

Interval harvests changes phytochemical traits and biological activities of elite transformed hairy root cultures of African geranium (*Pelargonium sidoides* DC.)

Zeynab Yousefian

University of Guilan

Yousef Hamidoghli (✉ hamidoghli@gmail.com)

University of Guilan

Pooran Golkar

Isfahan University of Technology

Mohammad Hossein Mirjalili

Medicinal Plants and Drugs Research Institute, Shahid Beheshti University

Article

Keywords:

Posted Date: November 14th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-2210993/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Tuberous roots of African geranium (*Pelargonium sidoides* DC.) with modern ethanolic formulation called EPs 7630 (Umckaloabo) have widely used in medicinal industries. The present study was aimed to explore changes in the phytochemical traits (i.e., phenolics, flavonoids, coumarins and hydroxycinnamic acid derivatives), protein content, enzymatic-antioxidants and antimicrobial activities of several high-yielding hairy root lines (HRLs) of *P. sidoides* harvested at different times. Among all HRLs, antioxidative responses (e.g., phenylalanine ammonia lyase, polyphenol oxidase and catalase contents) were increased in HRL₁₀ exposed to higher interval harvests. Moreover, HRL₁₀ exhibited a promising antimicrobial effect against *Escherichia coli*, with MIC equal to (2.61 mg/ml). In total 10 phytochemical compounds were identified through chromatographic (HPLC) technique including: umckalin (Um), gallic acid (GA), quercetin (QE), luteolin (LU) and the others. The extraction of HRs had high content of Um at 21 days (0.35 µg/ml). The maximal phenolic yield in particular GA was enhanced to (5194.5 µg/ml), about 10-fold in comparison with non-transformed roots (516 µg/ml) on the 35th day. HRs were able to produce the maximum improvement in flavonoid glycosides (13.96 µg QE/ml and 23.75 µg LU/ml) at the end of culture period. The most profitable transgenic lines offers an interesting option to enhance the productivity. These results support the *in vitro* cultivation of HRs for supply as a natural product to prevent inflammation and microbial related disorders.

Introduction

African geranium (*Pelargonium sidoides* DC., Geraniaceae), an excellent commercial and medicinal tuberous crop has been widely used as a therapeutic and palliative effects in the treatment of gastrointestinal disorders and traditional medicine for a long time^{1,2}. It has mainly grown in the South Africa especially in the parts of the Eastern Cape Province and the Free State^{3,4}. Certain *Pelargonium* species as having a definite place in the food industry^{5,6}.

The phenolics and chemical compositions (e.g., coumarins) in *P. sidoides* root extract (EPs7630®) were reported to have elite antioxidant and antiseptic properties⁷. A remarkable series of simple coumarins have been identified in *Pelargonium* species. The high degree of aromatic functionalization structure of coumarins depending on the hydroxyl and methoxyl groups (5,6,7- or 6,7,8-trihydroxycoumarin and 8-hydroxy-5,6,7-trimethoxycoumarin), which they are derived. Coumarins, in their oxygenation form, are very rare in the plant kingdoms, but they always coexist, in the different genera of *Pelargonium* (*P. sidoides* and *P. reniforme*)⁸.

Umckalin (Um) (5,6,7-trimethoxycoumarin) generally is known as an important coumarin which is especially observed in *P. sidoides*^{9,10}. It is a highly substituted benzopyranones found particularly in roots and is widely used in European market and commercially pharmaceutical products of *P. sidoides*¹¹. The Umckaloabo® (as a trade name of this medicine) is used for treating patients suffering from flu, colds, chest troubles and is effective for bronchitis in children and adults¹²⁻¹⁴.

The bioactive constituents of *P. sidoides* extracts were used as herbal remedies to treat various ailments (e.g., COVID-19, AIDS, H1N1 and SARS influenza), many of which involve viruses, bacteria, or fungi¹⁵⁻¹⁸. In several studies, high activity of *P. sidoides* crude root, and leaf extracts against a range of pathogenic bacteria such as multi-resistant *Staphylococcus aureus* strains and several fungal species associated with respiratory tract

infections (*Aspergillus niger*, *Fusarium oxysporum* and *Rhizopus stolonifer*) were shown by Mativandlela *et al.*¹⁴, Kayser and Kolodziej.¹⁹ and Kolodziej *et al.*²⁰.

In vitro production of valuable phytochemical compounds, is an ideal biotechnological platform for uniform production of secondary metabolites (SMs)^{21,22}. The consequence of Over-exploitation and uncontrolled harvesting of *P. sidoides* populations may affect its availability for future generations²³. Also with, considering the economic benefits and natural potential for the production of highly oxygenated coumarins (Um, fraxetin and scopoletin), in *P. sidoides*, greater attention is required for better conservation, and cultivation of *P. sidoides*²⁴.

Presently, hairy root cultures (HRCs) established via *Agrobacterium rhizogenes*-mediated transformation due to their rapid growth, large-scale biomass, biochemical, and genetic stability, and relatively high production without phytohormones are widely used as experimental models and as sources of beneficial compounds²⁵. Therefore, the results from this study support the use of naturally engineered HRs of *P. sidoides* in the production of high-value, complex molecules.

The aim of this work was to: (1) optimize the best interval times for effective harvesting in the viewpoint of *in vitro* production of (Um) and other major phytochemical compounds in *P. sidoides* HRs without detrimental effect of the tested stimulators on biomass growth; (2) to investigate the differential protein contents and enzymatic-antioxidant responses of *P. sidoides* hairy root lines (HRLs) at several harvest times (9 intervals) and determine whose lines better adapted to these conditions; (3) to evaluate the antimicrobial activity against Gram-negative, and Gram-positive bacteria in *P. sidoides* HRLs.

Methods

Plant materials, Liquid culture of *P. sidoides* hairy root lines and the time course of the study. Seeds of *P. sidoides* were obtained from the Pharmaceutical Garden of the Pharmaceutical Institute of the University of Kiel, Germany (Prof. Birgit Classen). A voucher specimen of the plant (identification number Ps 2012) has been deposited in the Herbarium of the Department of Pharmaceutical Biology at the University of Kiel. The HRC of *P. sidoides* was derived as described by Yousefian *et al.*²⁶ from leaves explants infected with a Ri-T-DNA-bearing *Agrobacterium tumefaciens* C58C1 (pRiA4) (Fig. 1A). HRLs (15, 4 and 10) were selected for this experiment as they accumulated a higher biomass and biological compound content. Experiments in this study were carried out in the shake-flask culture of the three selected HRLs. The Erlenmeyer flasks (150 ml) were placed on an orbital shaker running at 110–120 rpm and at 25 ± 2°C in the dark. Each flask was filled with 50mL hormone-free liquid Murashige and Skoog,²⁷ medium (Duchefa, Netherland), 30 g/l of sucrose and inoculated with 2 g fresh weight of HRLs. Cultures were incubated during 35 days of growth and then interval harvested in the 9 cycles, respectively, after (7, 14, 16, 17, 18, 19, 21, 28 and 35) days of growth, and kept at – 80°C until use. Tissue culture shoot and root plants along with growth chamber plantlets were used as control of the experiment. All plant materials used in this study were complied with institutional, national, and international guidelines and legislation.

Protein extraction. For estimation of total protein content, 0.5 gr of frozen three elite HRLs compared to 0.5 gr of each control sample including (the normal root of plants grown in chamber room, root and shoot tissue culture plantlets) combined from three biological replicates were extracted with liquid nitrogen. The samples were ground with 2.5 ml of extraction buffer (50 mM Tris-HCl, 15 mM β-mercaptoethanol and pH = 7.5). The homogenates were centrifuged at 4000 rpm for 30th minutes at 4°C and supernatants were kept at – 80°C, and

used for protein determination and enzyme assays. The protein content was determined based on the method of Bradford ²⁸ using bovine serum albumin (BSA, Sigma-Aldrich, USA) as a standard.

Phenylalanine Ammonia Lyase (PAL) activity assay. The phenylalanine ammonia lyase (PAL) activity was determined by the method of Wang *et al.* ²⁹. According to this method, 0.5 ml of the extraction buffer, 0.5 ml L-phenylalanine (10 mM), 0.4 ml of double distilled water, and 0.1 ml of enzyme extract were added and incubated at 37°C for 1 hour. The reaction was inactivated by adding 0.1 ml HCl (6 M), and the cinnamic acid (CA) was extracted three times with ethyl acetate, followed by evaporation to remove the extracting solvent. The solid remnant was dissolved in 3 ml of sodium hydroxide (0.05 M), and the CA concentration was quantified spectrophotometrically (Unico2100S UV-VIS, USA) at 290 nm.

Polyphenol Oxidase activity (PPO) assay. Polyphenol oxidase (PPO) activity was measured according to the method of Raymond *et al.* ³⁰. The reaction mixture (3 ml final volume) consisted of 0.2 ml pyrogallol (20 mM), 2.5 ml of potassium phosphate buffer (20 mM, pH = 7.0), and 20 μ l of enzyme extract, and the increase in absorbance at 430 nm was measured per min.

Catalase activity (CAT) assay. Catalase (CAT) activity was determined by the method described by Aebi ³¹. To 20 μ l root enzyme extract, 2.5 ml potassium phosphate buffer (50 mM, pH = 7.0) containing 20 μ l hydrogen peroxide (H_2O_2) was added, and left at 25°C for 1 minute. Enzyme activity was estimated by calculating the decomposition of μ M H_2O_2 per min per g protein in absorbance at 240 nm for 1 minute.

