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Research Article

Effects of Cadmium on Phenolic Composition and Antioxidant Activities of *Erica andevalensis*

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We evaluated the effects of cadmium on phenolic composition of *Erica andevalensis*, an endemic protected heather that grows in mine affected soils. Plants cultivated under laboratory-controlled conditions were exposed to acute doses of cadmium to investigate the mechanisms this species possesses to survive in the presence of toxic metals in its natural habitat. Cadmium increased the total levels of phenolics and flavonoids compounds, and the total antioxidant capacity. Cinnamic acid derivatives, epicatechin, and rutin were increased in the presence of cadmium when applied in levels that did not alter the ratio of chlorophylls. Phenolic compounds play an important role in the metabolism of *E. andevalensis* to survive in heavy metal polluted soils.

1. Introduction

Erica andevalensis Cabezudo and Rivera is an endemic species of South Western Iberian Peninsula, which only grows on soils affected by mining activity (outskirts of the mines, river banks of Tinto and Odiel Rivers) [1–3]. *E. andevalensis* flowers in soils and sediments highly polluted by a broad range of metals, which include cadmium [4–6]. Cadmium has no known physiological function in plants, and it is a highly toxic metal due to its reactivity with S and N atoms in amino acids [7]. It is also known to affect cellular structures and produce membrane damage, disruption of the electron transport, inhibition/activation of enzymes, and alteration of DNA [8]. Although cadmium does not belong to the Fenton-type class of metals, it is known to trigger lipid peroxidation in tissues at early exposure times, and several studies have shown the activation of antioxidative defences against cadmium toxicity [9].

Phenolic compounds have been described as electron-donating agents, and therefore they can act as antioxidants [10], acting as reducing agents, hydrogen donors, and singlet oxygen quenchers and preventing the evolution of oxidant-free radical and reactive species derived from metal catalysis by Fenton-like reactions [11, 12]. Moreover, it has been suggested that phenolics may act as biomarkers of metal exposure [13]. Previous studies detected a different phenolic composition in *E. andevalensis* compared with other heathers growing both in polluted and unpolluted soils [14], and, when grown under controlled conditions in the laboratory, acute exposure to cadmium-induced changes in the total antioxidant activity [15] and in the ascorbate and glutathione levels, and also the activity of the antioxidant enzymes [16].

The aim of the present study is to determine the phenolic composition of *E. andevalensis* in the absence of metal and in the presence of different cadmium concentrations, and to look deeper into the role of phenolic compounds in the heavy metal tolerance of *E. andevalensis*.

2. Materials and Methods

E. andevalensis seeds were collected from the field in autumn, picking mature fruits and extracting and cleaning the seeds

in the laboratory. The seeds were cleaned by hand to remove foreign materials such as flowers, leaves, and stems, and they were placed in distilled water. The seeds that sank were identified as viable. Subsequently, they were imbibed in distilled water and kept at 4°C in the dark for 20 days. After this time, they were air dried and stored at 4°C in dark, a procedure described previously for this species [17] to break the physiological dormancy of the seeds.

The seeds were sown in commercial acid soil (Floragard Rhodohum), a mixture of sphagnum peat based with 75% of organic matter, 4.5–5.5 of pH (H_2O), and 200–500 μS cm^{-1} of conductivity, and they were placed in a plant growth chamber with a photoperiod of 16 h of light, 24–19°C day/night and 250 μmol m^{-2}s of PAR radiation. The soil was watered regularly with tap water. Plants were growing for four and a half months. Immediately prior to the cadmium addition ($CdSO_4 8/3H_2O$), the plants were separated in five groups (three pots per group), and each pot was watered with 25 cm^3 of tap water (control) or 25 cm^3 of the following cadmium solutions: 0.53 μM, 5.3 μM, 53 μM, and 530 μM (equivalent to 0.05, 0.5, 5, and 50 μg cadmium g^{-1} soil and labelled as T1, T2, T3, and T4). Five days later, leaves were collected from each pot, weighed, frozen in liquid N_2, and stored at −80°C until the analysis. Material from three different pots per treatment were sampled for each chemical or biochemical analysis of each treatment (C, T1, T2, T3, and T4). All the measurements were carried out in triplicate.

