Polyphenols and antioxidant activity of Sulla pallida from mining sites of Morocco: Implication for phytoremediation of heavy metal-contaminated pastures

Anass EL YEMLAHI

elyemlahi@hotmail.fr

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

Hamass ZERRAD

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

Giovanni Caprioli

CHemistry Interdisciplinary Project (CHip), School of Pharmacy, University of Camerino, Via Madonna delleCarceri, 62032 Camerino, Italy

Laura Acquaticci

CHemistry Interdisciplinary Project (CHip), School of Pharmacy, University of Camerino, Via Madonna delleCarceri, 62032 Camerino, Italy

Houssam Annaz

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco; Research Laboratory Biology, Environment and Sustainable Development. ENS. Abdelmalek Essaadi University, Tetouan, Morocco

Mounir HASSANI ZERROUK

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

Amin LAGLAOUI

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

Abdelhay ARAKRAK

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

Mohammed BAKKALI

Research Team of Biotechnology and Biomolecular Engineering, FSTT, Abdelmalek Essadi University Tetouan, Morocco

---

Research Article

Keywords: S.pallida, heavy metals, polyphenolic, antioxidant activity, HPLC-ESI-MS/MS, and phytoremediation

Posted Date: January 30th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-5506939/v1

Abstract

An on-field survey was conducted to assess the physiological and symbiotic response of *Sulla pallida* in an abandoned mining site in Northeast Morocco. Results indicated that *S. pallida* maintained high nodulation and nitrogen efficiency even at late growth stages while accumulating significant levels of Fe, Pb, Zn, and Cu in its aerial parts. Phenol content varied significantly ($p<0.05$) by phenological stage and plant part, with a high concentration of total extractable polyphenols (7.73 %DM) and tannins (6.97 %DM) recorded during the vegetative stage. Antioxidant activity, as shown by DPPH radical scavenging (IC_{50} of 0.126 mg/mL), was comparable to the control (BHT, IC_{50} of 0.110 mg/mL). Anti-lipid peroxidation and ferric reduction potential were highest during flowering, with values outperforming controls in some assays. HPLC-ESI-MS/MS analysis identified 38 compounds, with high levels of 4-Hydroxy benzoic acid, rutin, vanillic, gallic, and p-coumaric acids. These findings highlighted *S. pallid* as a promising forage species for remediating heavy metal-contaminated pastures and promoting sustainable land use.

Introduction

Environmental pollution by heavy metals from is becoming a real issue with a large effect on agricultural productivity, particularly when they depend on symbiotic relationships. Variousanthropogenic activities have persistently contributed to soil contamination, particularly with heavy metals, which often remain in the environment and have detrimental effects on living organisms (Leila et al. 2020; Barillaro et al. 2024).

Such a situation requires the development of methods, such as soil flushing, vapor extraction, landfilling, leaching, and soil fixation, have been extensively implemented to address metal contamination in soil (Marques et al. 2009). However, these methods are expensive, labor-intensive, and often environmentally disruptive, highlighting the need for more sustainable and cost-effective alternatives. In this context, bioremediation involves plants with a high capacity to bio-concentrate and/or bio-immobilize toxic metals from soil, appears as a good and eco-friendly strategy to minimize the impact of heavy metals in soil.

In Mediterranean pastures, species of the genus Sulla (a Fabaceae) represent

a good asset in agricultural economics because of their quality as forage crops (Kadi et al. 2011; Issolah et al. 2014; Errassi et al. 2018) and ability to improve soil fertility (Molinu et al. 2023). Those species, composed of various annual and perennial species, display a high level of genotypic diversity (Duan et al. 2015; Liu et al. 2017), as well as the capacity to form specialized symbiotic relationships with soil bacteria (Kishinevsky et al. 2003; Benhizia et al. 2004).

Among Sulla species, *Sulla pallida* Desf. synonym *Hedysarum pallidum* Desf. (Choi and Ohashi 2003), is a north-African endemic species, preferentially allogamous (Neila and Mohamed 2001), recognized for its nutritional richness and ability to in arid zones such as those located in northeastern Morocco (El Yemlahi et al. 2024). The plant steel grows spontaneously in quite diverse environments (Abdelguerfi-Berrekia et al. 1991; Moore et al. 2006), including those of mining area located in Algeria (Benhizia et al. 2004; Benhamdi et al. 2014; Kassa-Laouar et al. 2019) and in Morocco (Smouni et al. 2010; Lamin et al. 2021), and suggesting eco-physiological adaptation to this particular environment.

Therefore, this original research aims to evaluate the phenolic content and antioxidant activity of natural populations of *S. pallida*, a forage legume growing spontaneously on mining sites in Northeast of Morocco.The findings could provide a basis for improving soil health and promoting sustainable land use in polluted environments.

Material and methods

Plant sampling and nodulation assessment

Plant samples (stems and roots) of *S.pallida*, were collected from an abandoned mining site in the North-eastern of Morocco. Samples were hand-clipped at the two growth stages, early vegetative and flowering stages. Nodulation ability was evaluated using a nodule-scoring chart proposed by Howieson and Dilworth (2016). Effectiveness in nitrogen fixation was assessed at the flowering stage, by measuring shoot dry weight and total N using the Kjeldahl method (Bremner 1965).

Heavy metal analysis

Plants were oven-dried at 60°C, then grounded using POLYMIX® PX-MFC 90 D Hammer mill. Furthermore, a mixture of soil samples was taken from the surface layer (0-15 cm), air-dried, and ground using a porcelain mortar and pestle. The samples were homogenized and sieved for heavy metal analysis, using an Axios X-ray fluorescence (XRF) spectrometer at the Units of Technical Support to Scientific Research (UATRS) of the National Centre for Scientific and Technical Research (CNRST) in Rabat, Morocco. The transfer factor (TF) of heavy metal from soil to plants was determined using the following formula:

$$\% \text{ TF } = \frac{\text{MP}}{\text{MS}} \times 100$$

Where “MP” is themetal concentration in the plant, and “MS” is themetal concentration in the soil.

Phenolics compounds analysis

Plant samples were dried at 50°C and were tested for total extractable phenolics (TEP) and tannins (TET) employing the Folin Ciocalteu method following the procedures outlined by (Makkar 2003).Total extractable condensed tannins (ECT) were determined using the butanol-HCl-Fe^{3+} method (Porter et al. 1985). The liquid extracts were further dried at 40°C and the solid residue was then solubilized in distilled water to determine total flavonoid content (TFC) using the aluminum chloride colorimetric method as outlined by (Nurcholis et al. 2021).