Guaiacol peroxidase activity (GPX) assay. Guaiacol peroxidase (GPX) activity was assayed following the formation of tetraguaiacol according to the methods of Zhang *et al.* ³². The reaction mixture was made of 2.5 ml potassium phosphate buffer (50 mM, pH = 7.0), 100 μ l hydrogen peroxide (H_2O_2) 1%, 100 μ l guaiacol 1% (v/v) and 250 μ l enzyme extract. The change in the optical density (ΔOD) of the solution was recorded spectrophotometrically at 470 nm, and GPX kinetics were reported as Units/mg protein/min. GPX content was determined by using a specific absorption coefficient value of 25.5 mM/cm.

Ascorbate peroxidase activity (APX) assay. For measurement of ascorbate peroxidase (APX) activity, the reaction mixture consisted of 2 ml potassium phosphate buffer (50 mM, pH 7.0), 100 μ l EDTA Na_2 (0.1 Mm), 100 μ l ascorbate (0.5 mM), 200 μ l H_2O_2 (3%) and 100 μ l enzyme extract in a final assay volume of 2.5 ml. The reduction in absorbance at 290 nm was recorded, and enzyme activity was computed using an extinction coefficient of 2.8 mM/cm ³³.

Superoxide dismutase (SOD) activity assay. Superoxide dismutase (SOD) activity was determined using the protocol of (Beauchamp and Fridovich, ³⁴) by rate inhibition of nitro blue tetrazolium (NBT) at 560 nm in a UV-Vis spectrophotometer.

Hydrogen peroxide (H_2O_2) assay. To determine the H_2O_2 concentration according to Alexieva *et al.* ³⁵, (0.1 g) of fresh roots were homogenized in 1.5 ml of TCA (trichloroacetic acid) (0.1%) and centrifuged at 4000 rpm for 15 minutes at 4°C. Then, 500 μ l of the supernatant was added to 500 μ l of potassium phosphate buffer (10 mM, pH 7.0) and 500 μ l potassium iodide (1 M). After 1 hour of incubating in the darkness, the absorbance of the mixture was read at 390 nm.

Measurement of phytochemical products by HPLC analysis. The sample preparation of phytochemical products of HRs, and control plants were extracted according to Başkan *et al.*³⁶ with slight modifications. To this end, briefly washed roots were dried at 50°C until constant weight and then dried HRs and control samples (0.1 g) were ground into a fine powder (about 1 mm), homogenized with 10 ml methanol (Merck, Germany), and followed by 30 min extraction with an ultrasonic and overnight incubation at room temperature. The homogenate was then centrifuged at 4500 rpm. The supernatant was collected and filtered through (0.22 µm pore size) into clean vials. For quantitative and qualitative determination of phenolic compounds, a High Performance Liquid Chromatography (HPLC) program equipped with a Diode-Array Detection (DAD) (Shimadzu Nexera X2, Kyoto, Japan) was used. The HPLC system was equipped with an LC-30AD pump, a SPD-20Avp UV/VIS detector, and an autosampler SIL-30AC was used. An aliquot of 20 µl of the filtered sample was injected into HPLC for phytochemical product analysis. The chromatographic separation was obtained by Zorbax eclipse (XDB) C₁₈, 5µm, 4.6 × 250 mm, with mobile phase methanol-water (80:20; v/v) and flow rate of 1 ml/min. The UV spectra of phenolic compounds were recorded at 280 and 320 nm. The determination of different phenolic compounds was measured according to methods^{26,37}. Standards of quercetin (QE), gallic acid (GA), caffeic acid (CAF), luteolin (LU), chlorogenic acid (CGA), rutin hydrate (RH), tannic acid (TA), ferulic acid (FA) and p-coumaric acid (P-COA) were calibrated by this HPLC instrument. All the standards were purchased from Sigma Inc. The (Um) content of samples was determined by comparison with a standardized extract with a content of 470 ppm of (Um), purchased from Afrigetics (batch number NANPSO03). The amounts of metabolites were expressed as (µg/ml) (Fig. 1B). For each experiment, two replicates were evaluated.

Antimicrobial assays. The antibacterial activities of *P. sidoides* extract were determined by the broth microdilution assay using 96-well microtiter plates according to The European Committee on Antimicrobial Susceptibility (EUCAST)³⁸ recommendations and CLSI,³⁹ guidelines (Fig. 1C). The antimicrobial effects of samples (mg/ml), were evaluated based on the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), towards the following reference strains from the American Type Culture Collection (ATCC): Gram-positive *Staphylococcus aureus* ATCC 6538 and; Gram-negative *Escherichia coli* ATCC 8739.

Statistical analysis. Statistical analyses were conducted with SPSS 19.0 and SAS (Ver. 9.3.1) software system. The study was carried out as a factorial based on a completely randomized design with three replications. Correlations between the tested activities in HRLs were established using the test of Pearson. The means ± SE (standard error) are presented for all graphs. The significant difference between treatments was evaluated using Duncan's test at $P \leq 0.05$.

Results

Total protein content. The results indicate that the mean of protein content was about (1.2 and 1.1) times higher in HRL₁₀ (0.52 ± 0.04 mg/g FW) than that found in HRL₄ (0.41 ± 0.02 mg/g FW) and HRL₁₅ (0.45 ± 0.04 mg/g FW) (Fig. 2A). The means of total protein contents had a significant decrease at harvested times of 4th and 5th cycles in all HRLs (Fig. 3A). Overall, the protein content of *P. sidoides* HRLs was higher in days (19th, 21th, 28th, and 35th) than on the other interval times, and reached to the maximum level (0.92 ± 0.06 mg/g FW) of its in HRL₁₀ in day 19th (Fig. 4A1). Also in the control shoot samples of *P. sidoides*, such as leaves of tissue culture plantlets were detected with higher concentrations of total protein as compared to the other organ types (Fig. 4A2).

Phenylalanine Ammonia Lyase (PAL) activity. The enzymatic antioxidants evaluated in the HRLs are represented in Table 1. The pattern of PAL activity differed substantially between the three transgenic RLs. Comparing the HRLs revealed that PAL was more active in HRL₁₀ than the other HRLs (Fig. 2B). Moreover, the lowest PAL levels were noted in the 8th cycles, in contrast, the highest PAL activity was achieved within the 7th cycle (Fig. 3B). The maximum activity of the PAL in the HRL₁₅ was detected under 21 days of treatment ($0.96 \pm 0.02 \mu\text{g CA/s/mg pro}$) and simultaneously correlated with the highest protein accumulation in this variant of culture. The activity of PAL enzyme increased significantly in all treatments compared with normal roots. The most significant activity of the PAL enzyme was observed in the leaf control samples that were planted in tissue culture media ($0.68 \pm 0.14 \mu\text{g CA/s/mg pro}$) (Fig. 4B).

Table 1

Analysis of variance for different harvest times of incubation, on several studied traits in *P. sidoides* suspension-HRLs.

Source of variability	D.F.	Mean of sum of squares							
		Pr	PAL	PPO	CAT	SOD	APX	GPX	H ₂ O ₂
Treatment	26	0.104**	0.12**	0.003**	145.31**	0.003**	0.0007**	0.04**	0.074**
Line	2	0.091**	0.085**	0.0004 ^{ns}	92.15 ^{ns}	0.001*	0.0016**	0.024**	0.051**
Time	8	0.046**	0.115**	0.004**	257.32**	0.005**	0.0007**	0.09**	0.126**
Line × Time	16	0.134**	0.127**	0.003**	95.97 ^{ns}	0.002**	0.0007**	0.017**	0.05**
Residual	54	0.006	0.005	0.0006	61.696	0.0003	0.00008	0.002	0.36
CV(%)		17.43	18.17	17.76	21.44	17.44	24.26	18.10	11.57
D.F. degree of freedom; CV. Coefficient of variation; **, and * significant at $P < 0.01$ and $P < 0.05$ respectively; ns: not significant									
Abbreviations: protein (Pr) content, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) activity, and hydrogen peroxide (H ₂ O ₂) concentration.									

Polyphenol Oxidase (PPO) activity. The HRL₁₀ had the highest PPO activity (0.14 Units/ mg pro) during growth of suspension culture (Fig. 2C). The greatest mean for PPO activity was observed in the HRLs that were harvested in the 6th cycle ($0.17 \pm 0.015 \text{ Abs Units/ mg pro}$), but it showed a decrease after the 8th cycle (0.11 Units/ mg pro) (Fig. 3C). A significant decrease of PPO enzyme activities also was observed at the 4th and 5th cycles in all HRLs of the treatments and it followed a similar pattern with protein content (Fig. 3A and C). Our results showed that all HRLs had a considerable effect on increasing the activity of PPO, as compared to the control samples (Fig. 4C).

Catalase (CAT) activity. No significant difference in CAT activity was detected in the HRLs of *P. sidoides* between the 9 studied cycles for any harvested times, except at the 8th cycle, which had the lowest content (Fig. 3D). Although between HRLs and interval times were not observed a significant effect, but harvested times changes had considerable impact (Table 1). As shown in (Fig. 2D) the highest mean activity of HRLs were measured in

HRL₁₀ (38.35 ± 1.5 Units/ mg pro). In contrast, the lowest activity was observed in HRL₁₅ (34.67 ± 2.07 Units/ mg pro). The results showed that the control shoot tissue plantlet contained more significant of individual CAT activity than the other control samples (Fig. 4H).

Guaiacol peroxidase (GPX) activity. The activity of GPX, an oxidative enzyme of phenolic compounds, was significantly enhanced by different harvested times, especially at 2th and 6th cycles, but gradually declined with increasing exposure times (Fig. 3E). In the HRL₁₅, a reduction in the GPX activity was founded after 21 days of exposure time (0.12 ± 0.01 Units/mg pro/ min). On the contrary, GPX activity was enhanced at 19th days in HRL₁₅ (0.55 ± 0.02 Units/mg pro/ min), which was higher significantly than the other HRLs. After 19 days in the HRs, no increasing significant changes in GPX activity were found in any of the HRLs (Fig. 4D1). The highest and lowest contents of GPX enzyme (1.05 ± 0.01 and 0.11 ± 0.01 Units/mg pro/ min, respectively) were observed in control stem plant from tissue culture growing and normal roots of the plant in a growth chamber (Fig. 4D2). The highest enzymatic activity of GPX was found in the HRL₁₅, followed by the HRL₄ and then the HRL₁₀ (Fig. 2E).

