:

- A550 nm sample: median absorbance of eight wells containing cells treated with extracts

- A550 nm BG: median background absorbance of eight empty wells

- A550 nm control: median absorbance of eight wells containing cells grown in control cell culture medium + 1% DMSO.

Data are expressed as GI(%) + sem (%) from three independent assays.

2.8 Isolation of blood plasma and preparation of samples

Blood from healthy volunteers was purchased from the Regional Centre of Blood Donation and Blood Treatment in Lodz, Poland. Plasma samples were preincubated for five minutes at 37˚C with TR and ATPAP1 root extracts at final concentrations of 5, 25 or 50 µg/ml. The control samples were not treated either extract.

2.9 Evaluation of DPPH and ABTS radical scavenging abilities

The anti-free radical activities of the examined TR and AtPAP1 extracts were assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH*) (Janaszewska and Bartosz 2002) and ABTS* (derived from oxidation of 2,200-azinobis-(3-ethylbenzothiazoline-6- sulfonic acid)) reduction assays (Re et al., 1999).

2.10 Measurements of the ferric-reducing ability of plasma (FRAP)

This method was also used for the evaluation of ferric reducing ability of different concentrations of the tested TR and AtPAP1 root extracts of *L. sibiricus* . The obtained results were then calculated from a standard curve, ranging from 0 to 1mM of $FeSO_4$, and expressed as equivalents of Fe^{2+}.

2.11 Statistical analysis

Continuous data was presented as mean with standard deviation (SD). As the data was found to have a non-normal distribution, it was compared using the Mann-Whitney U-test. *P*-values <0.05 were considered as statistically significant. Analyses were performed using STATISTICA software v.13 for Windows (StatSoft Inc).

3. Results

3.1 Biomass accumulation of AtPAP1 transgenic roots grown in shake flasks and 5 L nutrient sprinkle bioreactor

The biomass accumulation of AtPAP1 transgenic roots grown in different liquid volume of SH medium (80 mL, 500 mL, 1.5 L and 2.5 L) (Figure 1 and 2) in flasks and a 5 L nutrient sprinkle bioreactor (in 1 L medium) was determined after 30 days. The increase in fresh root biomass ranged from 71 to 312 g/L for all tested cultures. The greatest final fresh root weight was obtained for the roots from the bioreactor, this biomass being 312 g/L: an approximately 50-fold increase over the initial inoculum (6.0 g/L DW). The

increase of dry root biomass ranged from 4.5 to 20.8 g/L. Again, the highest dry root weight was observed for the roots grown in the bioreactor (20.83g/L) and the 1 L flask (13.2 g/L), these values being about 34- and 22-fold greater than the inoculum biomass (0.6 g/L). In turn, the lowest dry root weight was observed for the roots cultured in the 300 mL, 3 L and 5 L flasks with final values of 4.54, 4.88 and 4.63 g/L, respectively. The biomass of the AtPAP1 transformed roots are described in Table 1.

3.2 Phenolic acid content of the AtPAP1 transgenic roots of L. sibiricus growing in the shake flasks and 5 L nutrient sprinkle bioreactor

The concentrations of five phenolic compounds (neochlorogenic acid, chlorogenic acid, caffeic acid, p-coumaric acid and ferulic acid) in the AtPAP1 transgenic roots of *L. sibiricus* growing in the various shake flasks and 5 L bioreactor were determined by HPLC analysis (Table 2). The results showed that the chlorogenic acid and caffeic acid were produced in the highest amounts (Table 2). The highest levels of the chlorogenic acid were observed in the cultures grown in the 1L flask (23 mg/g DW) and the bioreactor (21.5 mg/g DW). However, the bioreactor root culture also produced higher amounts of caffeic acid (14.5 mg/g DW) than those growing in the flasks (9.2-12.1 mg/g DW). Additionally, the roots cultured in the bioreactor achieved a higher biomass (20 g/L of the dry weight) than the flask cultures. Therefore, the yields of the chlorogenic acid and the caffeic acid (mg per liter of medium) were highest (448 and 302 mg/L) in the bioreactor roots culture (Table 3). The phenolic acid content and the productivity of the AtPAP1 transformed roots are described in Table 3.

3.3 Gene expression in AtPAP1 transgenic roots and TR roots

The study examined the transcript levels of the metabolic genes *C4H, PAL, 4CL* involved in the phenylpropanoid pathway after enhancing the expression of AtPAP1. The expression of the *C4H, PAL, 4CL* genes was greater in the transformed roots than the roots without AtPAP1 transcriptional factor, indicating that the production of phenolic acids in transgenic roots could be enhanced by AtPAP1 transcriptional factor (Figure 3).

3.4 Antiproliferative activity of TR and AtPAP1 transgenic root extracts in melanoma cells

The inhibition of melanoma cell growth was determined after 72-hour incubation with TR and AtPAP1 *L. sibiricus* root extracts in the concentration range 2.5 to 40 µg/mL in cell culture medium (Figure 4). Both extracts were found to exhibit a dose-dependent antiproliferative effect and the AtPAP1 root extract demonstrated a high growth inhibition in the melanoma cell line at 40 $\mu g.mL^{-1}$ (53.3±8.1 %) (Table 4).