Antioxidant activity evaluation

Free radical scavenging activity

The radical-scavenging activity was investigated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) bioassay following the procedure approved by (Mensor et al. 2001). A concentrated solution of extracts used to determinetotal flavonoid contentwas prepared and then diluted to 2 mg/mL, 1 mg/mL, and 0.5 mg/mL. Briefly, 2.5ml of the serial dilution was transferred into glass test tubes and mixed with 1 mL of 0.3 mM DPPH solution newly prepared in methanol. The mixture was then incubated at room temperature in the dark for 30 minutes. Methanol and BHT (butyl hydroxytoluene) were utilized as negative and positive controls respectively. The absorbance was measured at 517 nm and the antioxidant activity was then calculated as percent inhibition as follows:

$$\% \text{ inhibition} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100$$

Where “Acontrol” is the absorbance of the control experiment and “Asample” is the absorbance of the sample.

Ferric reducing antioxidant power

The antioxidant activity of *S. pallida* extracts was additionally analyzed using the ferric reducing antioxidant power (FRAP assay) as earlier stated by (Lfitat et al. 2021). Briefly, 2.5 ml of extract solutions prepared at different concentrations werecombined with equal volumes of phosphate buffer (0.2 M, pH 6.6) and 1% $K_3Fe[CN]_6$. The obtained solution was incubated in a water bath at 50°C for 20 min,thencentrifuged at 3000 rpm for 10 min after adding 2.5 ml of 10% TCA (Trichloroacetic Acid). 2.5 mL was taken from the supernatant and mixed with 2.5 mL of distilled water and 100 µL of 0.1% iron chloride ($FeCl_3$). The absorbance of the reaction mixture was measured at 700 nm. An increase in the absorbance indicates the high ferric-reducing ability of the extract. Distilled water was used as a blank and rutin as a positive control.

Antioxidant assessment utilizing a β-carotene-linoleate method

The anti-lipid peroxidation activities of the samples were assessed by the b-carotene/linoleate model system (Miller 1971). A stock solution of β-carotene and linoleic acid was prepared with 2 mL of β-carotene (2 mg in 10 mL of chloroform) added to a tube containing 40 mg of linoleic acid and 400 mg of Tween 40. After combining the two phases, the chloroform was completely evaporated, and then 50 mL of oxygen-saturated distilled water was added to the mixture with stirring.Five milliliters of the prepared emulsion were placed into a set of tubes, each containing 0.2 mL of various concentrations of the extracts or BHT. Immediately after the emulsion was added, the absorbance was measured at 470 nm using a UV spectrophotometer. Finally, the tubes were incubated in a water bath at 50°C, and the absorbance was taken after 120 minutes. The antioxidant activity was then calculated as inhibition % of the β-carotene, using the following formula:

$$I\% = \frac{\text{Aantiox120} - \text{Acontrol120}}{\text{Aantiox0} - \text{Acontrol120}} \times 100$$

Where: “$Aantiox_0$” and “$Aantiox_{120}$” are the absorbance of the sample at 0 and 120 min respectively, and “$Acontrol_{120}$” is the absorbance of the control at 120 min.

Half minimal inhibitory concentration (IC_{50})

The extract concentration corresponding to 50% inhibition of DPPH radicals scavenging was extrapolated from the curves of the percentage inhibition against the concentration used.The results are reported as half minimal inhibitory concentration (IC_{50}), which indicates the quantity of extract or standard required to reduce the initial concentration of DPPH radicals by 50%. Similarly, the sample concentration providing 50% β-carotene bleaching (IC_{50}) was determined by plotting the inhibition percentages against the sample concentrations. Finally, the extract concentration needed to reach 0.5 of absorbance for ferric reducing power was calculated from the graph of absorbance, and the results are presented as half minimal inhibitory concentration (IC_{50}). The lower (IC_{50}) value is related to a higher antioxidant activity.

HPLC-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS/MS) analysis

Reagents and standards

Cyanidin-3-glucoside chloride, delphinidin-3,5-diglucoside chloride, delphinidin-3-galactoside chloride, petunidin-3-glucoside chloride, malvidin-3-galactoside chloride, quercetin-3-glucoside and kaempferol-3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The remaining 31 analytical standards of the 38 phenolic compounds were supplied by Sigma-Aldrich (Milan, Italy). Individual stock solutions of each analyte, at a concentration of 1000 mg L^{-1}, were prepared by dissolving pure standards in HPLC-grade methanol and storing them in glass stoppered bottles at 4 °C, except for anthocyanins, which were stored at − 15 °C until analysis. Standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solutions with HPLC-grade methanol. Formic acid (99%) was obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric acid (37%) was obtained from Carlo Erba Reagents (Milan, Italy). HPLC-grade methanol was supplied by Sigma-Aldrich (Milano, Italy). Deionized water (> 18 MΩ cm resistivity) was further purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 µm syringeless filter, Phenomenex (Castel Maggiore, BO, Italy).

HPLC-ESI-MS/MS

HPLC-ESI-MS/MS analysis was conducted on the aerial parts of *S. pallida* using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in negative and positive ionization modes. The instrument was allowed to perform a single run with polarity switching without any problems. MS/MS parameters of each analyte were optimized in flow injection analysis (FIA) (1 µL of a 10 mg L^{-1} individual standard solution) by using Optimizer Software (Agilent). The separation of target compounds was achieved on a Synergi Polar–RP C18 analytical column (250 mm x 4.6 mm, 4 µm) from Phenomenex (Chesire, UK). The column was preceded by a Polar RP security guard cartridge (4 mm x 3 mm ID). The mobile phase was a mixture of (A) water and (B) methanol, both with formic acid 0.1%, at a flow rate of 0.8 mL min^{-1} in gradient elution mode. The composition of the mobile phase varied as follows: 0–1 min, isocratic condition, 20% B; 1–25 min, 20–85% B; 25–26 min, isocratic condition, 85% B; 26–32 min, 85–20% B. All solvents and solutions were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 µL. The temperature of the column was 30 °C, and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 12 L/min, the nebulizer pressure was 55 psi, and the capillary voltage was 4000 V. Detection was performed in the dynamic-multiple reaction monitoring (dynamic-MRM) mode, and the dynamic-MRM peak areas were integrated for quantification. The most abundant product ion was used for quantitation, and the others for qualification. The specific time window for each compound (Δ retention time) was set at 2 min. The selected ion transitions and the mass spectrometer parameters for the analyzed compounds are reported in Table 7.