Ascorbate peroxidase (APX) activity. The activity of the APX enzyme increased under all control samples compared with those in different harvested times and HRLs treatments (Fig. 4E). The mean value activity of the APX enzyme shows a significant difference in the first 14 days of treatments and between all HRLs (Fig. 3F). The activity of APX enzyme showed the highest value in HRL₄ at 14th days of growth (0.06 Units/mg pro) compared with other HRLs treatments (Fig. 4E1). While the activity of this enzyme was reduced in HRL₁₀ at 19th days of treatment (0.014 Units/mg pro) and in HRL₁₅ at 21th days of treatment (0.012 Units/mg pro) (Fig. 4E1). A reduction was observed in HRLs of *P. sidoides* at the highest exposure times. The mean activity of HRLs ranged from 0.02 to 0.04 Units/mg pro (Fig. 2F).

Superoxide dismutase (SOD) activity. Superoxide dismutase (SOD) activity was found to be significantly higher ($P < 0.05$) in HRL₄ at 19th days of growth (0.28 ± 0.008 Units/mg pro) as compared to other studied HRLs (Fig. 4F1), whereas the least SOD activity in HRLs was assessed in 2th and 9th cycles of harvests (Fig. 3G). However, HRL₄, HRL₁₀, and HRL₁₅, respectively, had possessed significantly higher SOD activity during growth treatments (Fig. 2G). The enzymatic activity of SOD increased from 0.04 to 0.27 ± 0.05 Units/mg pro in control samples (Fig. 4F2).

Hydrogen peroxide (H₂O₂) scavenging activity. The results of the hydrogen scavenging activity of the extracts are as shown in Table 1. The extracts of the HRL₄ used, the highest scavenging activity among all the other extracts used (Fig. 2H). In the 6th and 7th cycles of culture, the mean value activity of H₂O₂ increased between all HRLs (Fig. 3H). The HRL₁₅ extract exhibited the highest scavenging activity at the 19th and 21th days of growth (1.07 ± 0.18 and 1.03 ± 0.22 μ M/ g FW), followed by HRL₄ extracts at 6th and 9th cycles of harvested times (1.026 ± 0.08 and 0.92 ± 0.23 μ M/ g FW respectively). The lowest content of H₂O₂ was founded in HRL₁₅ at the 14th and 35th days of growth (0.53 ± 0.02 and 0.52 ± 0.07 μ M/ g FW) (Fig. 4G1). Furthermore, an increase in H₂O₂ activity was observed in the control shoot plant from tissue culture growing compared to the normal roots of the plant in a growth chamber (Fig. 4G2).

Correlations between different enzymes and protein contents. Pearson's correlation coefficients were calculated to determine whether a linear relationship existed between any of the enzymatic antioxidant assays (Table 2). The highest positive and significant correlations ($r = 0.43^{**}$) was observed between the PAL and PPO activity

(Table 2). A negative and significant correlations between PPO and APX assays ($r=-0.32^{**}$) and between PAL and GPX ($r=-0.25^*$) have also been observed.

Table 2
Correlation between the biochemical traits in control plants and HRs of *P. sidoides*

Trait	APX	H ₂ O ₂	SOD	CAT	PPO	PAL	GPX	Pr
APX	1							
H ₂ O ₂	-0.12	1						
SOD	-0.18	0.28 ^{**}	1					
CAT	-0.02	0.29 ^{**}	0.09	1				
PPO	-0.32 ^{**}	0.32 ^{**}	0.12	0.08	1			
PAL	-0.18	0.25 [*]	-0.11	0.26 [*]	0.43 ^{**}	1		
GPX	0.09	0.010	-0.14	0.007	-0.09	-0.25 [*]	1	
Pr	-0.13	-0.017	-0.09	-0.18	0.31 ^{**}	0.41 ^{**}	-0.17	1
**, and * Correlation is significant at $P < 0.01$ and $P < 0.05$ respectively								
Abbreviations: ascorbate peroxidase (APX), hydrogen peroxide (H ₂ O ₂), superoxidedismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), guaiacol peroxidase (GPX), and protein (Pr) content.								

Time course of polyphenolic compounds accumulation during suspension treatment and correlation between them. The HPLC chromatogram analysis indicated that the predominant compound in the ethanolic extracts of transformed HRs of *P. sidoides* was Um. Its level ranged from 0.06 ± 0.005 to $0.29 \pm 0.003 \mu\text{g/ml}$ with the highest production observed in HRs (Table 3). These levels were 1.33 to 6.21 times higher than in the roots of plantlets cultivated in tissue culture ($0.048 \mu\text{g/ml}$). The other bioactive compounds, such as (QE, GA, CAF, LU, CGA, RH, TA, FA and P-COA), were observed in HPLC chromatograms of methanolic extracts of transformed HRs treated with different interval of harvests (Fig. 1D and E). As shown in Table 3, QE content of HRs treatment harvested in a 35th days of culture period was $13.96 \pm 0.19 \mu\text{g QE/ml}$, whereas the first, and middle period of growth resulted on QE accumulation of $0.19 \pm 0.01 \mu\text{g/ml}$. The content of GA detected in HRs harvested with different times was $5194.5 \pm 7.7 \mu\text{g GA/ml}$ at 35th day of treatment. Harvesting HRs at the 7th day of treatment resulted in lower levels of GA, at $495 \pm 10 \mu\text{g GA/ml}$ (Table 3). CAF content in 5 different harvested times of *P. sidoides* HRs changed from 1.29 ± 0.02 to $92.7 \pm 0.56 \mu\text{g CAF/ml}$, and with the selection of the superior line, CAF content was about 10.14-times higher than non-transgenic roots. Transgenic and non-transgenic roots were also compared regarding CGA, RH, TA, FA, Lu and p-COA contents. It was found that the CGA, RH, TA, FA, LU, and p-COA contents of transgenic roots were more significant than the non-transgenic roots in the HRs screened. Results obtained revealed that, the content of detected phenolic acids has been different in variants subculture times with different sources of HRCs. It was found that, HRC has been characterized by higher production of identified phenolic acids compared with control samples with shoot or roots. Generally, all the HRCs of the leaves sources have shown high production of phenolic acids compared to the control.

Table 3

Comparative analysis of SM contents of tissue culture-grown plant and HRCs of *P. sidoides* Values are the means $\pm$ standard deviation (n = 2). Means followed by the same letter are not significantly different at the 5% probability level ($p \leq 0.05$) by the LSD (least significant difference) test

Polyphenolic compounds	Effect of different harvested times on the phytochemical compounds in HRs					Control root plant	Control shoot plant
	Harvest time (days)						
	7th	14th	21th	28th	35th		
Umckalin	0.122 ± 0.03 ^b	0.109 ± 0.002 ^c	0.2995 ± 0.003 ^a	0.064 ± 0.005 ^d	0.1275 ± 0.002 ^b	0.052 ± 0.0 ^e	0.03 ± 0.001 ^f
Quercetin	0.190 ± 0.01 ^e	0.190 ± 0.01 ^e	0.1950 ± 0.005 ^e	4.75 ± 0.25 ^b	13.96 ± 0.2 ^a	1.32 ± 0.1 ^c	0.75 ± 0.03 ^d
Gallic acid	495 ± 10 ^d	625.5 ± 4.5 ^b	625.5 ± 9 ^d	561 ± 4 ^c	5194.5 ± 7.7 ^a	516 ± 4 ^d	178 ± 7 ^e
Caffeic acid	1.3 ± 0.02 ^f	7.9 ± 0.37 ^d	5.04 ± 0.16 ^e	5.83 ± 0.16 ^e	92.7 ± 0.56 ^a	9.13 ± 0.1 ^c	21.76 ± 0.2 ^b
Chlorogenic acid	184.5 ± 8 ^d	250 ± 8 ^c	231 ± 4 ^c	467.5 ± 9.5 ^b	4352.5 ± 38.9 ^a	425.5 ± 12.5 ^b	48.5 ± 1.5 ^e
<i>P</i> -coumaric acid	7.5 ± 0.4 ^e	11.65 ± 0.35 ^d	17.6 ± 0.43 ^c	9.14 ± 0.71 ^e	86.05 ± 0.48 ^a	7.93 ± 0.7 ^e	39.80 ± 0.2 ^b
Luteolin	0.61 ± 0.08 ^d	2.990 ± 0.1 ^b	2.4150 ± 0.2 ^{bc}	1.86 ± 0.1 ^c	23.75 ± 0.5 ^a	2.26 ± 0.14 ^c	1.8 ± 0.2 ^c
Ferulic acid	11.36 ± 0.6 ^c	7.4100 ± 0.6 ^d	27.10 ± 0.9 ^b	6.155 ± 0.8 ^d	132.1 ± 2.4 ^a	12.5 ± 0.8 ^c	25.3 ± 0.7 ^b
Tannic acid	846.5 ± 10.5 ^g	2954 ± 6 ^c	2993.5 ± 6.5 ^b	246.4 ± 6 ^d	23244 ± 22.6 ^a	2318.5 ± 7.5 ^f	2422 ± 4 ^e
Rutin hydrate	7.44 ± 0.8 ^d	3.2850 ± 0.3 ^d	4.35 ± 0.6 ^d	467.5 ± 9.5 ^b	4352.5 ± 38.9 ^a	38.2 ± 1.06 ^c	5.15 ± 0.8 ^d

For polyphenolic compounds of HRs, and control sample extracts, there was a strong correlation between LU with both TA ($r = 0.99^{**}$) and GA ($r = 0.99^{**}$), and between CGA with both TA ($r = 0.99^{**}$), GA ($r = 0.99^{**}$) and as well as RH ($r = 0.99^{**}$) (Supplementary Table S1). Also, a strong correlation was observed between TA and GA ($r = 0.99^{**}$) (Supplementary Table S1). However, the non-significant correlation was found in the case of Um with RH, p-COA, TA, GA, CGA, FA, and Lu (Supplementary Table S1).