3.5 Antioxidant properties of human blood plasma after treatment with TR and AtPAP1 root extracts of L. sibiricus

The free radical scavenging (ABTS and DPPH) and the ferric reducing (FRAP) properties of the examined TR and AtPAP1 root extracts on plasma were demonstrated. Three concentrations were administered: 5, 25, and 50 µg/ml (Figure 5). The DPPH and ABTS radical assays revealed that while both types of *L. sibiricus* extract increased blood antioxidant potential at all examined concentrations (5-50 µg/mL) (Figure 6), the AtPAP1 root extract demonstrated better activity. Similar results were found for the FRAP assay, with AtPAP1 root extract being more effective.

4. Discussion

Plant molecules have greater bioactivity than synthetic drugs, and can be safer and more effective, possibly because human beings have co-evolved with plants over the past few million years Sivakumar 2006; Atanasov et al., 2015). For several decades, plant *in vitro* cultures have been investigated to identify cheap and plentiful sources of plant compounds and pharmaceuticals. In order to increase the production of plant compounds, different techniques of biotechnology and engineering genetics have been used. One such approach is based on obtaining transformed root cultures by infection with *Agrobacterium rhizogenes* (Skała et al., 2015). Many studies have been based on the introduction of various transcription factors involved in metabolic pathways which can be implicated in a wide variety of plant-specific processes, including those associated with secondary metabolism, cell shape determination, cell differentiation and stress responses (Luo et al., 2008; Qiu et al., 2014). The transformed or transgenic root cultures obtained in this way are typically genetically stable, are able to grow in a medium without growth regulators, and lack secondary growth or geotropism (Srivastava and Srivastava 2007). Long-term, stable production of significant amounts of secondary metabolites, including those important from the pharmacological point of view, can be achieved using bioreactors (Sivakumar 2006). Plant bioreactors are attractive expression systems for economical production of bioactive compounds and pharmaceuticals. Various plant expression systems or platforms have been tested with certain degrees of success over the past years (Bannerjee et al., 2012; Sivakumar 2006). Bioreactors are essential components in adventitious and hairy root engineering, not only because they provide an aseptic environment mimicking controlled conditions for the growth of roots, but also because they enable high-tech systematic development of the responses of higher root biomass and biopharmaceutical production (Baque et al., 2012).

The aim of the present study was to compare the production of phenolic acids in transgenic *L. sibiricus* roots treated with an AtPAP1 transcription factor in different volumes of shake flasks (300 mL, 1 L, 3 L, 5 L) and 5 L bioreactor. It also assessed the biological activity of the extract. Our present findings conform previous observations that over-expression of the AtPAP1 transcriptional factor takes place in various

clones of transgenic roots (Sitarek et al., 2018), and that this could be related to activation of the phenylpropanoid pathway genes. They also indicate that the overexpression of *AtPAP1* induced the expression of phenylopropanoid pathway genes including *PAL, C4H, 4CL*. These findings confirm those of Anh Tuan et al.. which note that the introduction of the *AtPAP1* gene dramatically increased the expression levels of all examined biosynthetic genes (*PgPAL1*, *PgPAL2*, *PgC4H*, *Pg4CL*, *PgC3H*, *PgHCT*, and *PgHQT)* in *Platycodon grandiflorum* transgenic hairy roots (Anh Tuan et al., 2014).

Of the different culture vessels, the 5 L bioreactor was found to offer the greatest biomass acquisition (21 DW). The dry weight of the transgenic roots from the 300 mL, 3L and 5L shake flasks was similar, ranging from 4.54 to 4.63 g L^{-1}. In contrast, Du et al found the greatest dry weight accumulation for the culture in the 250 mL shake flask. Similar dry weights were found for the 1 L, 10L and 30L flasks: 9.4 to 11.5 g/L (Du et al., 2003). In the present study, efficient accumulation of secondary metabolites was observed for all volumes and culture vessels; however, the greatest yield of phenolic acids was observed for the transgenic roots cultured in the 5 L bioreactor.

This is the first report to show the content and yield of phenolic acids for *L. sibiricus* cultures in various volumes of shake flasks and a bioreactor. Similarly, Chattopadhyay et al report 27% greater productivity of bioactive compounds (paclitaxel) in a 3 L bioreactor than a shake flask (Chattopadhyay et al., 2002). Similarly, Verma et al report greater secondary metabolite production in a 5 L bioreactor than a shake flask (Verma et al., 2015). Conversely, Kuzma et al note that diterpene production was more efficient in shake flask root culture than the 5 L bioreactor (Kuzma et al., 2017).