Statistical analysis

All experiences were conducted in three triplicatesand expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) was carried outusing (Proc GLM) procedure of SAS (SAS Institute Inc., Cary, NC, USA), following the model equation:

$$Y = \mu + s + e$$

Where “μ” is the overall mean, “s” is the growth of the stage, and “e“ is a random error.

Results

Symbiosis and nodulation evaluation

Primary results showed the occurrence of *S. pallida* on alkaline (8.62) silty soil, rich in potassium (289 ppm) and poor in phosphorus (9.50 ppm) and nitrogen (0.15%).Furthermore, the on-field survey of examined plants showed that *S. pallida* had a great symbiosis ability and forms extremely abundant, large, and pink root nodules (Howieson and Dilworth 2016). Results (Table 1) showed high productivity (up to 41.74% of dry matter at the flowering stage) and high nitrogen content (4.14% at the vegetative stage) of *S. pallida* at a mining site located in Northeast Morocco.

Heavy metal analysis

From the elemental analysis point of view, the results of five soil heavy metal analyses are depicted in Table 2. Results showed low concentrations of manganese (1100 mg/kg) and iron (39450 mg/kg) in comparison with maximum permissible limits set by official organizations such as World Health and Food and Agricultural Organization (Table 2). By contrast, high concentrations of lead (8755 mg/kg), followed by zinc (3710 mg/kg) and copper (471 mg/kg) were recorded in the studied zone, generally above the threshold limits (Table 2). Similarly, high levels of iron (4270 mg/Kg), lead (1010.25 mg/kg), zinc (693.75 mg/kg), and copper (129.00 mg/kg), notably above the critical limits, were observed in aerial parts of *S. pallida* evaluated at two maturity stage (Table 2). On the other hand,a low concentration of manganese was recorded (184.50 mg/kg).

Phenolic contents analysis

Regarding thephenolics content of *S. pallida*, results (Table 3) disclosed notable differences (*P<0.05*) of total extractable phenols (TEP) and tannins (TET) content. The higher concentration was noted in the aerial parts at the vegetative stage (7.73 %DM and 6.97 %DM, respectively). Similarly, the amount of total extractable condensed tannin (ECT) was significantly (*P<0.05*) higher at the vegetative stage (4.50 %DM) in comparison with the flowering stage (2.50 %DM) and the root part (0.55 %DM). Total flavonoid content (TFC) was higherat the vegetative stage (9.34 mg/100g DM) compared with the flowering stage (4.88 mg/100g DM) and the roots part (3.19 mg/100g DM).

Antioxidant activity analysis

DPPH radical scavenging activity

The antioxidant properties of *S. pallida* extracts were assessed using DPPH free radical scavenging activity assay (Table 4). The results revealed a dose-dependent (*p<0.05*) variation in the extract activity of *Sulla* evaluated at two growth stages. The (IC_{50}) value which is the concentration of the extracts needed to scavenge 50% of the initial DPPH radicals, was higher (*p<0.05*) at the vegetative stage (IC_{50}=0.126) compared with the flowering stage (IC_{50}=0.416). At 1 mg/mL of the extract, *S. pallida* at the vegetative stage demonstrated a high percentage of free radical scavenging ability up to 81.88%, similar to that obtained by the synthetic one (BHT) (82.1%).

Ferric reducing antioxidant power

Likewise, the extracts of *S. pallida* set at three different concentrations (0.25, 0.5, and 1 mg/mL) showed high ferric reducing power (IC_{50}=0.179 mg/mL) at the vegetative stage and generating (OD700) values of 0.590, 0.975 and 1.032 respectively (Table 5). These values are slightly lower (*P<0.005*) in comparison with those recorded at the flowering stage (0.765, 1.001 and 1.121) with (IC_{50}=0.112 mg/mL). However, they were much higher in comparison with the root part (0.192, 0.347 and 0.548) (IC_{50}=0.964 mg/mL) or with the positive control (0.430, 0.674 and 0.961) (IC_{50}=0.310 mg/mL).

β-carotene bleaching test

The inhibitory effect of *S. pallida* extract on lipid peroxidation was assessed using the beta-carotene/linoleic acid bleaching assay, which founded on the reduction of the yellow color of β-carotene due to its interaction with radicals generated during the oxidation of linoleic acid. Results show that the upper parts of *S. pallida* extract set to 1 mg/mL, possessed a higher antioxidant effect at vegetative (84.83%) and flowering stage (83.00%) than BHT (63.30%) with an IC_{50} equal to 0.39 mg/mL and 0.56mg/mL respectively (Table 6).

HPLC-ESI-MS/MS profile

The phenolic compounds in the aqueous extract of S. pallida were identified through HPLC-ESI-MS/MS, detecting 38 compounds (Table 7). The findings show that 16 phenolic compounds were present with a total concentration of 2297.17 mg/kg, including 4-Hydroxy benzoic acid (401.30 mg/kg) and rutin (313.51 mg/kg), along with vanillic acid (245.26 mg/kg), gallic acid (243.44 mg/kg), and p-coumaric acid (209.29 mg/kg) were the most abundant compounds, representing over 61% of the extract (Table 8).

Discussion

The current data showed that *S. pallida* is able to maintain high symbiotic efficiency in term of nitrogen content and dry matter accumulation, similar and/or higher in comparison with other Sulla species evaluated under field conditions (Fitouri et al. 2012; El Yemlahi et al. 2019). Such findings indicate the aptitude of *S. pallida* to establish symbiosis withsoil microorganismsand promoting sustainable agriculture (El Yemlahi et al. 2019; M'saouar et al. 2020). In fact, earlier study revealed that the bacteria isolated from root-nodules of *S. pallida*, collected from the same minging site, had a great similarity to rhizobial bacteria (El Yelmlahi et al., 2019). Those strains are able to fix atmospheric nitrogen in a symbiotic relationship with the host plants, thereby enriching soil fertility and reducing the need for synthetic fertilizers.