Antimicrobial activity. *P. sidoides* extract presented moderate antibacterial activity (Fig. 1B). the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) are shown in Table 4. The strongest bactericidal activity was found in HRL₁₀ against the Gram-negative *E. coli*, with MIC equal to 2.61 mg/ml and MBC as high as 2.61 mg/ml (Table 4). The tested extract in HRL₁₀ showed a slightly weaker bacteriostatic effect against Gram-positive bacteria, with MIC of 5.25 mg/ml for *S. aureus* with a slightly better bactericidal effect (MBC) equal to 1.5 mg/ml. HRL₁₅ and HRL₄ extracts were found to exhibit similar antibacterial activity against *E.*

coli (MIC/MBC: 3.73–3.71 mg/ml/15.12–NA mg/ml), and weaker towards Gram-positive bacteria in HRL₄ (MIC/MBC: 7.5 mg/ml/NA mg/ml). The MICs of methanol extracts from *P. sidoides* shoots and roots against two different Gram-negative and Gram-positive bacteria were also in the range of 0.125–0.625 mg/ml; however, in the same experiment, HRL₁₅ showed a much stronger effect against *S. aureus* (MIC 0.93 ± 0.02 mg/ml). The methanolic extracts of *P. sidoides* shoot (0.31 mg/ml) also demonstrated weaker activity against selected Gram-positive bacteria, while the root extract was more active against *Staphylococcus* strain (0.125 ± 0.002 mg/ml). The Gram-negative bacterium, *E. coli*, showed microbial resistance to all the tested HRLs except for HRL₄, but the HRL₁₀ methanolic extract displayed the greatest antimicrobial activity against the Gram-negative bacteria (2.61 ± 0.005 mg/ml).

Table 4

Antibacterial activity of the methanol extracts of the shoots, roots and HRLs of *P. sidoides* shown as minimal inhibitory concentration (MIC), and minimal bactericidal concentration (MBC).

sample	Gram-positive bacteria		Gram-negative bacteria	
	<i>S. aureus</i>		<i>E. coli</i>	
	MIC(mg/ml)	MBC(mg/ml)	MIC(mg/ml)	MBC (mg/ml)
Control root	0.125 ± 0.002^e	na	0.625 ± 0.006^c	na
Control shoot	0.312 ± 0.0005^d	na	0.311 ± 0.005^d	na
HRL ₁₅	0.937 ± 0.02^c	0.918 ± 0.02	3.735 ± 0.015^a	15.12 ± 0.12
HRL ₄	7.5 ± 0.006^a	na	3.715 ± 0.035^a	na
HRL ₁₀	5.25 ± 0.02^b	1.5 ± 0.007	2.615 ± 0.005^b	2.615 ± 0.005
*Each treatment contained three replicates with standard errors data are expressed as mean $\pm$ SE within columns from Duncan's multiple range test (DMRT) and different lowercase letters indicates significantly different at a 5% level; na, no activity				

Discussion

HRCs system is one of the best functional criteria for the development of this crop and sustainable supply of plant materials with quality assurance to the market^{37,40}. Despite the economically important, promising features and advancements, the production of *P. sidoides*-derived pharmaceuticals by HRs has not yet been commercially exploited. In the present study, three elite HRLs of *P. sidoides*, were evaluated during 35 days of suspension culture for their antioxidant potential to support the modern claims of their medicinal uses. The HRs were tested for preliminary phytochemical screening, enzymatic-antioxidants responses and antibacterial activities.

In this investigation, suspension culture treatment resulted in enhanced PAL activities, antioxidant enzyme accumulation, and stress levels (H₂O₂) in HRLs of *P. sidoides* derived by genetic transformation (*Agrobacterium tumefaciens* C58C1 (pRiA4)), particularly at the highest time of harvesting. PAL activity had an increased activity on the 21th day, stronger at (0.96 ± 0.019 µg CA/s/mg pro; HRL₁₅) than at other harvesting times. This work showed that both the PAL activity and phenolics accumulations were enhanced dramatically during the final

culture period treatment, indicating that PAL is the critical enzyme in regulating the phenolic acid accumulations in the *P. sidoides* HRCs. The CAT activity in treated HRLs reached the maximum on the 7th and 19th days, as well as SOD activity, reached to its peak on the 19th day. Similarly, Karatoprak *et al.*⁴¹ displayed higher levels of SOD activity (5.50 U/ml and 5.67 U/ml) compared to the control roots (4.48 U/ml). In this regard, Shi *et al.*⁴² showed that SOD and CAT activities in sorbitol-treated HRs of *Salvia miltiorrhiza* reached the maximum in the first days of treatment, and there was no correlation between tanshinone accumulation and enzyme activity. Chayjarung *et al.*⁴³ demonstrated that HRCs of peanut in an early stage of elicitor exposure (at 24 h), had significant increase in the antioxidant enzymes (CuZn-SOD, APX, and GPX).

This research also revealed the accumulation of PPO, GPX, and APX levels in three HRLs of *P. sidoides*, declined significantly with increasing exposure times. Whereas, different times of harvesting, especially at the 6th and 9th cycles cause particular enhanced H₂O₂ accumulation in HRLs by decreasing the activity of H₂O₂-metabolizing enzymes. A search of the literature did not reveal any information about the hydrogen peroxide scavenging capacity of HRLs of *P. sidoides*. According to previous results, Izuegbuna *et al.*⁴⁴ showed the scavenging ability of *P. inquinans* leaves was high and dose-dependent, which could be a result of high levels of phenolic compounds and flavonoids with high amounts of polyphenol, and antioxidant activities⁴⁵. The overall results showed that, HRL₁₀ cultured on MS medium exhibited higher means of enzymatic activity (PAL, PPO and CAT contents) than the other HRLs. On the other hand, a comparison with the means of H₂O₂ activity showed that low concentration in HRL₁₀ at the same tested times (35 days, Fig. 3). These changes could be attributed to its phenolic compounds which act as synthetic antioxidants to prevent lipid oxidation and decomposition of hydroperoxides in living tissues⁴⁶.

Pelargonium contains a rich spectrum of tannin, flavonoid, phenolic acids and coumarin derivatives^{47,48}, whose accumulation is influenced by the activities of enzymes related to their synthesis and oxidation. Strong correlations ($r = 0.43^{**}$) between the PAL activity, and PPO, demonstrated on a similar physiological basis metabolism for these two enzymes as phenylperpanoids pathway. These results are in a good line with previous reports that showed a positive relationship between PAL and PPO enzymes activity in *Calotropis procera* HRs after addition of magnetic nanoparticles (MNPs) and salicylic acid (SA)⁴⁹. Positive and significant correlation between H₂O₂ with PPO ($r = 0.32^{**}$), PAL ($r = 0.25^*$), SOD ($r = 0.28^{**}$) and CAT ($r = 0.29^{**}$), indicating at high antioxidant capacity of these enzymes to scavenging peroxy radicals in HRCs of *P. sidoides*. The lack of correlation between the antioxidant enzymes (APX, and GPX) with PAL, as a marker of phenolics metabolism, suggests that the determination of these enzymes is not an indicator for evaluating the changes in phytochemical marker compounds of HRCs in *P. sidoides*.

Here, the HRs and control samples (untransformed root and shoot plants) express ten compounds, including distinct phenolic acids (GA, CGA, CAF, and TA), hydroxycinnamic acid derivatives (FA, and p-CoA), coumarins (Um), and flavonoids (QE, LU, and RH) variations. An analytical HPLC method for the detection of TA, CGA, LU, and FA in ethanolic HR extracts has not been published previously.

The phytochemical screening of HRCs of *P. sidoides* resulted in an increased production of Um, CGA, RH, TA, FA, LU, and p-CoA contents in the HRs extracts compared with untransformed roots, control shoots and roots. Results obtained revealed that, the content of detected phenolic acids has been different in variants harvest times with different sources of HRs. Generally, all the HRCs of the leaves sources have been shown high production of

phenolic acids compared to the control. The alterations occurring in SMs production of transformed plants may be due to the content of polyamine metabolism, irritation to auxins, agropine synthesis (ags) and co-expression of (*rol*) genes, *rolA*, *rolB*, *rolC*, and *rolD* ⁵⁰. As previously reported, molecular analysis of these RLs confirmed the presence of T_L and T_R T-DNA parts, represented by *rolB*, *rolC*, *aux* and *ags* respectively ²⁶. This suggests that biosynthetic capacity for accumulation of different classes of SMs can be due to the *rolC* gene-induced from the *A. rhizogenes* plasmid ⁵¹. But the real mechanism of advancement is still not well-founded and needs more systematic studies of expression of critical enzymes involved in superior metabolic pathways.