The dominant phenolic acids in the *L. sibiricus* transgenic roots were chlorogenic and caffeic acid, with productivity increasing with culture vessel size, the highest productivity being obtained in the 5 L bioreactor: chlorogenic acid (448 mg/L) and caffeic acid (302 mg/L). The greatest accumulation of chlorogenic acid has been reported in *Echinacea purpurea* roots grown in a 1000 L bioreactor (Wu et al., 2007); a considerable increase in chlorogenic acid and caffeic acid accumulation was observed in the pilot-scale 5 L bioreactor with AtPAP1 transgenic root culture of *L. sibiricus* studies used in the present work. These bioactive compounds are reported to be potent antioxidant, anti-inflammatory, anticancer compounds and can be used in nutraceuticals intended for weight loss (Farah et al., 2008).

Our findings suggest that the metabolic engineering of key regulatory enzymes may enable control of nutrient flux to a desired compound or allow the selective control of a desired pathway. This type of genetic engineering, combined with proper reactor design, may ensure the success of large-scale secondary metabolite production.

The biological properties of the extract obtained from the roots grown in the 5 L bioreactor were then tested, this being the highest-yielding culture. It was found to inhibit the growth melanoma cells in low concentrations, which may be attributed to its high phenolic acid content. De Oliveira Melo et al. report that European mistletoe (*Viscum album* L.) extract, which is rich chlorogenic acid, caffeic acid and other phenolic compounds, showed selective tumoral cytotoxicity with apoptosis induction and cell cycle effect

on melanoma cells in a dose-dependent manner (De Oliveira Melo., 2018). Similarly, chlorogenic acid was found to significantly suppressed melanoma cell proliferation (Li et al., 2014), and *M. nigra* leaf extract, rich in chlorogenic acid, rutin and isoquercitrin, induced cytotoxicity on melanoma cells following 24-hour incubation (De Freitas et al., 2016). Similarly, our tested extract was also rich in chlorogenic acid and caffeic acid.

As little is known of the antioxidant properties of root extracts from *L. sibiricus* on human plasma, the present study uses the DPPH, FRAP and ABTS assays to examine the effect of the extracts on plasma. A key novel finding of the present study is that treatment with the extract of the roots with AtPAP1 transcriptional factor increased the antioxidant potential of human plasma. This result may be attributed to its high polyphenolic compound content. Phenolic compounds and flavonoids are secondary metabolites of plants bearing a poly-hydroxyl group, which is known to possess antioxidant activity (Shahidi and Chandrasekara 2010). Our findings are consistent with those of Kolodziejczyk-Czepas et al., who note that Trifolium extracts (contain phenolic acids, flavonoids or isoflavones) also have antioxidant effects on human blood plasma, as identified by ABTS and DPPH radical tests Kolodziejczyk-Czepas et al., 2015a), as do polyphenolic-polysaccharide conjugates isolated from *Matricaria chamomilla*, as determined by the DPPH, ABTS and FRAP tests Kolodziejczyk-Czepas et al., 2015b). The same tests found that *Hedeoma multiflorum* extract, which is rich in polyphenol compounds (0.96 ± 0.08 μmol equivalent caffeic acid/mg dry matter), also has significant antioxidant activity Dade et al., 2011). However, due to insufficient knowledge of biological effects of *L. sibiricus* roots, further studies are needed to realise its great potential as a pharmaceutical compound.

5. Conclusion

Plant material grown *in vitro* is often a cheap and effective system for the production of bioactive compounds. As bioreactor technology can dramatically improve yields, bioreactor root cultures should be used in the nutraceutical industry. Our findings confirm that high phenolic acid production can be achieved from transgenic *L. sibiricus* roots transformed with AtPAP1 transcriptional factor in a 5 L bioreactor, specifically that of chlorogenic acid and caffeic acid. They also confirm that the extract obtained from the most productive culture grown in the bioreactor axerts an antiproliferative activity in human chemoresistant melanoma cells. Finally, the tested extract was found to demonstrate potent antioxidant potential for human blood plasma.

Declaration of interest

All authors declare that they do not have any conflict of interest that could inappropriately influence this manuscript.

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References

Sayed, A., Alam, A., Islam, S., Ali, T., Ullah, E., Zaffar Shibly, A., Ali,A., Hasan-Olive, M., 2016. *Leonurus sibiricus* L. (honeyweed): A review of its phytochemistry and pharmacology. Asian Pac J Trop Biomed. 6(12), 1076–1080

Oliveira, AS., Cercato, LM., de Santana Souza, MT., Melo, AJO., Lima, BDS., Duarte, MC., Araujo, AAS., de Oliveira E Silva, AM., Camargo, EA., 2017. The ethanol extract of *Leonurus sibiricus* L. induces antioxidant, antinociceptive and topical anti-inflammatory effects. J Ethnopharmacol. 206, 144-151. doi: 10.1016/j.jep.2017.05.029.

Sitarek, P., Rijo, P., Garcia, C., Skała, E., Kalemba, D., Białas, AJ., Szemraj, J., Pytel, D., Toma, M., Wysokińska, H., Śliwiński, T., 2017. Antibacterial, Anti-Inflammatory, Antioxidant, and Antiproliferative Properties of Essential Oils from Hairy and Normal Roots of *Leonurus sibiricus* L. and Their Chemical Composition. Oxid Med Cell Longev. 2017, 7384061. doi: 10.1155/2017/7384061.