On the other hand, the field survey revealed the great capacity of *S. pallida* to accumulate high concentrations ofiron (Fe), lead (Pb), zinc (Zn), and copper (Cu), in its upper part. Some of those metals such as Fe, Zn, and Cu, are essential to maintain various biochemical and physiological functions. While others, such Pb, pose a significant health concern due to their toxic effects (Yang et al. 2022).

Similar findings have been advanced by (Benhamdi et al. 2014; Kassa-Laouar et al. 2019), who reported the ability of *S. pallida* growing wild in the old mining sites located in Algeria, to accumulate antimony (Sb) and arsenic (As) in shoots and roots. Particular results have been attributed to the presence of heavy metal-resistant soil bacteria (Benhizia et al. 2004; Hamane et al. 2020; Oubohssaine et al. 2022),and ability of the plant to induce significant increases in phenolic compoundsand enzymes, with high antioxidant activities (Benhamdi et al. 2014; Kassa-Laouar et al. 2019; Usman et al. 2020; Oubohssaine et al. 2022). In fact, phenolic compounds, such as tannins, frequently play a master role in responding to various abiotic and biotic stresses (Dehghanian et al. 2022; Kumar et al. 2023).

To emphasize, several chemical compounds, including flavonoidsand condensed tannins, have been identified in the genus Sulla, which confer antioxidant, anti-tumor, anti-aging, and anti-diabetic properties (Tibe et al. 2011; Dong et al. 2013; Molinu et al. 2023). In this study, high concentration of total extractable phenolics, mainly consisting of tannins, were measuredwhen compared with related species such as *S. flexeuosa* grown in different habitats (Errassi et al. 2018), or with those obtained for *S.coronaria* examined at different phonological stages (Amato et al. 2005), using different preservation methods (Rufino-Moya et al. 2019). A study carried out by (Lavid et al. 2001) demonstrated that waterlily (Nymphaea) rich in tannin are tolerant to Pb by directly chelating the Pb.Results indicated by (Jańczak-Pieniążek et al. 2022) showed that heavy metals such as (Cu) and (Pb) caused an improvementin the activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia lyase (TAL), a key enzyme involved in the biosynthesis of phenolic compounds with an antioxidant function (Tian and Lei 2006).

Furthermore, the results of measured phenolic compounds disclose high concentration of soluble condensed tanninin the aerial partsof *S. pallida* in comparison with *S. flexeuosa* (Errassi et al. 2018) or *S. coronaria* (Molinu et al. 2023)over the same growth of stage. Furthermore, the results ofHPLC-ESI-MS analysis of the aerial parts of *S. pallida* showed a high concentration of coumaric acid with great scavenging and antioxidative properties (Roychoudhury et al. 2021). Additionally, the high concentration of hydroxybenzoic acid detected in this study has been reported to exhibit various biological activities including antioxidant, antibacterial, and antifungal properties (Cueva et al. 2010), as well as to modulate the effect of short-term drought and freezing stress on *Triticum aestivum* L. plants (Horváth et al. 2007). Studies conducted by (Yang et al. 2008) shed light on rutin's potent ability to scavenge free radicals, superoxide anion radicals, and hydroxyl radicals, as well as its effect on lipid peroxidation.

Remarkably, significant amounts of vanillic acid and gallic acid werealso detected in the extract of *Sulla pallida.* The phenolic compound gallic acid has been shown to possess strong antioxidant activity and to enhance the activity of endogenous antioxidant enzymes (Singh et al. 1999), such as those evaluated by (Benhamdi et al. 2014).Vanillic acid, on the other hand, can be effective upon different environmental stresses byinducing a plant antioxidant defense, methylglyoxal detoxification system, and osmoregulation (Parvin et al. 2024). Equally important, the syringic acid, revealed by HPLC-ESI-MS analysis, plays a crucial role in the communication between plants and soil microorganisms (Zhou et al. 2014). In this regard, the findings obtained by (Hamane et al. 2020) highlighted the presenceof high diversity soil bacteria, with great plant growth-promoting rhizobacteria (PGPR) activities, isolated from root-nodules of *S. pallida* collected from the same studied site.

Such compounds might serve collectively as antioxidants to detoxify ROSs generated under heavy metal stress conditions (Sakihama et al. 2002; Kumar et al. 2023). In this study, the antioxidant activities evaluation of *S. pallida* extract showed high radical scavenging capacity, and ferric-reducing ability, in comparison with results reported by (Hafsi et al. 2017) for *S. carnosa* grown under salinity and potassium deficiency, whichtended to be lower as the plant progressed from vegetative to flowering stage (Molinu et al. 2023).

Conclusion

The findings of the present study demonstrated a significant potential for *S. pallida* as a forage legume due to its strong mean DPPH radical scavenging activity (IC_{50} 0.271 $mg.mL^{-1}$), ferric reducing power (IC_{50} 0.145 $mg.mL^{-1}$), and ability to inhibit lipid peroxidation (IC_{50} 0.374 $mg.mL^{-1}$). Additionally, its great ability to accumulate high concentration of heavy metals, particularly for Pb (1610 mg/Kg DM), and to maintain remarkable symbiosis with soil microorganisms, suggests that *S. pallida* could be an effective plant for remediating and rehabilitating heavy metal-contaminated pastures and maintaining a consistent, long-term land use in heavy metal polluted environments.

Declarations

Acknowledgments

The authors would like to thank the National Centre for Scientific and Technical Research (CNRST) for mineral analysis.

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

All authors contributed to the study conception and design. Data collection was conducted by Mounir HASSANI ZERROUK, Amin LAGLAOUI, Abdelhay ARAKRAK, and Mohammed BAKKALI. Material preparation, and analysis were performed by Anass EL YEMLAHI, HamassZERRAD, Giovanni Caprioli, Laura Acquaticci. The first draft of the manuscript was written by Anass EL YEMLAHI. All authors read and approved the final manuscript.