Recently, Panara *et al.* ¹² identified chemical characterization of *P. sidoides* root originated from several classes via UPLC-QToF-MS. Studies have shown that the concentration of Um of field-selected plants is regulated by the annual rainfall of the collection sites ⁵². Due to difficulties in synthesizing of Um, there is a need to identify alternative methods for the production of the compound. Thus, *in vitro* cultures of *P. sidoides* have been studied as viable sources of Um. The analysis of HRs extracts from the three HRLs revealed that increasing root Um concentrations were strongly related to both increasing exposure times of culture, especially in the 3th week of growing in the suspension culture. Furthermore, the HRs from the 3th week of culture is potentially the most economical for harvesting owing to their high Um yield. Additionally, average HRs Um concentration varied significantly different from the Um concentrations of shoot and root plant samples of the tissue culture. This suggests that it may be possible to use HRCs of the *P. sidoides* to identify best lines with high root Um concentrations and hence bioactivity. This would be valuable for sourcing the best biotechnological propagation material for cultivation studies and commercial operations. The suspension culture application of the *P. sidoides* has been demonstrated to increase coumarin production in HRCs previously by (Yousefian *et al.* ²⁶), showing that eliciting treatments can be used to manipulate plant bioactive contents. However, in this study, applications of HR suspension culture had a significantly effect in the content of the root coumarins in particular Um. According to Colling *et al.* ³⁷, the concentrations of main phytochemical compounds such as (QE, GA, CAF, and p-COA) in transgenic roots of *P. sidoides* were found absent or present at deficient levels compared to our results. Our results confirmed various earlier studies, which expounded that the HRs declared higher polyphenolic compounds, e.g., in *Hypericum perforatum* ⁵³, and *Ligularia fischer* ⁵⁴. The authors showed that both the level of CGA, 3-feruloylquinic acid, and the total amount of identified phenolic acids in the transformed shoots were 1.3 times higher than in control shoots of *Hypericum perforatum* ⁵⁵. Furthermore, in the other species, such as Bitter melon (*Momordica charantia* L.), (Cucurbitaceae) the concentration of CGA, GA, FA, CAF, p-COA, and t-cinnamic acid was significantly increased in jasmonic acid (JA) and salicylic acid (SA) elicited HRs in compared to non-elicited HRs ⁵⁶.

Although the mechanism of HRC on Um content needs to be further clarified. These results suggested that the metabolic response of *P. sidoides* HRs to different harvest intervals may be associated with allocation of the carbon flow between the various conflicting pathways ⁵⁷.

The accumulation of various compounds in HRs of *P. sidoides* is influenced by the activation of enzymes related to their synthesis and oxidation ^{39,40}. Correspondingly, Hayashi *et al.* ⁵⁸ showed that in cultured cells of *Glycyrrhiza glabra* regulation of various SMs are at least partially due to activation of key enzymes, which catalyze their biosynthesis. Similar results have also been reported in the establishment and maintenance of long-term cell cultures of *Thymus persicus*, which the highest and the lowest content of terpenoids, were obtained during different times of culture ⁵⁹.

In this study, the HRCs exhibit higher antibacterial activities. These results support the *in vitro* cultivation of HRs to supply as a natural product to prevent inflammation, oxidative stress and microbial related disorders, similar to the previous reports ^{2,14,60}. Antimycobacterial and immunomodulatory efficacy of *P. sidoides* could be attributed to the presence of highly oxygenated coumarins (7-hydroxy-5, 6-di-methoxycoumarin; 6,8-dihydroxy-5,7-dimethoxycoumarin) gallic acid-derivatives, flavonoids, phenolic and hydroxycinnamic acid-derivatives were tested for this particular biological activity ^{2,9}. Here we confirmed the presence of some of these compounds in the transgenic lines. Bacterial inhibition by this HR clone extract is likely in most of the HR clones analyzed by Colling *et al.* ³⁷ which exhibited moderate to high activity against the two Gram-positive (*Bacillus subtilis* and *S. aureus*) and two Gram-negative bacteria (*E. coli* and *Klebsiella pneumoniae*). Similarly, HRs of *Brassica rapa* ssp. *rapa* ⁶¹, *Momordica charantia* ⁶², *Fagopyrum tataricum* Gaertn ⁶³ and *Raphanus raphanistrum* ⁶⁴ have also been contained high amounts of phenolic and flavonoid compounds which exhibited high antioxidant activities.

In the present study, there are differences in the antimicrobial effects of various extracts of HRLs and control samples in *P. sidoides*. Similarly, the study of Mativandlela *et al.* ^{14,16}, reported higher antimicrobial and especially antitubercular activities of *P. sidoides* root extracts against some human and plant pathogens, with ethanol and acetone root extracts rather than chloroform extracts. Lewu *et al.* ⁶⁵ reported that there was a broad-spectrum inhibitory effect of acetone and methanol extracts of the shoot and root of the *P. sidoides* herb on the Gram-negative and Gram-positive bacterial and fungal species. The *Pelargonium* methanolic extracts of HRs showed a more selective antibacterial action against the tested Gram-positive micro-organisms *S. aureus* and Gram-negative micro-organisms *E. coli*. The HRLs displayed reduced antimicrobial activity compared to the methanolic control extracts. The antibacterial activity of HRL₄ against *S. aureus* and *E. coli* suggested that HRL₄ may delay (lower) microbial contamination. *P. sidoides* extract, with its toxicological properties, could be employed as an indigenous medical system for the successful phytopharmaceutical. It also has the potential as a treatment for acute bronchitis and symptoms of the common cold ⁶⁶.

Furthermore, flavonoid derivatives may contribute to the anti-oxidant activity of the *Pelargonium* extracts. Therefore, *in vitro*-regulation of phenolic compounds biosynthesis in *P. sidoides* HR may offer additional advantages of quantitative and qualitative improvements of these medicinally important metabolites.

Conclusions

The results obtained from this study also suggest that the differences in enzymatic-antioxidants (including: PAL, PPO, CAT, GPX, APX, SOD and H₂O₂) activities and biochemical responses in 3 examined *P. sidoides* HRLs were affected under different harvest intervals during 35 days of culture. Using the elite HRLs through higher capacity for maintaining and enhancing the phytochemical compounds, reduce H₂O₂ production and improve in activity of defensive enzymes under different harvest time conditions could be a new strategy for improving tolerance in *P. sidoides* HRLs during culture situations. It should be noted that the HRL₁₀ could be introduced as a promising line for enhanced protein content, PAL, CAT, and PPO responses, and defense enzyme (H₂O₂) status of *P. sidoides* plant under 35 days of suspension culture. Based on the availability data, we conclude that the relationship between individual phenolic compounds, modes-of-actions, and the activity of their corresponding biosynthetic enzymes are still more complex, and poorly understood. Thus, further work for molecular mechanism of these enzymes with relation to phytochemical accumulation in *Pelargonium* are needed for more understanding of their potential physiological roles, and warrant further investigation.

Declarations

Data availability

All data are available in the main text, extended data figures, tables and supplementary data. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

We are special thankful to the Department of Pharmaceutical Biology at the University of Kiel, Germany (Professor Birgit Classen) for providing seeds of *Pelargonium sidoides*. The authors thank Pars Gita Daru Company (Iran) and would like to extend their appreciation to Dr. Mesgarzadeh, for umckalin analysis. The authors gratefully acknowledge the facilities support provided for this survey by the Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran. This study is part of Miss. Zeynab Yousefian PhD's thesis Guilan University, Iran.

Author contributions

ZY designed research, performed all the experiments, acquisition of data, analysis and wrote the drafted manuscript. Dr. YH supervised the stages of experiments and revised the manuscript. Dr. PG supervised all the experiments, contributed to the experimental work especially to biological assay, provided the compounds, partly participated in data analysis, revised the manuscript. Dr. MHM revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

References

1. Kolodziej, H. Traditionally used *Pelargonium* species: chemistry and biological activity of umckaloabo extracts and their constituents. *Curr Top. Phytochem.* **3**, 77–93 (2000).
2. Kolodziej, H. Antimicrobial, antiviral and immunomodulatory activity studies of *Pelargonium sidoides* (EPs® 7630) in the context of health promotion. *J. Pharm.* **4**, 1295–1314 (2011).
3. Dreyer, L. L. & Marais, E. M. Section Reniformia, a new section in the genus *Pelargonium* (Geraniaceae). *South African J. Bot.* **66**, 44–51 (2000).
4. Van der Walt, J. J. A. & Vorster, P. J. *Pelargoniums of Southern Africa*. National Botanic Gardens, Kirstenbosch. Juta, Cape Town. Vol. 3 (1988).
5. Lalli, J. Y. Y. *in vitro* pharmacological properties and composition of leaf essential oils and extracts of selected indigenous *Pelargonium* (Geraniaceae) species. MPharm Thesis, University of Witwatersrand, Johannesburg, South Africa Latte Kp and KolodziejH Pelargoniins, new ellagitannins from *Pelargonium reniforme*. *Phytochem.* **54**, 701–710 (2006).
6. Hsouna, A. B. & Hamdi, N. Phytochemical composition and antimicrobial activities of the essential oils and organic extracts from *Pelargonium graveolens* growing in Tunisia. *Lipids Health Dis.* **11**, 167 (2012).
7. EMA/HMPC/444251/2015. Committee on Herbal Medicinal Products (HMPC). Assessment report on *Pelargonium sidoides* DC and/or *Pelargonium reniforme* Curt., radix. Based on Article 16d (1), Article 16f and Article 16h of Directive 2001/83EC (traditional use) (2018).