Sitarek, P., Skała, E., Toma, M., Wielanek, M., Szemraj, J., Skorski, T., Białas, AJ., Sakowicz, T., Kowalczyk, T., Radek, M., Wysokińska, H., Śliwiński, T,. 2017. Transformed Root Extract of *Leonurus sibiricus* Induces Apoptosis through Intrinsic and Extrinsic Pathways in Various Grades of Human Glioma Cells. Pathol Oncol Res. 23(3),679-687. doi: 10.1007/s12253-016-0170-6.

Sitarek, P., Skała, E., Wysokińska, H., Wielanek, M., Szemraj, J., Toma, M., Śliwiński, T.,2016. The Effect of *Leonurus sibiricus* Plant Extracts on Stimulating Repair and Protective Activity against Oxidative DNA Damage in CHO Cells and Content of Phenolic Compounds. Oxid Med Cell Longev. 2016,2016:5738193. doi: 10.1155/2016/5738193.

Sitarek, P., Skała, E., Toma, M., Wielanek, M., Szemraj, J., Nieborowska-Skorska, M., Kolasa, M., Skorski, T., Wysokińska, H., Śliwiński, T., 2016. A preliminary study of apoptosis induction in glioma cells via alteration of the Bax/Bcl-2-p53 axis by transformed and non-transformed root extracts of *Leonurus sibiricus* L. Tumour Biol. 37(7),8753-64. doi: 10.1007/s13277-015-4714-2.

Raghavendrarao, S., Anil Kumar, M., Ranjitha, K., Bollipo, D,. 2017. A review on advanced methods in plant gene targeting. JGEB. Volume 15, Issue 2, Pages 317-321

Rivera, A.L., Gómez-Lim, M., Fernández, F., Loske, A.M., 2012. Physical methods for genetic plant transformation. Physics of Life Reviews. 9, 308–345

Qiu, J., Sun, S., Luo, S. et al., 2014. Arabidopsis AtPAP1 transcription factor induces anthocyanin production in transgenic *Taraxacum brevicorniculatum* Plant Cell Rep. 33, 669. doi:10.1007/s00299-014-1585-8

Zhang, Y., Yan, YP., Wang, ZZ., 2010. The Arabidopsis PAP1 transcription factor plays an important role in the enrichment of phenolic acids in *Salvia miltiorrhiza*. J. Agric. Food Chem. 58, 12168–12175

Anh Tuan, P., Yeon Kwon, D., Lee, S., Arasu, MF., Al-Dhabi, NA., Park, NI., Park, SU., 2014. Enhancement of Chlorogenic Acid Production in Hairy Roots of *Platycodon grandiflorum* by Over-Expression of An *Arabidopsis thaliana* Transcription Factor *AtPAP1* . Int J Mol Sci. 15(8), 14743–14752.

Srivastava, S., Srivastava, AK., 2007. Hairy root culture for mass-production of high-value secondary metabolites. Crit Rev Biotechnol. 27(1),29-43.

Banerjee, S., Singh, S., Ur, Rahman, L.,2012. Biotransformation studies using hairy root cultures - A review. Biotechnol Adv. 30(3),461-8. doi: 10.1016/j.biotechadv.2011.08.010.

Sitarek, P., Kowalczyk, T., Rijo, P., Białas, AJ., Wielanek, M., Wysokińska, H., Garcia, C., Toma, M., Śliwiński, T., Skała, E., 2018. Over-Expression of AtPAP1 Transcriptional Factor Enhances Phenolic Acid Production in Transgenic Roots of *Leonurus sibiricus* L. and Their Biological Activities. Mol Biotechnol. 60(1),74-82. doi: 10.1007/s12033-017-0048-1.

Sivakumar, G., 2006. Bioreactor technology: A novel industrial tool for high-tech production of bioactive molecules and biopharmaceuticals from plant roots. Biotechnol. J. 1, 1419–1427 DOI 10.1002/biot.200600117

Kusakari, K., Yokoyama, M., Inomata, M., Gozu, Y., Katagiri, C., Sugimoto,Y., 2012. Large-scale production of saikosaponins through root culturing of *Bupleurum falcatum* L. using modified airlift reactors. Journal of Bioscience and Bioengineering. VOL. 113 No. 1, 99–105

Chmiel, A., Ledakowicz, S., Pawlowska, B., 2001. The mist bioreactor for cultures of transformed roots. In: 28th Conference SSCHE, Proceedings on CD ROM, May 21–25, Tatranske Matliare (SK)

Schmittgen, TD., Livak, KJ., 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 3,1101–1108

Janaszewska, A., Bartosz, G., 2002. Assay of total antioxidant capacity: Comparison of four methods as applied to human blood plasma. Scand J Clin Lab Invest. 62,231–6

Re, R., Pellegrini, N., Proteggente, A., et al. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 26,1231–7.