Funding

Not applicable

Competing Interests

The authors declare that they have no conflicting interests

Availability of data and materials

Not applicable

References

1. Abdelguerfi-Berrekia R, Abdelguerfi A, Bounaga N, Guittonneau GG (1991) Répartition des espèces spontanées du genre *Hedysarum* L. en Algérie, en relation avec certains facteurs du milieu. *Fourrages* 126:187–207.
2. Amato G, Di Miceli G, Giambalvo D, Scarpello C, Stringi L (2005) Condensed tannins content in Sulla (*Hedysarum coronarium* L.) as affected by environment, genotype and growth stage. In: Bullitta S (ed) *Bioactive compounds in pasture species for phytotherapy and animal welfare*. CNR-ISPAAM, Sassari, pp 41–54. http://hdl.handle.net/10447/8477
3. Barillaro J, Soto da Costa L, Gómez-Corea WN, et al (2024) Landscape degradation drives metal bioaccumulation in bats from Atlantic Forest cacao region, Brazil. Environmental Science and Pollution Research 1–15. https://doi.org/10.1007/S11356-024-35478-X/METRICS
4. Benhamdi A, Bentellis A, Rached O, et al (2014) Effects of antimony and arsenic on antioxidant enzyme activities of two steppic plant species in an old antimony mining area. Biol Trace Elem Res 158:96–104.

https://doi.org/10.1007/S12011-014-9917-7

5. Benhizia Y, Benhizia H, Benguedouar A, et al (2004) Gamma proteobacteria can nodulate legumes of the genus Hedysarum. Syst Appl Microbiol 27:462–468. https://doi.org/10.1078/0723202041438527
6. Bremner JM (1965) Total nitrogen. In: Norman AG (ed) *Methods of soil analysis*. Agron Monogr 9:1149–1178. https://doi.org/10.2134/agronmonogr9.2.c32
7. Choi BH, Ohashi H (2003) Generic criteria and an infrageneric system for Hedysarum and related genera (Papilionoideae-Leguminosae). Taxon 52:567–576. https://doi.org/10.2307/3647455
8. Cueva C, Moreno-Arribas MV, Martín-Álvarez PJ, et al (2010) Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. Res Microbiol 161:372–382. https://doi.org/10.1016/J.RESMIC.2010.04.006
9. Dehghanian Z, Habibi K, Dehghanian M, et al (2022) Reinforcing the bulwark: unravelling the efficient applications of plant phenolics and tannins against environmental stresses. Heliyon 8:e09094. https://doi.org/10.1016/J.HELIYON.2022.E09094
10. Dong Y, Tang D, Zhang N, et al (2013) Phytochemicals and biological studies of plants in genus Hedysarum. Chem Cent J 7:1–13. https://doi.org/10.1186/1752-153X-7-124/TABLES/1
11. Duan L, Wen J, Yang X, et al (2015) Phylogeny of Hedysarum and tribe Hedysareae (Leguminosae: Papilionoideae) inferred from sequence data of ITS, matK, trnL-F and psbA-trnH. Taxon 64:49–64. https://doi.org/10.12705/641.26
12. El Yemlahi A, Arakrak A, Laglaoui A, Bakkali M (2019) Preliminary characterization of root-nodule bacteria isolated from forage legumes of the genus *Hedysarum* in northern Morocco. Moroccan J Biol 16:30–34.
13. El Yemlahi A, Abdelhay A, Laglaoui A, et al (2024) Potential contribution of *Sulla spinosissima* subsp. *capitata* and *Sulla pallida* as a forage crop in arid Mediterranean regions. Arid Land Research and Management 38:382–408. https://doi.org/10.1080/15324982.2024.2306618
14. Errassi A, Ayadi M, Chabbi M, Noutfia A, Jaber A (2018) Phenolic Composition of *Hedysarum flexuosum* (Sulla) in Northwestern Morocco. *J Mater Environ Sci* 9:1662–1667.
15. Fitouri SD, Trabelsi D, Saïdi S, et al (2012) Diversity of rhizobia nodulatingsulla (Hedysarum coronarium L.) and selection of inoculant strains for semi-arid Tunisia. Ann Microbiol 62:77–84. https://doi.org/10.1007/S13213-011-0229-2/METRICS
16. Hafsi C, Falleh H, Saada M, et al (2017) Potassium deficiency alters growth, photosynthetic performance, secondary metabolites content, and related antioxidant capacity in Sulla carnosa grown under moderate salinity. Plant Physiology and Biochemistry 118:609–617. https://doi.org/10.1016/J.PLAPHY.2017.08.002
17. Hamane S, Zerrouk MH, Lyemlahi AE, et al (2020) Screening and Characterization of Phosphate-Solubilizing Rhizobia Isolated from Hedysarum pallidum in the Northeast of Morocco. 113–124. https://doi.org/10.1007/978-981-15-2576-6_7
18. Horváth E, Pál M, Szalai G, et al (2007) Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. Biol Plant 51:480–487. https://doi.org/10.1007/S10535-007-0101-1/METRICS
19. Howieson JG, Dilworth MJ (2016) *Working with rhizobia*. Australian Centre for International Agricultural Research (ACIAR), ACIAR Monograph No. 173, Canberra, Australia, 312p.
20. Issolah R, Tahar A, Alane F, et al (2014) Analysis of the growth and the chemical composition within some algerian populations of Sulla. Journal of Biological Sciences 14:220–225. https://doi.org/10.3923/JBS.2014.220.225