8. Kayser, O. & Kolodziej, H. Highly oxygenated coumarins from *Pelargonium sidoides*. *Phytochem.* **39**, 1181–1185 (1995).
9. Brendler, T. & van Wyk, B. E. A historical, scientific and commercial perspective on the medicinal use of *Pelargonium sidoides* (Geraniaceae). *J. Ethnopharmacol.* **119**, 420–433 (2008).
10. Kolodziej, H. *Pelargonium reniforme* and *Pelargonium sidoides*: their botany, chemistry and medicinal use. In: Lis-Balchin, M. (Ed.), *Geranium and Pelargonium*. Taylor & Francis, London, pp. 262–290 (2002).
11. Hudaib, M., Khdaib, A., Salah, R. & Alkhatib, H. S. A simple and rapid HPLC-UV method for the determination of umckalin, as an herbal marker, in the cough syrup of *Pelargonium* extract. *Jordan J Pharm Sci.* **3**(2), 109–116 (2010).
12. Panara, A., Aalizadeh, R. & Thomaidis, N. S. Chemical characterisation of *Pelargonium sidoides* root based on LC-QToF-MS non-target screening strategies. *Phytochem. Anal.* 40–56.
13. Riley, D. S. *et al.* Treatment with EPs 7630, a *Pelargonium sidoides* root extract, is effective and safe in patients with the common cold: results from a randomized, double blind, placebo- controlled clinical trial. *Integr Med (Encinitas)*. **18**(1), 42–51 (2019).
14. Mativandlela, S. P. N., Lall, N. & Meyer, J. J. M. Antibacterial, antifungal and antitubercular activity of (the roots of) *Pelargonium reniforme* (CURT) and *Pelargonium sidoides* (DC) (Geraniaceae) root extracts. *South African J. Bot.* **72**, 232–237 (2006).
15. Lis-Balchin, M. *Geranium and Pelargonium*. Taylor & Francis. London. pp, 317 (2002).
16. Mativandlela, S. P. N., Meyer, J. J. M., Hussein, A. A. & Lall, N. Antitubercular activity of compounds isolated from *Pelargonium sidoides* DC. *Pharm. Biol.* **45**, 645–650 (2007).
17. Galanakis, C. M. The food systems in the Era of the Coronavirus (COVID-19) pandemic crisis. *Foods*. **9**(4), 523 (2020).
18. Keck, T., Strobl, A., Weinhaeusel, A., Funk, P. & Michaelis, M. *Pelargonium* Extract EPs 7630 in the treatment of human corona virus-associated acute respiratory tract infections – a secondary subgroup-analysis of an open-label, uncontrolled clinical trial. *Front Pharmacol.* **12**, 666546 (2021).
19. Kayser, O. & Kolodziej, H. Antibacterial activity of extracts and constituents of *Pelargonium sidoides* and *Pelargonium reniforme*. *Planta Med.* **63**, 508–510 (1997).
20. Kolodziej, H., Kayser, O., Radtke, O. A., Kiderlen, A. F. & Koch, E. Pharmacological profile of extracts of *Pelargonium sidoides* and their constituents. *Phytomedicine* **10**, 18–24 (2003).
21. Srivastava, V., Mehrotra, S., Mishra, S. *Biotechnology, P. Hairy roots: an effective tool of plant biotechnology*. Springer Nature Singapore Pte Ltd. pp, 342 (2018).
22. Smetanska, I. Production of secondary metabolites using plant cell cultures. *Adv. Biochem Engin. Biotechnol.* **111**, 187–228 (2008).
23. Lewu, F. B., Adebola, P. O. & Afolayan, A. J. Commercial harvesting of *Pelargonium sidoides* in the Eastern Cape, South Africa: striking a balance between resource conservation and rural livelihoods. *J. Arid Environ.* **70**, 380–388 (2007).
24. Gödecke, T., Kaloga, M. & Kolodziej, H. A phenol glucoside, uncommon coumarins and flavonoids from *Pelargonium sidoides* DC. *Z Naturforsch B.* **60**, 677–682 (2005).
25. Chandra, S. & Chandra, R. Engineering secondary metabolite production in hairy roots. *Phytochem Rev.* **10**, 371–395 (2011).

26. Yousefian, Z., Golkar, P. & Mirjalili, M. H. Production enhancement of medicinally active coumarin and phenolic compounds in hairy root cultures of *Pelargonium sidoides*: the effect of elicitation and sucrose. *J. Plant Growth Regul.* **40**(2), 628–641 (2020).
27. Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassays with Tohaoco tissue cultures. *Physiol. Plant.* **15**, 473–497 (1962).
28. Bradford, M. M. *et al.* A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).
29. Wang, J. W., Zheng, L. P., Wu, J. Y. & Tan, R. X. Involvement of nitric oxide in oxidative burst, phenylalanine ammonia-lyase activation and Taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. *Plant Cell Physiol.* **14**, 391–397 (2006).
30. Raymond, J., Rakariyatham, N. & Azanza, J. Purification and some properties of polyphenoloxidase from sunflower seeds. *Phytochem.* **34**(4), 927–931 (1993).
31. Aebi, H. E. "Catalase," in *Methods in enzymology* (ed. Packer, L) vol. 105, Academic Press, Orlando, Fla, USA. pp. 121–126 (1974).
32. Zhang, J. F. *et al.* Responses of the antioxidant defenses of the Goldfish *Carassius auratus*, exposed to 2,4-dichlorophenol. *Environ. Toxicol. Pharmacol.* **19**(1), 185–190 (2005).
33. Nakano, Y. & Asada, K. Purification of ascorbate peroxidase in spinach chloroplast: in inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *J. Plant Cell Physiol.* **28**, 131–140 (1987).
34. Beauchamp, C. H. & Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287 (1971).
35. Alexieva, V., Sergiev, I., Mapelli, S. & Karanov, E. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell Environ.* **24**, 1337–1344 (2001).
36. Başkan, S., Öztekin, N. & Erim, F. B. Determination of carnosic acid and rosmarinic acid in sage by capillary electrophoresis. *Food Chem.* **101**, 1748–1752 (2007).
37. Colling, J., Groenewald, J. H. & Makunga, N. P. Genetic alterations for increased coumarin production lead to metabolic changes in the medicinally important *Pelargonium sidoides* DC (Geraniaceae). *Metab. Eng.* **12**, 561–572 (2010).
38. EUCAST. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) technical notes on antimicrobial susceptibility testing. (2015).
39. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing, informational supplement. CLSI document M100. Clinical and Laboratory Standards Institute, USA, 32nd ed (2022).
40. Tanga, M., Lewu, F. B., Oyedeji, O. A. & Oyedeji, O. O. Cultivation of medicinal plants in South Africa: a solution to quality assurance and consistent availability of medicinal plant materials for commercialization. *Acad. J. Med. Plants.* **6**(7), 168–177 (2018).
41. Karatoprak, G. Ş., Göger, F. & Yerer, M. B. Chemical composition and biological investigation of *Pelargonium endlicherianum* root extracts. *Pharm. Biol.* **55**(1), 1608–1618 (2017).
42. Shi, M., Kwok, K. W. & Wu, J. Y. Enhancement of tanshinone production in *Salvia miltiorrhiza* Bunge (red or Chinese sage) hairy-root culture by hyperosmotic stress and yeast elicitor. *Biotechnol Appl Biochem.* **46**,

191–196 (2007).

43. Chayjarung, P. *et al.* Influence of peanut hairy root cultivars on prenylated stilbenoid production and the response mechanism for combining the elicitors of chitosan, methyl jasmonate, and cyclodextrin. *Planta*. **6**, 256–232 (2022).
44. Izuegbuna, O., Otunola, G. & Bradley, G. Estimation of phytochemical, vitamins composition and antioxidant activity of *Pelargonium inquinans* leaves. *Pharmacogn J.* **11**(2), 237–244 (2019).
45. Lin, J. T., Liu, S. C., Kuo, L. C., Yang, D. J. Composition of phenolic compounds and anti-oxidant attributes of *Cyclea gracillima* Diels extracts. *J. Food Drug Anal.* **26**(1), 193–200 (2018).
46. Martins, N., Barros, L. & Ferreira, I. C. F. R. *In vivo* antioxidant activity of phenolic compounds: Facts and gaps. *Trends Food Sci. Technol.* **48**, 1–12 (2016).
47. El-Sherei M. M, El-Sakhawy F. S, Kaseem H. A, Meselhy K. M & Sleem A. A. Bioactive phenolics from *Pelargonium denticulatum* cultivated in Egypt. *Egypt J Biomed Sci.* **15**, 285–300 (2004).
48. Hauer, H., Germer, S., Elsässer, J. & Ritter, T. Benzopyranones and their sulfate esters from *Pelargonium sidoides*. *Planta Med.* **76**(4), 350–352 (2010).
49. Adabavazeh, F., Pourseyedi, SH., Nadernejad, N., Razavizadeh, R. & Mozafari, H. Evaluation of synthesized magnetic nanoparticles and salicylic acid effects on improvement of antioxidant properties and essential oils of *Calotropis procera* hairy roots and seedling. *Plant Cell, Tissue Organ Cult.* **151**, 133–148 (2022).
50. Chaudhuri, K. N., Ghosh, B., Tepfer, D. & Jha, S. Spontaneous plant regeneration in transformed roots and calli from *Tylophora indica*: Changes in morphological phenotype and tylophorine accumulation associated with transformation by *Agrobacterium rhizogenes*. *Plant Cell Rep.* **25**, 1059–1066 (2006).
51. Roychowdhury, D., Chaubey, B. & Jha, S. The fate of integrated Ri T-DNA rol genes during regeneration via somatic embryogenesis in *Tylophora indica*. *J. Bot.* **707831**, 1–16 (2015).
52. White, A. G., Davies-coleman, M. T. & Ripley, B. S. Measuring and optimising umckalin concentration in wild-harvested and cultivated *Pelargonium sidoides* (Geraniaceae). *S Afr J Bot.* **74**, 260–267 (2008).
53. Tusevski, O., Stanoeva, J. P., Stefova, M. & Simic, S. G. Phenolic Profile of dark-grown and photoperiod-exposed *Hypericum perforatum* L. Hindawi Publishing Corporation. *Sci World J.* **602752**, 9 (2013).
54. Ansari, M. A. *et al.* Evaluation of polyphenolic compounds and pharmacological activities in hairy root cultures of *Ligularia fisheri* Turcz. f. *spiciformis* (Nakai). *molecules.* **24**, 1586 (2019).
55. Tusevski, O., Stanoeva, J. P., Stefova, M., Pavokovic, D. & Simic, S. G. Identification and quantification of phenolic compounds in *Hypericum perforatum* L. transgenic shoots. *Acta Physiol. Plant.* **36**, 2555–2569 (2014).
56. Chung, I., Thiruvengadam, M. & Rekha, K. Elicitation Enhanced the Production of Phenolic Compounds and Biological Activities in Hairy Root Cultures of Bitter melon (*Momordica charantia* L.). **59**, 1–10 (2016).
57. Rogowska, A. & Szakiel, A. Enhancement of phytosterol and triterpenoid production in plant hairy root cultures – simultaneous stimulation or competition ? *Plants.* **10**, 2028 (2021).
58. Hayashi, H., Huang, P. & Inoue, K. Up-regulation of soyasaponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*. *Plant Cell Physiol.* **44**, 404–411 (2003).
59. Bakhtiar, Z. & Mirjalili, M. H. Long-term cell suspension culture of *Thymus persicus* (Lamiaceae): A novel approach for the production of anti-cancer triterpenic acids. *Ind. Crop. Prod.* **181**, 114818 (2022).