Atanasov, AG., Waltenberger, B., Pferschy-Wenzig EA., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, EH., Rollinger, JM., Schuster, D., Breuss, JM., Bochkov, V., Mihovilovic , MD., Bauer, R., Dirsch, VM., Stuppner, H., 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnology Advances. Volume 33, Issue 8, Pages 1582-1614

Skała, E., Kicel, A., Olszewska, MA., Kiss, AK., Wysokińska, H., 2015. Establishment of hairy root cultures of *Rhaponticum carthamoides* (Willd.) Iljin for the production of biomass and caffeic acid derivatives. BioMed Res. Int. 181098, 1-11

Luo, J., Butelli, E., Hill, L., Parr, A., Niggeweg, R., Bailey, P., Weisshaar, B., Martin, C., 2008. AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: Expression in fruit results in very high levels of both types of polyphenol. Plant J 56, 316–326

Qiu, J., Sun, S., Luo, S. et al. 2014. Arabidopsis AtPAP1 transcription factor induces anthocyanin production in transgenic *Taraxacum brevicorniculatum* Plant Cell Rep 33, 669. doi:10.1007/s00299-014-1585-8

Baque, A., Sang-Hyun, M., Lee, EJ., Zhong, JJ., Paek K-Y., 2012. Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. Biotechnology Advances. 30, 1255–1267

Du, M., Wu, XJ., Ding, J., Hu, Z.B., White, K.N., Branford-White, C.J., 2003. Astragaloside IV and polysaccharide production by hairy roots of *Astragalus membranaceus* in bioreactors. Biotechnology Letters 25, 1853–1856.

Chattopadhyay, S., Srivastava, AK., Bhojwani, S.S., Bisaria, V.S., 2002. Production of podophyllotoxin by plant cell cultures of *Podophyllum hexandrum* in bioreactor. Journal of Bioscience and Bioengineering. Volume 93, Issue 2, 215-220

Verma, PC., Singh, H., Negi, A.S., Saxena, G., Rahman, L., Banerjee, S., 2015. Yield enhancement strategies for the production of picroliv from hairy root culture of *Picrorhiza kurroa* Royle ex Benth., Plant Signaling & Behavior. 10:5 DOI: 10.1080/15592324.2015.1023976

Kuźma, Ł., Kaiser, M., Wysokińska, H., 2017. The production and antiprotozoal activity of abietane diterpenes in *Salvia austriaca* hairy roots grown in shake flasks and bioreactor. Prep Biochem Biotechnol. 2,47(1):58-66. doi: 10.1080/10826068.2016.1168745.

Wu, CH., Murthy, HN., Hah, EJ., Paek, K-Y., 2007. Large-scale cultivation of adventitious roots of *Echinacea purpurea* in airlift bioreactors for the production of chichoric acid, chlorogenic acid and caftaric acid. Biotechnol Lett. 29,1179–1182 DOI 10.1007/s10529-007-9399-1

Farah, A., Monteiro, M., Donangelo, C. M., Lafay, S., 2008. Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans. The Journal of Nutrition, 138, 2309–2315.

de Oliveira Melo, MN., Passos Oliveira, A., Wiecikowski, AF., Sampaio Carvalho, R., de Lima Castro, J., Alves Gomesde Oliveira, F., Marcelo Gualberto Pereira, H., Feoda Veiga, V., Marques Alves Capella, M., Rocha, L., Holandino, C., 2018. Phenolic compounds from *Viscum album* tinctures enhanced antitumor activity in melanoma murine cancer cells. Saudi Pharmaceutical Journal. Volume 26, Issue 3, Pages 311-322

Li, H-R., Habasi, M., Xie, L-Z,. Ais, H-A,.2014. Effect of Chlorogenic Acid on Melanogenesis of B16 Melanoma Cells. Molecules. *19*, 12940-12948; doi:10.3390/molecules190912940

de Freitas, M.M., Ribeiro Fontes,P., Monteiro Souza, P., Fagg, C.W., Neves Silva Guerra, E., de Medeiros Nóbrega, YK., Silveira, D., Fonseca-Bazzo, Y., Simeoni, L.A., Homem-de-Mello, M.,

Oliveira Magalhães, P.,2016. Extracts of *Morus nigra* L. Leaves Standardized in Chlorogenic Acid, Rutin and Isoquercitrin: Tyrosinase Inhibition and Cytotoxicity. PLoS One. 11(9): e0163130.

Shahidi, F., Chandrasekara, A., 2010. Hydroxycinnamates and their in vitro and in vivo antioxidant activities. Phytochem Rev. 9,147–70.

Kolodziejczyk-Czepas, J., Nowak, P., Moniuszko-Szajwaj, B., Kowalska, I., Stochmal, A., 2015. Free radical scavenging actions of three Trifolium species in the protection of blood plasma antioxidant capacity in vitro. Pharm Biol. 1–8 DOI: 10.3109/13880209.2014.974064

Kolodziejczyk-Czepas, J., Bijak, M., Saluk, J., Ponczek, MB., Zbikowska, HM., Nowak, P.,Tsirigotis-Maniecka, M., Pawlaczyk, I., 2015. Radical scavenging and antioxidant effects of *Matricaria chamomilla* polyphenolic-polysaccharide conjugates. Int J Biol Macromol. 72,1152-8. doi: 10.1016/j.ijbiomac.2014.09.032.