21. Jańczak-Pieniążek M, Cichoński J, Michalik P, Chrzanowski G (2022) Effect of Heavy Metal Stress on Phenolic Compounds Accumulation in Winter Wheat Plants. Molecules 28:. https://doi.org/10.3390/MOLECULES28010241
22. Kadi SA, Guermah H, Bannelier C, et al (2011) Nutritive value of sun-dried sulla (hedysarumflexuosum) and its effect on performance and carcass characteristics of growing rabbits. World Rabbit Science 19:151–159. https://doi.org/10.4995/WRS.2011.848
23. Kassa-Laouar M, Mechakra A, Rodrigue A, et al (2019) Antioxidative Enzyme Responses to Antimony Stress of Serratia marcescens – an Endophytic Bacteria of Hedysarum pallidum Roots. Pol J Environ Stud 29:141–152. https://doi.org/10.15244/PJOES/100494
24. Kishinevsky BD, Nandasena KG, Yates RJ, et al (2003) Phenotypic and genetic diversity among rhizobia isolated from three Hedysarum species: H. spinosissimum, H. coronarium and H. flexuosum. Plant Soil 251:143–153. https://doi.org/10.1023/A:1022967213088/METRICS
25. Kumar K, Debnath P, Singh S, Kumar N (2023) An Overview of Plant Phenolics and Their Involvement in Abiotic Stress Tolerance. Stresses 2023, Vol 3, Pages 570-585 3:570–585. https://doi.org/10.3390/STRESSES3030040
26. Lamin H, Alami S, Bouhnik O, et al (2021) Identification of the endosymbionts from Sulla spinosissima growing in a lead mine tailings in Eastern Morocco as Mesorhizobiumcamelthornisv. aridi. J Appl Microbiol 130:948–959. https://doi.org/10.1111/JAM.14834
27. Lavid N, Schwartz A, Yarden O, Tel-Or E (2001) The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (Nymphaeaceae). Planta 212:323–331. https://doi.org/10.1007/S004250000400
28. Leila B, Loubna B, Ali B (2020) Impacts of mining activities on soil properties: case studies from Morocco mine sites. Soil Sci Ann 71:395–407. https://doi.org/10.37501/soilsa/133011
29. Lfitat A, Zejli H, Bousraf FZ, et al (2021) Comparative assessment of total phenolics content and in vitro antioxidant capacity variations of macerated leaf extracts of Olea europaea L. and Argania spinosa (L.) Skeels. Mater Today Proc 45:7271–7277. https://doi.org/10.1016/J.MATPR.2020.12.990
30. Liu PL, Wen J, Duan L, et al (2017) Hedysarum L. (Fabaceae: Hedysareae) Is Not Monophyletic – Evidence from Phylogenetic Analyses Based on Five Nuclear and Five Plastid Sequences. PLoS One 12:e0170596. https://doi.org/10.1371/JOURNAL.PONE.0170596
31. Makkar HPS (2003) Quantification of Tannins in Tree and Shrub Foliage. Quantification of Tannins in Tree and Shrub Foliage. https://doi.org/10.1007/978-94-017-0273-7
32. Marques APGC, Rangel AOSS, Castro PML (2009) Remediation of Heavy Metal Contaminated Soils: Phytoremediation as a Potentially Promising Clean-Up Technology. Crit Rev Environ Sci Technol 39:622–654. https://doi.org/10.1080/10643380701798272
33. Mensor LL, Menezes FS, Leitão GG, et al (2001) Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 15:127–130. https://doi.org/10.1002/PTR.687
34. Miller HE (1971) A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc 48:91–91. https://doi.org/10.1007/BF02635693
35. Molinu MG, Sulas L, Campesi G, et al (2023) Subterranean Clover and Sulla as Valuable and Complementary Sources of Bioactive Compounds for Rainfed Mediterranean Farming Systems. Plants (Basel) 12:. https://doi.org/10.3390/PLANTS12020417
36. Moore G, Sanford P, Wiley T (2006) Herbaceous perennial legumes. *Perennial pastures for Western Australia*. Department of Agriculture and Food Western Australia, Bulletin 4690, Perth, Australia.

37. M'saouar R, Bakkali M, Laglaoui A, Arakrak A (2020) Isolation and characterization of the roots and soil endomycorrhizae of *Hedysarum pallidum* Desf. in the northeast of Morocco. In: Kumar M, Kumar V, Prasad R (eds) *Phyto-Microbiome in Stress Regulation*. Environmental and Microbial Biotechnology. Springer, Singapore. https://doi.org/10.1007/978-981-15-2576-6_4
38. Neila TF, Mohamed M (2001) Hedysarum phylogeny mediated by RFLP analysis of nuclear ribosomal DNA: RFLP and Hedysarum phylogeny. Genet Resour Crop Evol 48:339–345. https://doi.org/10.1023/A:1012051410501/METRICS
39. Nurcholis W, Sya'bani Putri DN, Husnawati H, et al (2021) Total flavonoid content and antioxidant activity of ethanol and ethyl acetate extracts from accessions of Amomum compactum fruits. Annals of Agricultural Sciences 66:58–62. https://doi.org/10.1016/J.AOAS.2021.04.001
40. Oubohssaine M, Sbabou L, Aurag J (2022) Native Heavy Metal-Tolerant Plant Growth Promoting Rhizobacteria Improves Sulla spinosissima (L.) Growth in Post-Mining Contaminated Soils. Microorganisms 2022, Vol 10, Page 838 10:838. https://doi.org/10.3390/MICROORGANISMS10050838
41. Parvin K, Hasanuzzaman M, Mohsin SM, et al (2024) Vanillic Acid Modulates Antioxidant Defense and Methylglyoxal Detoxification Systems to Combat Drought Stress in Tomato Seedlings. Plants 2024, Vol 13, Page 3114 13:3114. https://doi.org/10.3390/PLANTS13223114
42. Porter LJ, Hrstich LN, Chan BG (1985) The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223–230. https://doi.org/10.1016/S0031-9422(00)94533-3
43. Roychoudhury S, Sinha B, Choudhury BP, et al (2021) Scavenging Properties of Plant-Derived Natural Biomolecule Para-Coumaric Acid in the Prevention of Oxidative Stress-Induced Diseases. Antioxidants (Basel) 10:. https://doi.org/10.3390/ANTIOX10081205
44. Rufino-Moya PJ, Blanco M, Bertolín JR, Joy M (2019) Effect of the method of preservation on the chemical composition and in vitro fermentation characteristics in two legumes rich in condensed tannins. Anim Feed Sci Technol 251:12–20. https://doi.org/10.1016/J.ANIFEEDSCI.2019.02.005
45. Sakihama Y, Cohen MF, Grace SC, Yamasaki H (2002) Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. Toxicology 177:67–80. https://doi.org/10.1016/S0300-483X(02)00196-8
46. Sbabou L, Idir Y, Bruneel O, Le Quere A, Aurag J (2016) Characterization of root-nodule bacteria isolated from *Hedysarum spinosissimum* L., growing in mining sites of northeastern region of Morocco. *SOJ Microbiol Infect Dis* 4:1–8. https://doi.org/10.15226/sojmid/4/3/00156
47. Singh K, Khanna AK, Chander R (1999) Protective effect of ellagic acid on *t*-butyl hydroperoxide-induced lipid peroxidation in isolated rat hepatocytes. *Ind J Exp Biol* 37:939–943.
48. Smouni A, Ater M, Auguy F, Laplaze L, El Mzibri M, Berhada F, Filali-Maltouf A, Doumas P (2010) Évaluation de la contamination par les éléments-traces métalliques dans une zone minière du Maroc oriental. *Cah Agric* 19:273–279. https://doi.org/10.1684/agr.2010.0413
49. Sulas L, Seddaiu G, Muresu R, Roggero PP (2009) Fixation de l'azote du sulla en conditions méditerranéennes. *Agron J* 101:1470–1478. https://doi.org/10.2134/agronj2009.0150
50. Tian X, Lei Y (2006) Nitric oxide treatment alleviates drought stress in wheat seedlings. Biol Plant 50:775–778. https://doi.org/10.1007/S10535-006-0129-7/METRICS
51. Tibe O, Meagher LP, Fraser K, Harding DRK (2011) Condensed tannins and flavonoids from the forage legume sulla (*Hedysarum coronarium*). J Agric Food Chem 59:9402–9409. https://doi.org/10.1021/JF2014759