60. Kolodziej H. & Kiderlen, A. F. *In vitro* evaluation of antibacterial and immunomodulatory activities of *Pelargonium reniforme*, *Pelargonium sidoides* and the related herbal drug preparation EPs® 7630. *Phytomedicine*. **14**(1), 18–26 (2007).
61. Chung, I. M., Rekha, K. & Thiruvengadam, M. Production of glucosinolates, phenolic compounds and associated gene expression profiles of hairy root cultures in turnip (*Brassica rapa* ssp. *rapa*). *3 Biotech*. **6**, 175 (2016).
62. Thiruvengadam, M. *et al.* Establishment of *Momordica charantia* hairy root cultures for the production of phenolic compounds and determination of their biological activities. *Plant Cell. Tissue Organ Cult.* **118**, 545–557 (2014).
63. Huang, X. *et al.* Efficient rutin and quercetin biosynthesis through flavonoids-related gene expression in *Fagopyrum tataricum* gaertn. hairy root cultures with UV-B irradiation. *Front. Plant Sci.* **7**, 63 (2016).
64. Muthusamy, B. & Shanmugam, G. Analysis of flavonoid content, antioxidant, antimicrobial and antibiofilm activity of *in vitro* hairy root extract of radish (*Raphanus sativus* L.). *Plant Cell, Tissue Organ Cult.* **140**, 619–633 (2020).
65. Lewu, F. B., Grierson, D. S. & Afolayan, A. J. Extracts from *Pelargonium sidoides* inhibit the growth of bacteria and fungi. *Pharm. Biol.* **44**, 279–282 (2006).
66. Moyo, M. & Van Staden, J. Medicinal properties and conservation of *Pelargonium sidoides* DC. *J. Ethnopharmacol.* **152**, 243–255 (2014).

Figures

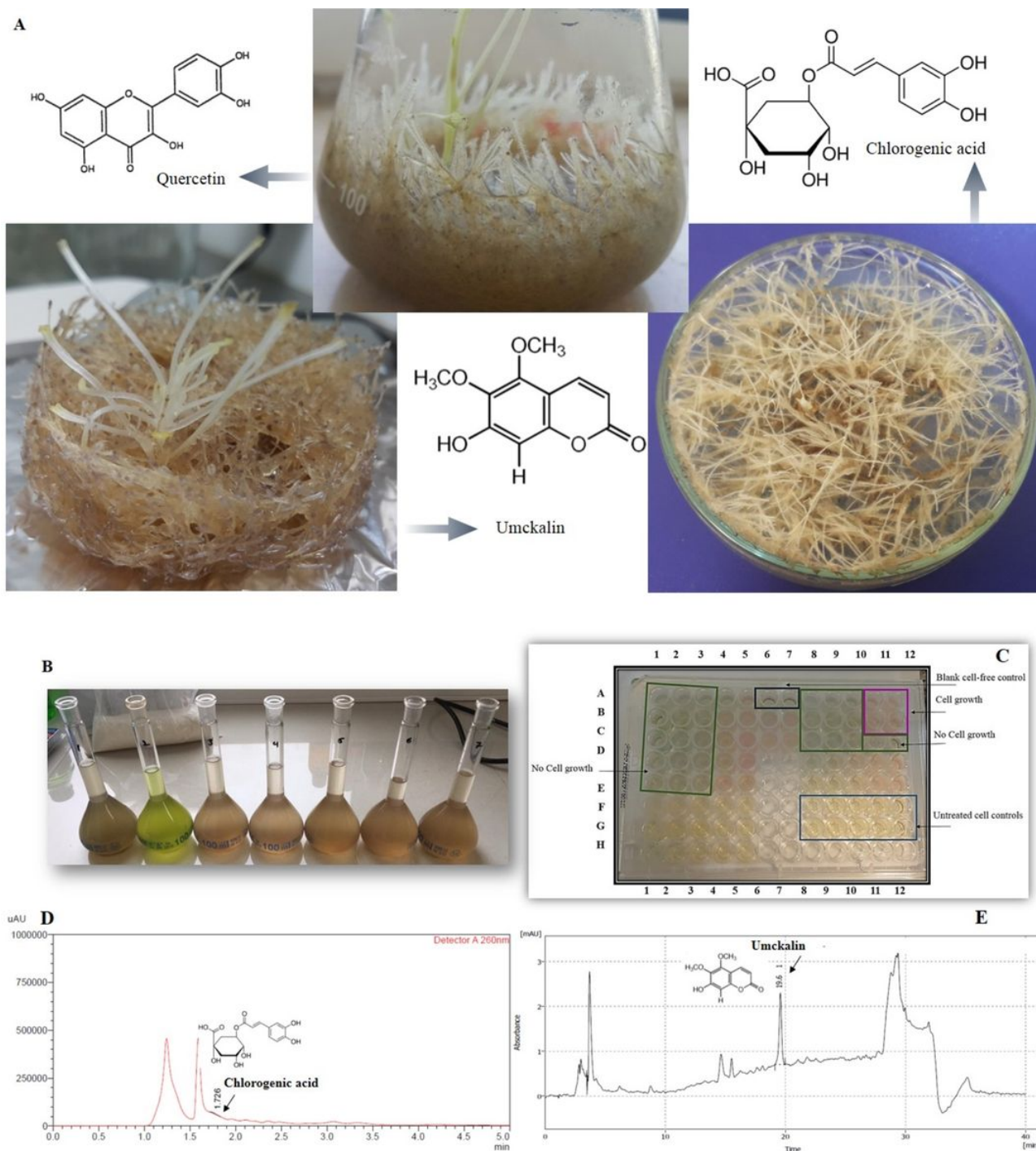


Figure 1

(A) Production of some specific bioactive compounds, such as umckalin, quercetin, and chlorogenic acid in the HRCs of *P. sidoides*. (B) Preparation of different solvent extracts of the HRs, and control samples. (C) Antibacterial activity of HRLs by (Microdilution assay). (D and E) HPLC chromatogram of the *P. sidoides* HRs extracts correspond to Chlorogenic acid, and Umckalin peak in harvested HRs from 3th weeks of culture.

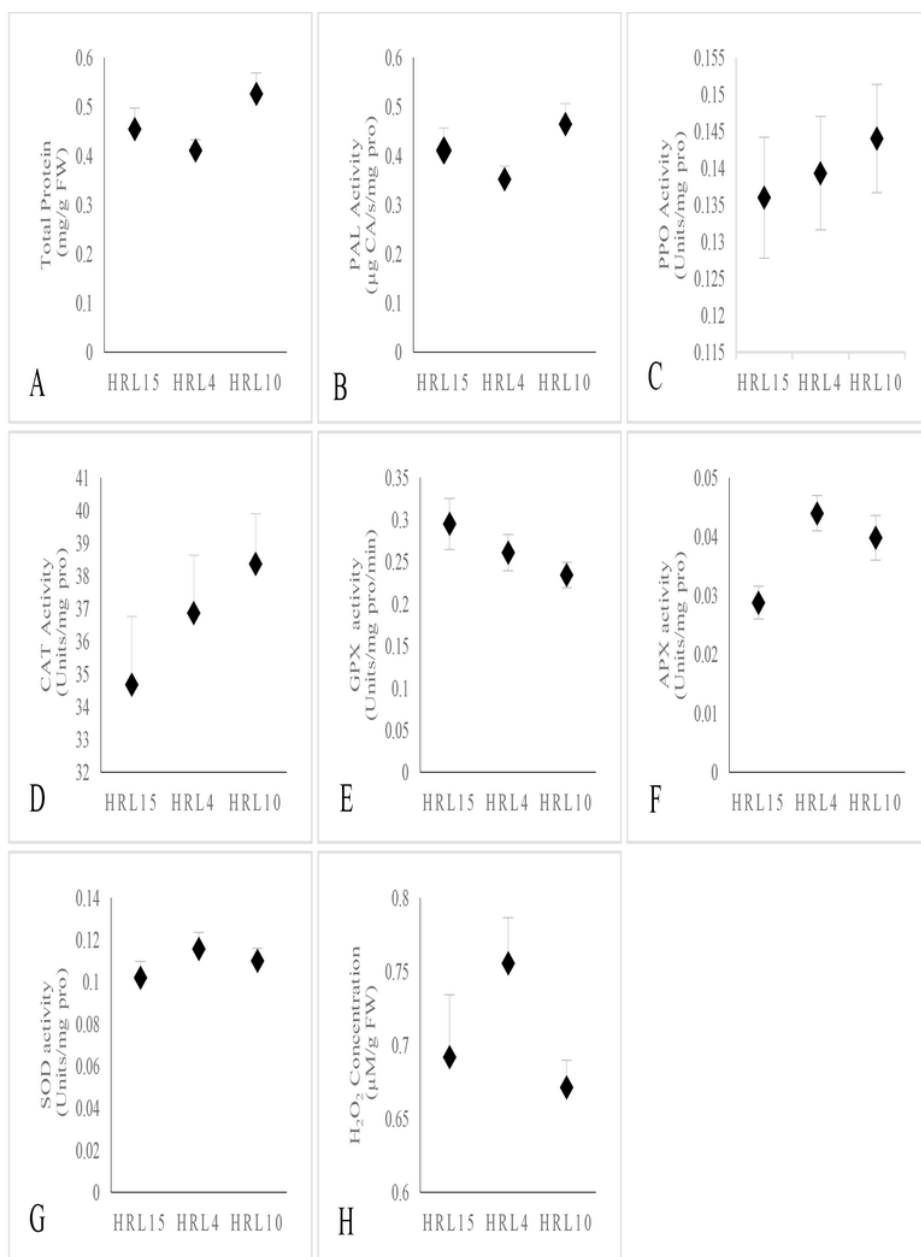


Figure 2.