Dadé, MM., Schinella, GR., Fioravanti, DE., Alfiotournier, H., Antioxidant and cytotoxic properties of an aqueous extract from the Argentinean plant *Hedeoma multiflorum*. Pharm Biol.49(6),633-9. doi: 10.3109/13880209.2010.526949.

Figures/Tables

Fig. 1. Leonurus sibiricus AtPAP1 transgenic roots cultured in A) 300 mL shake flask con-taining 80 mL SH medium, B) 1 L shake flask containing 500 mL SH medium, C) 3 L shake flask containing 1.5 L SH medium, D) 5 L shake flask containing 2.5 L SH medium, E) 5 L nu-trient sprinkle bioreactor in 1 L SH medium after 30 days. Bars = 1 cm.

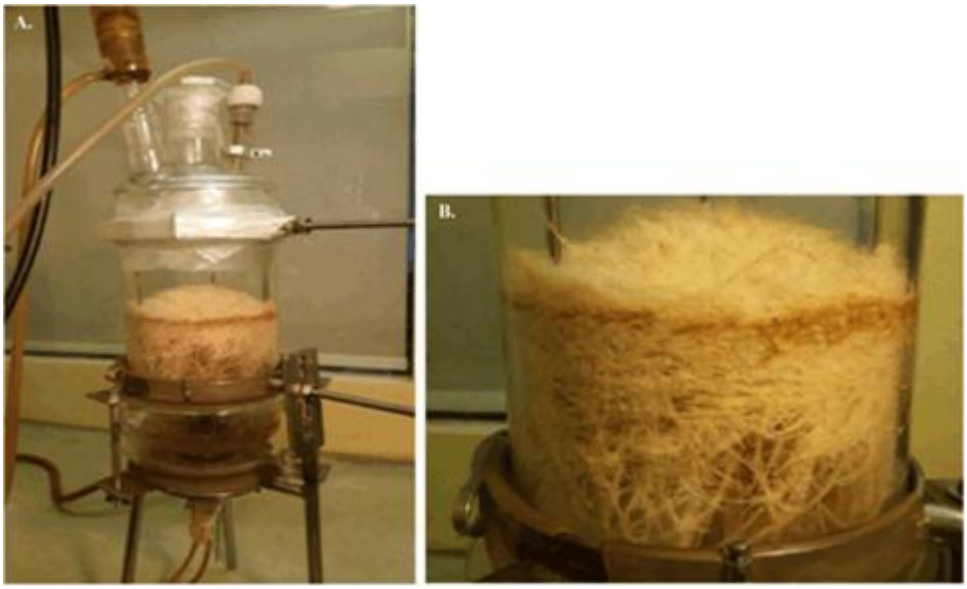

Fig. 2. Leonurus sibiricus AtPAP1 transgenic roots cultured in 5 L nutrient sprinkle bioreactor in 1 L SH medium after 30 days.

Table 1
Fresh and dry weight biomass production of AtAP1 transgenic roots of L. sibircus after 30-day culture in 300 mL, 1 L, 3 L, 5 L flask and 5 L bioreactor cultures.

Parameter	300 mL (80 mL medium)	1 L (500 mL medium)	3 L (1.5 L medium)	5 L (2.5 L medium)	Bioreactor 5 L (1 L medium)
			Root biomass (g/L)		
Fresh mass (FW)	91.99 ± 8.88^{b}	141.25 ± 4.20^{d}	104.98 ± 1.36^{c}	71.89 ± 1.09^{a}	312.66 ± 2.60^{e}
Dry mass (DW)	4.54 ± 1.12^{a}	13.20 ± 0.60^{b}	4.88 ± 0.45^{a}	4.63 ± 0.44^{a}	20.83 ± 1.01^{c}

Data with the same letter in the row indicated insignificant differences $p < 0.05$.

Table 2

Phenolic acids content in AtAP1transgenic roots of L. sibircus after 30-day culture in 300 mL, 1 L, 3 L, 5 L flask and 5 L bioreactor cultures.

Parameter	300 mL (80 mL medium)	1 L (500 mL medium)	3 L (1.5 L medium)	5 L (2.5 L medium)	Bioreactor 5 L (1 L medium)
			Phenolic acid content (mg/g DW)		
Neochlorogenic acid	0.021 ± 0.005[b]	0.03 ± 0.005[b]	0.02 ± 0.006[b]	0.03 ± 0.001[b]	0.007 ± 0.001[a]
Chlorogenic acid	18.65 ± 1.07[b]	23.15 ± 0.26[d]	16.91 ± 0.73[ab]	15.60 ± 0.85[a]	21.52 ± 1.16[c]
Caffeic acid	10.82 ± 0.62[b]	9.2 ± 0.43[a]	12.02 ± 0.64[c]	12.11 ± 1.27[c]	14.50 ± 0.60[d]
p-coumaric acid	0.05 ± 0.006[b]	0.07 ± 0.01[c]	0.05 ± 0.01[b]	0.04 ± 0.008[b]	0.02 ± 0.004[a]
Ferulic acid	1.17 ± 0.01[a]	2.40 ± 0.23[d]	1.41 ± 0.06[b]	1.36 ± 0.08[b]	1.87 ± 0.16[c]

Data with the same letter in the row indicated insignificant differences $p < 0.05$.