52. Usman K, Abu-Dieyeh MH, Zouari N, Al-Ghouti MA (2020) Lead (Pb) bioaccumulation and antioxidative responses in Tetraenaqataranse. Scientific Reports 2020 10:1 10:1–10. https://doi.org/10.1038/s41598-020-73621-z
53. Yang J, Guo J, Yuan J (2008) In vitro antioxidant properties of rutin. LWT - Food Science and Technology 41:1060–1066. https://doi.org/10.1016/J.LWT.2007.06.010
54. Yang Z, Li X, Tian L, et al (2022) Heavy metals exposure is associated with early liver dysfunction among rural residents aged 40-75 years in southwest China. J Appl Toxicol 42:1044–1056. https://doi.org/10.1002/JAT.4276
55. Zhou XG, Wu FZ, Xiang WS (2014) Syringic acid inhibited cucumber seedling growth and changed rhizosphere microbial communities. Plant Soil Environ 60:158–164. https://doi.org/10.17221/924/2013-PSE

Tables

Table 1. Symbiosis efficiency of *S. pallida* evaluated at mining site

	Aerial dry matter (%)	Nitrogen content (%)
Vegetative stage	19.99±0.91	4.14±0.01
Flowering stage	41.74±0.34	2.00±0.08
Mean	33.04	2.86
SEM	5.33	0.52
P-value	0.0001	0.0001

Values represent an average of triplicates ± standard deviation. SEM: standard error of the mean.

Table 2. Mean concentrations of heavy metals (mg/kg DM) in soil and plants samples of *S. pallida* at mining site

	Soil		Plants		Transfer factor (%)
	Samples	MPLS[a]	Samples	MPLF[a]	
Fe	39450±777.82	50000	4270±2035.89	1000	10.82
Pb	8755±162.63	100	1010.25±732.17	30	11.54
Zn	3710±127.28	300	693.75±449.24	60	18.70
Mn	1100±0.00	2000	184.50±75.66	1000	16.77
Cu	471±7.07	100	129.00±42.43	40	27.39

Fe: iron; Pb: lead; Zn: zinc; Mn: manganese; Cu: copper; MPLS: maximum permissible limits in soil. MPLF: maximum permissible limits in animal feeds.

[a]According to World Health Organization (WHO) and Food and Agricultural Organization (FAO) (2001; 2011)

Table 3. Polyphenols content of *S. pallida* at mining site

	Aerial parts		Root parts	SEM	P-value
	Vegetative stage	Flowering stage			
TEP[a]	7.73±0.21[a]	5.32±0.22[b]	1.32±0.06[c]	0.937	0.0001
TET[a]	6.97±0.23[a]	5.03±0.15[b]	0.95±0.13[c]	0.888	0.0001
NTP[a]	0.77±0.03[a]	0.28±0.06[b]	0.37±0.07[b]	0.077	0.0001
ECT[b]	4.50±0.29[a]	2.50±0.04[b]	0.55±0.07[c]	0.572	0.0001
TFC[c]	9.34±0.71[a]	4.88±0.91[b]	3.19±0.65[b]	1.186	0.0086

Different letters in the same row indicate significant differences ($p < 0.05$). Values represent an average of triplicates ± standard deviation.TEP: total extractable phenolics; TET: total extractable tannins; TFC: total flavonoid content; ECT: extractible condensed tannin; NTP: non-tannic phenolics; SEM: standard error of the mean.

[a] expressed as eq-g Tanic acid/100g DM.

[b] expressed eq-g Leucocyanidin/100g DM.

[c] expressed as eq-mg Rutin/100g DM.

Table 4. DPPH free radical scavenging activity of *S. pallida* at mining site

		DPPH free radical scavenging (%)			
Concentration (mg/mL)		**0.25**	**0.5**	**1**	**IC_{50}**
Aerial parts	Vegetative stage	50.24±0.60[a]	71.50±1.28[a]	81.88±0.20[a]	0.126
	Flowering stage	32.12±4.03[b]	67.18±1.41[ab]	79.70±1.33[ab]	0.416
Root parts		24.70±2.12[c]	46.40±1.00[c]	67.20±0.99[c]	0.655
BHT		50.70±0.97[a]	72.10±0.15[b]	82.10±0.47[b]	0.110

Different letters in the same row indicate significant differences ($p < 0.05$). Values represent an average of triplicates ± standard deviation. BHT was used as positive control.

Table 5. Ferric Reducing Power of *S. pallida* at mining site

		Absorbance at 700 nm			
Concentration (mg/mL)		**0.25**	**0.5**	**1**	**IC_{50}**
Aerial parts	Vegetative stage	0.590±0.007[b]	0.975±0.006[b]	1.032±0.004[b]	0.179
	Flowering stage	0.765±0.002[a]	1.001±0.003[a]	1.121±0.002[a]	0.112
Root parts		0.192±0.002[d]	0.347±0.009[d]	0.548±0.005[d]	0.964
Rutin		0.430 ±0.005[c]	0.674 ±0.008[c]	0.961±0.003[c]	0.310

Different letters in the same row indicate significant differences ($p < 0.05$). Values represent an average of triplicates ± standard deviation. BHT was used as positive control.

Table 6. Percentage of Inhibition of *S. pallida* extract using β -Carotene/linoleic Acid Bleaching System.