Figure 2

The means of protein (Pr) content, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD) activity, and Hydrogen peroxide (H_2O_2) concentration (A, B, C, D, E, F, G, and H) from three different HRLs; Data are means $\pm$ SE of three replicates.

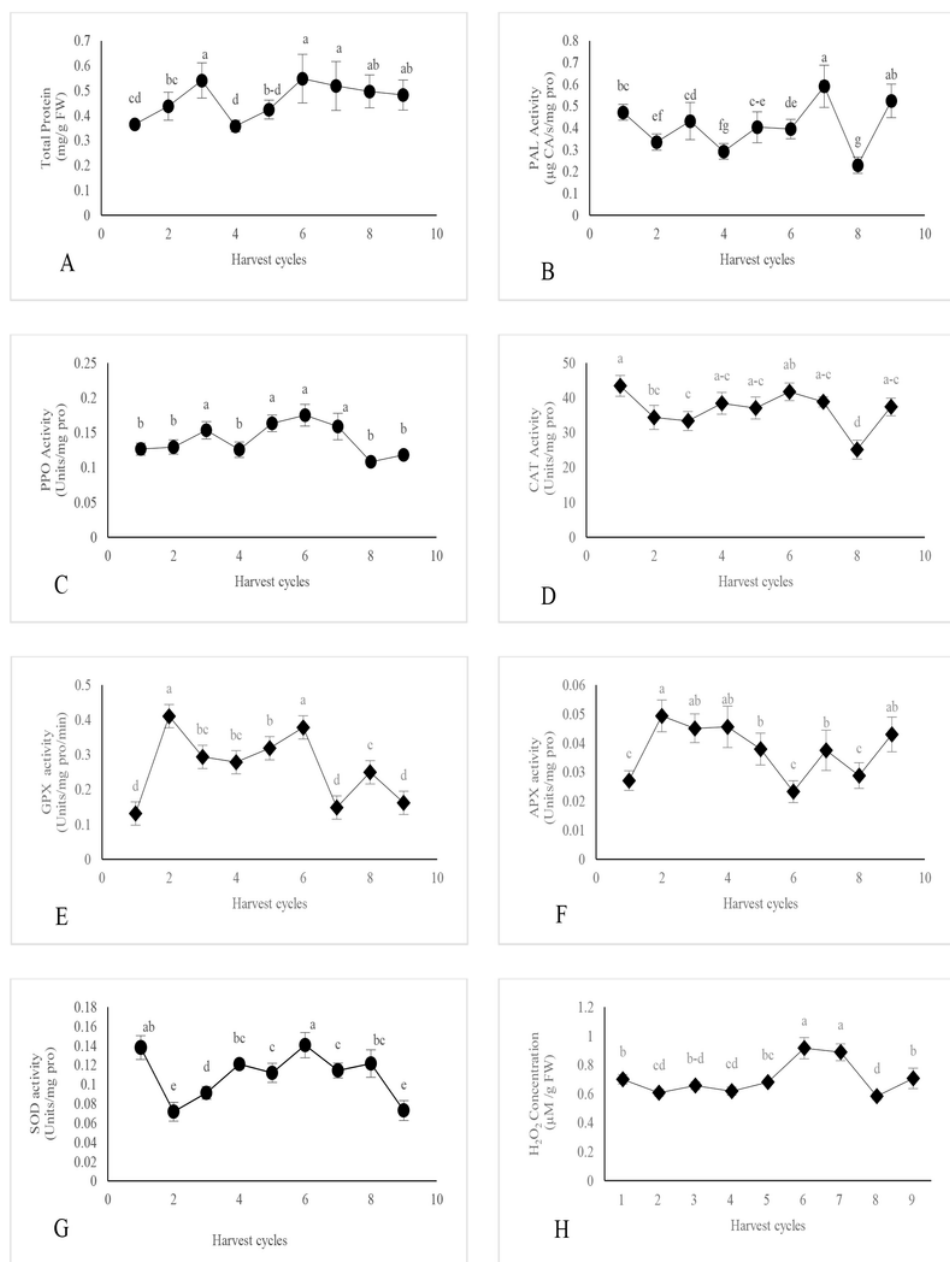


Figure 3.

Figure 3

The means of protein (Pr) content, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD) activity, and Hydrogen peroxide (H₂O₂) concentration (A, B, C, D, E, F, G, and H) under nine different harvest cycles; Data are means ± SE of three replicates. Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's test.

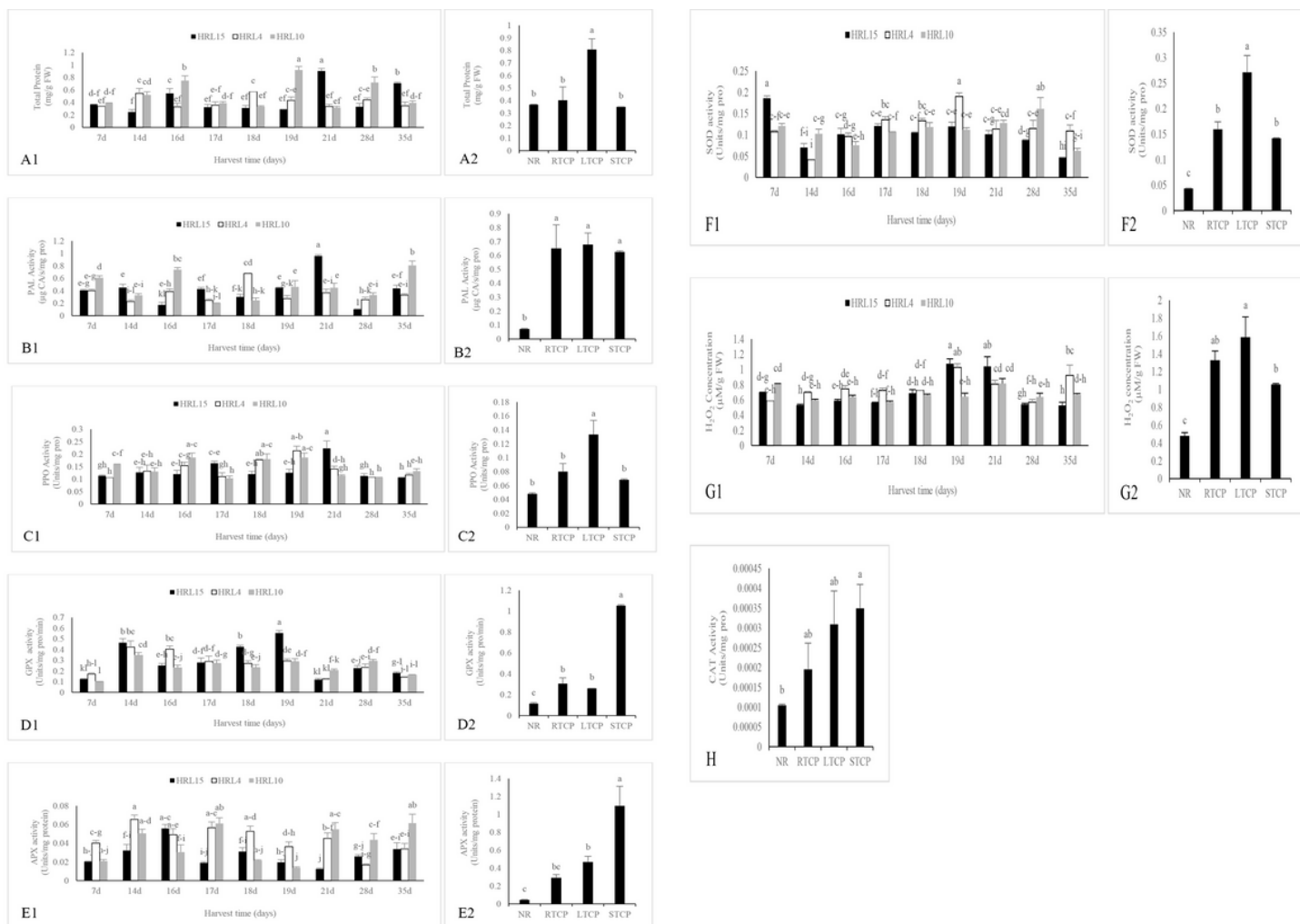


Figure 4.

Figure 4

Effect of different harvest times, during 35 days of incubation, on the protein (Pr) content, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD) activity, and Hydrogen peroxide (H₂O₂) concentration (A1, B1, C1, D1, E1, F1, and G1) in *P. sidoides* suspension-HRLs; concentrations of PRO, PAL, PPO, GPX, APX, SOD activity, H₂O₂ concentration, and catalase (CAT) activity (A2, B2, C2, D2, E2, F2, G2, and H) in control samples including, normal root (NR) of plants grown in chamber room, root tissue culture plantlets (RTCP) and shoot tissue culture plantlets (leaves (LTCP) and stems (STCP)) in *P. sidoides*; Vertical bars indicate mean values $\pm$ SE of three replicates. The mean values followed by the same letter are not significantly different at $P \leq 0.05$, according to the Duncan's test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1.docx](#)