Table 3

Phenolic acids yield (mg/L) in AtAP1 transgenic roots of L. sibircus after 30-day culture in 300 mL, 1 L, 3 L, 5 L flask and 5 L bioreactor cultures.

Parameter	300 mL (80 mL medium)	1 L (500 mL medium)	3 L (1.5 L medium)	5 L (2.5 L medium)	Bioreactor 5 L (1 L medium)
			Phenolic acid yield (mg/L)		
Neochlorogenic acid	0.09 ± 0.02[a]	0.456 ± 0.062[d]	0.113 ± 0.029[ab]	0.14 ± 0.03[bc]	0.15 ± 0.02[c]
Chlorogenic acid	84.82 ± 4.87[b]	305.77 ± 3.51[c]	82.64 ± 3.58[b]	72.26 ± 3.93[a]	448.39 ± 24.25[d]
Caffeic acid	49.22 ± 2.86[a]	121.49 ± 5.74[c]	58.76 ± 3.12[b]	56.09 ± 5.88[ab]	302.21 ± 12.55[d]
p-coumaric acid	0.23 ± 0.02[a]	0.92 ± 0.17[c]	0.27 ± 0.04[a]	0.21 ± 0.03[a]	0.40 ± 0.07[b]
Ferulic acid	5.35 ± 0.07[a]	31.72 ± 3.03[c]	6.90 ± 0.33[b]	6.30 ± 0.39[b]	39.12 ± 3.34[d]

Data with the same letter in the row indicated not significantly differences $p < 0.05$.

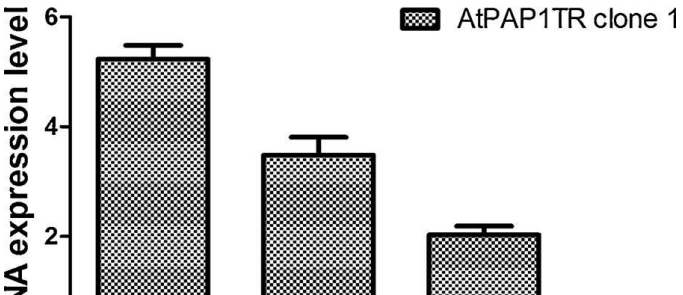

Fig. 3. Expression levels of PAL, C4H and 4CL in the L. sibiricus AtPAP1 TR transgenic root. Results are expressed as means ± SD relative to the control.

Table 4

Growth inhibition (%) after 72-h treatment with TR and AtPAP1 TR root ex-tracts of L. sibiricus.

	TR extract	AtPAP1 TR extract
2.5 μg/mL	5.53 ± 3.86[a]	8.09 ± 4.56[a]
5 μg/mL	4.45 ± 2.51[a]	17.8 ± 7.24[b]
10 μg/mL	7.53 ± 4.87[a]	9.52 ± 5.06[a]
20 μg/mL	9.57 ± 4.32a	12.5 ± 5.94[a]
40 μg/mL	29.64 ± 8.94[a]	53.3 ± 8.06[b]

Data with the same letter in the row indicated not significantly differences $p < 0.05$.

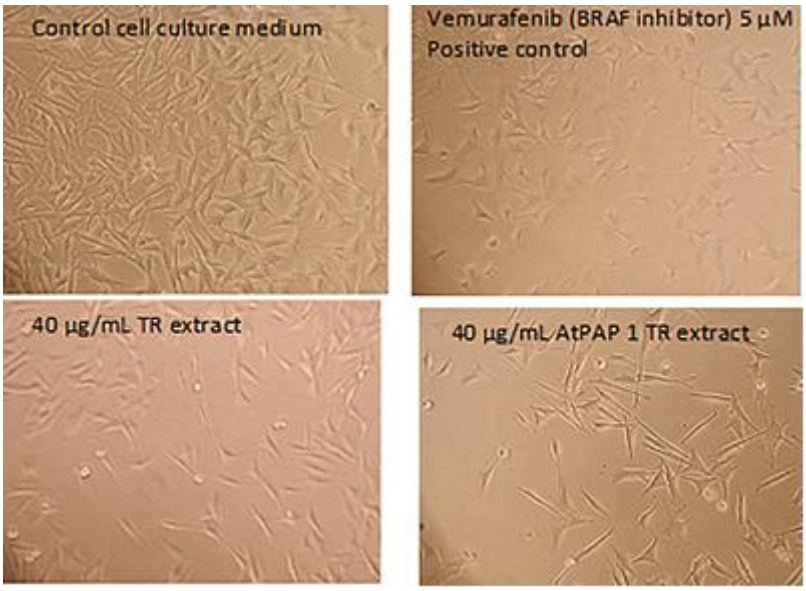

Fig. 4. Microscopic observation in A-2058 melanoma cells after 72-h exposure to control cell culture medium (A) or to medium containing 40 µgmL−1 of TR extract (C) or AtPAP1 TR extract (D). Vemurafenib (5 µM) was used as positive control (B).