		% of inhibition			
Concentration (mg/mL)		**0.25**	**0.5**	**1**	**IC_{50}**
Aerial parts	Vegetative stage	$37.87±1.85^{b}$	$61.73±1.10^{a}$	$84.83±3.21^{a}$	0.392
	Flowering stage	$45.90±1.75^{a}$	$55.40±1.40^{b}$	$83.00±1.39^{a}$	0.356
Root parts		$12.33±0.58^{c}$	$27.80±2.60^{c}$	$50.87±1.50^{c}$	0.971
BHT		$36.60±0.46^{b}$	$52.50±1.10^{b}$	$63.30±1.49^{b}$	0.560

Different letters in the same row indicate significant differences ($p < 0.05$). Values represent an average of triplicates ± standard deviation. BHT was used as positive control.

Table 7. HPLC–MS/MS acquisition parameters (dynamic-MRM mode) used for the analysis of the 38 marker compounds.

No.	Compounds	Precursor ion, *m/z*	Product ion, *m/z*	Fragm-entor, V	Collision energy, V	Polarity	Retention time (Rt, min)	Delta retention time (ΔRt)
1	Gallic acid	169	125.2*	97	12	Negative	6.96	2
2	Neochlorogenic acid	353	191.2*, 179	82	12, 12	Negative	9.52	2
3	Delphindin-3-galactoside	465.01	303*	121	20	Positive	11.36	2
4	(+)-Catechin	289	245.2*,109.2	131	8, 20	Negative	11.44	2
5	Procyanidin B2	576.99	576.99*, 321.2	160	0, 32	Negative	12.41	2
6	Chlorogenic acid	353	191.2*, 127.5	82	12, 20	Negative	12.42	2
7	*p*-Hydroxybenzoic acid	137	93.2*	92	16	Negative	12.86	2
8	(-)-Epicatechin	289	245.1*, 109.1	126	8, 20	Negative	13.03	2
9	Cyanidin-3-glucoside	449	287.3*, 255.6	121	20, 20	Positive	13.14	2
10	Petunidin-3-glucoside	479.01	317*, 302	121	20, 44	Positive	13.26	2
11	3-Hydroxybenzoic acid	137	93.2*	88	8	Negative	13.59	2
12	Caffeic acid	179	135.2*, 134.1	92	12, 24	Negative	13.65	2
13	Vanillic acid	167	152.4*, 108.1	88	12, 20	Negative	14.32	2
14	Resveratrol	227	185*	131	12	Negative	14.40	2
15	Pelargonidin-3-glucoside	433.01	271*, 121	116	24, 50	Positive	14.52	2
16	Pelagonidin-3-rutinoside	579.01	271*	145	32	Positive	14.56	2
17	Malvidin-3-galactoside	493.01	331*, 315.1	121	20, 50	Positive	14.64	2
18	Syringic acid	196.9	182.2*, 121.2	93	8, 12	Negative	15.28	2
19	Procyanidin A2	575	575*, 285	170	0, 20	Negative	16.18	2
20	*p*-Coumaric acid	163	119.2*, 93.2	83	12, 36	Negative	16.70	2
21	Ferulic acid	193	134.2*, 131.6	83	12, 8	Negative	17.10	2

22	3,5-Dicaffeoylquinic acid	514.9	353.1*, 191	117	8, 28	Negative	17.61	2
23	Rutin	609	300.2*, 271.2	170	32, 50	Negative	17.73	2
24	Hyperoside	465.01	303*, 61.1	97	8, 50	Positive	18.33	2
25	Isoquercitrin	463	271.2*, 300.2	155	44, 24	Negative	18.36	2
26	Delphindin-3,5-diglucoside	462.9	300.1*	165	24	Negative	18.38	2
27	Phloridzin	435.39	273*, 167	155	8, 28	Negative	18.83	2
28	Quercitrin	446.99	300.2*, 301.2	160	24, 16	Negative	19.61	2
29	Myricetin	316.99	179.1*, 182	150	16, 24	Negative	19.61	2
30	Naringin	578.99	271.3*, 151.3	170	32, 44	Negative	19.62	2
31	Kaempferol-3-glucoside	447	284.2*, 255.2	170	24, 40	Negative	19.77	2
32	Hesperidin	611.01	303*, 334.8	112	20, 12	Positive	20.19	2
33	Ellagic acid	301	301*, 229	170	0, 24	Negative	21.41	2
34	*trans*-cinnamic acid	149	131.2	74	4	Positive	21.44	2
35	Quercetin	300.99	151.2*, 179.2	145	16, 12	Negative	21.87	2
36	Phloretin	272.99	167*, 123	116	8, 20	Negative	22.30	2
37	Kaempferol	287.01	153*, 69.1	60	36, 50	Positive	23.84	2
38	Isorhamnetin	314.99	300.2*, 196.1	145	16, 4	Negative	24.57	2

Table 8. Concentration ($mg.kg^{-1}$ of dried extract) of bioactive compounds in the extract.

	Compounds	Concentration (mg/kg)
1	Gallic acid	243.44
2	Neochlorogenic acid	17.73
3	Delphindin3-galactoside	n.d.
4	Catechin	n.d.
5	procyanidin B2	n.d.
6	Chlorogenic acid	n.d.
7	4-Hydroxy benzoic acid	401.30
8	Epicatechin	n.d.
9	Cyanidin-3-glucoside	n.d.
10	Petunidin-3-glucoside	n.d.
11	3-Hydroxy benzoic acid	n.d.
12	Caffeic acid	189.90
13	Vanillic acid	245.26
14	Resveratrol	n.d.
15	Pelargonidin-3-glucoside	n.d.
16	Pelargonidin-3-rutinoside	n.d.
17	Malvidin-3-galactoside	n.d.
18	Syringic acid	165.63
19	Procyanidin A2	n.d.
20	P-Coumaric acid	209.29
21	Ferulic acid	115.59
22	3,5-Dicaffeoylquinic acid	n.d.
23	Rutin	313.51
24	Hyperoside	n.d.
25	Isoquercitrin	137.59
26	Delphinidin-3,5-diglucoside	178.30
27	Phloridzin	1.58
28	Quercitrin	n.d.
29	Myricetin	n.d.
30	Naringin	n.d.
31	Kaempferol-3-glucoside	16.02

32	Hesperidin	n.d.
33	Ellagic acid	n.d.
34	*trans*-cinnamic acid	n.d.
35	Quercetin	32.02
36	Phloretin	2.83
37	Kaempferol	n.d.
38	Isorhamnetin	26.18
Total compounds		2297.17

[a]n.d., not detectable. Relative standard deviation (RSD) for all compounds ranged from 2.11 to 7.32%.