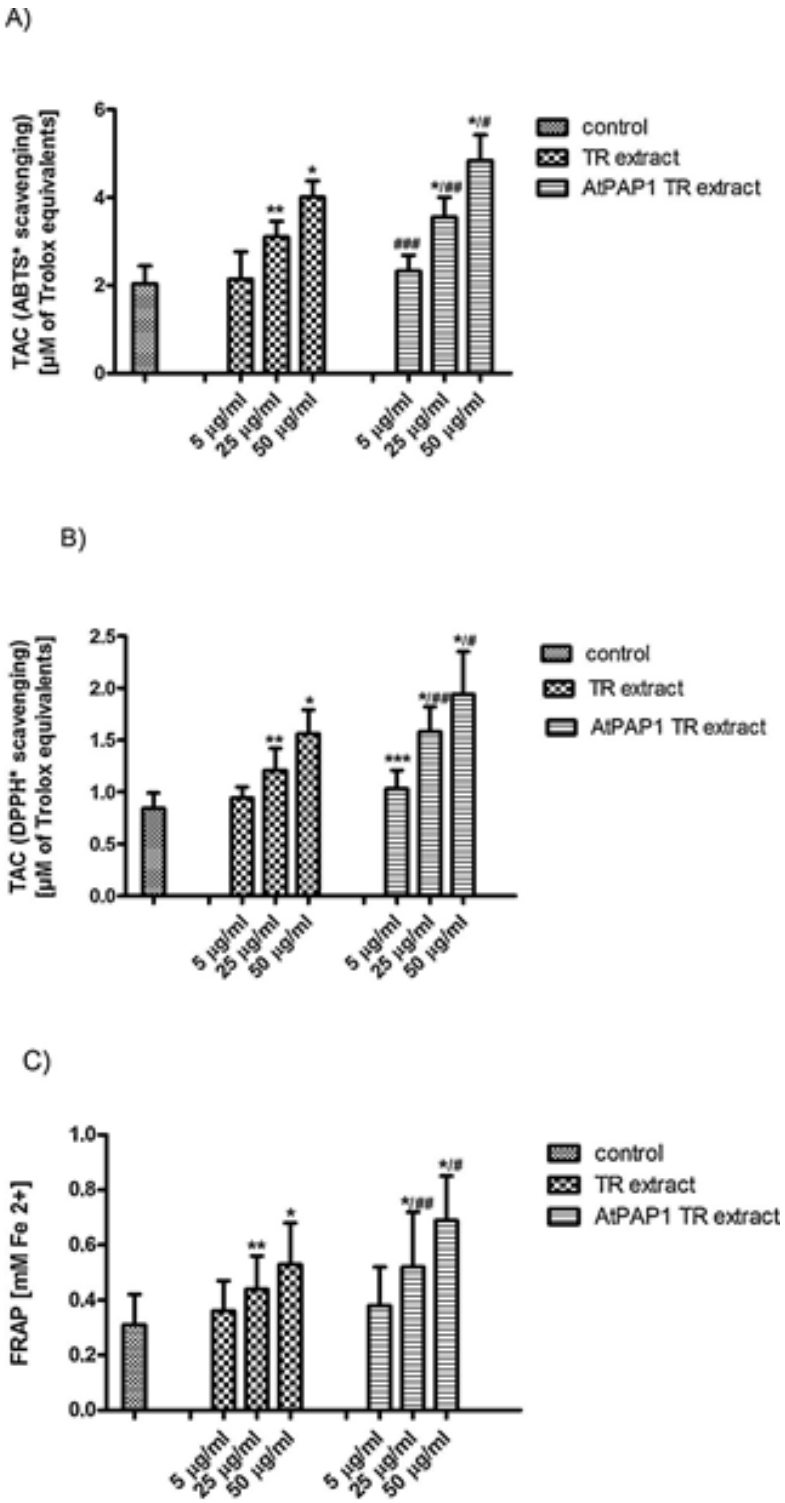

Fig. 5. Comparison effect of TR and AtPAP1 root extracts on the antioxidant capacity of blood plasma. The human blood plasma was determined spectrophotometrically by the ABTS (Panel A) and DPPH (Panel B) radicals decolourization methods, as well as by measurements of ferric reducing ability (the FRAP assay) (Panel C). Results are presented as means ± SD of three independent experiments. *p < 0.001 TR extracts and AtPAP1 TR extracts vs. Control;

**p < 0.01 TR extracts and AtPAP1 TR extracts vs. Control; *** p < 0.05 TR extracts and AtPAP1 TR extracts vs. Control; # p < 0.001 TR extracts vs. AtPAP1 TR extracts; ## p < 0.01 TR extracts vs. AtPAP1 TR extracts; ### p < 0.05 TR extracts vs. AtPAP1 TR extracts.