Cadmium concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Hewlett Packard 4500).

The cadmium content in soil was measured as described in previous studies [5]. Leaves from three different pots of each treatment were dried in oven at 50°C (0.3 g in dried weight per analysis). Samples were incubated overnight with 3 mL of HNO_3 and 60 μL HF in Teflon microwave vessels. Afterwards, a microwave extraction was carried out, which consisted of heating the samples to 165°C during 10 min of ramping and holding for further 20 min (1000 W). When samples were cooled, double-distilled water was added to give a final volume of 50 mL, and the cadmium content was determined by ICP-MS.

Leaves were homogenised into liquid N_2 followed by a 30% methanol extraction step. The total content of phenolic compounds was assayed spectrophotometrically [18], and the results were expressed as micrograms of gallic acid equivalents (GAEs) per milligram of fresh weight (μg GAE equivalents mg^{-1} FW), and after a calibration curve was obtained using gallic acid as standard (Sigma, St. Louis, MO, USA).

The total content of flavonoids was assayed spectrophotometrically [19], and the results were expressed as micrograms of rutin per milligram of fresh weight (μg rutin mg^{-1} FW).

Individual phenolic compounds in leaf extracts were analyzed by high performance liquid chromatography (HPLC) with direct injection of 50 μL of the filtered (45 micrometers) methanol-extracted sample [14]. An Agilent 1100 series (Palo Alto, CA, USA) chromatograph equipped with a diode array detector was used. A gradient of solvent A (water : acetic acid, 98 : 2 v/v) and solvent B (water : acetonitrile : acetic acid, 58 : 40 : 2 v/v/v) was applied to a reversed phase Nova-pack C18 column (300 × 3.9 mm I.D., particle size 5 μm) as follows: 0–25 min, 45% B linear; 25–45 min, 45% B isocratic; 45–60 min, 100% B linear; 60–70 min, washing and reequilibration of the column. The flow rate was 1.0 mL min^{-1}, and the temperature was set at 20°C. Detection was performed by scanning from 200 to 700 nm. Identification of individual compounds was carried out by comparing their retention times and spectra with those of original standards. Standards were obtained from Merck (Darmstadt, Germany), Fluka (Buch, Switzerland), and Sigma (St. Louis, MO, USA). Quantitative determinations were carried out with standard external calibration method. Wavelengths used for quantification were 260 nm for ellagic and vanillic acid, 280 nm for benzoic acids, tyrosol and flavan-3-ol, 320 nm for cinnamic acids and their tartaric esters, and 360 nm for flavonols. The identification of unknown phenolic compounds not included as standards was based on retention times and spectra obtained from the literature. These compounds were assayed by assuming that their molar absorptivities were the same as those of their corresponding free standard molecules.

The radical scavenging activity of the extracts was determined using 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH$^\bullet$) method [20], with some modifications [21, 22]. The degree of radical scavenging activity was calculated as described [23]. A thirty percent methanol solution was chosen for background correction.

The total antioxidant capacity of the extracts was determined by phosphomolybdenum method described [24].

The pigment content (chlorophylls *a* and *b*) was quantified using the equations given by Lichtenthaler [25]. The ratio between chlorophyll *a* and chlorophyll *b* was calculated as the quotient of *a*/*b*.

A Beckman Coulter DU 800 Spectrophotometer with 1 cm of pathway was used for all absorbance measurements.

Statistical analyses were achieved with an independent samples *t*-test, and significant differences in relation to the control plants were indicated with $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results and Discussion

In the present work, the effect of cadmium on the phenolic composition and the antioxidant activities of *E. andevalensis*, a metal-tolerant species whose natural habitat is the mining area from SW Iberian [1, 3, 6] is described. Little is known about the effects of cadmium in the phenolic metabolism of Ericaceae family.

As expected, the cadmium content in the soil-increased proportionally to the cadmium added (Figure 1(a)). Simultaneously, *E. andevalensis* took up cadmium from the soil, and it accumulated in the leaves, in relation to the levels in soils (Figure 1(b)). No visual effects were observed in the plants under any of the treatments applied, and the leaves stayed green in all the treatments, with no differences in the chlorophyll *a* to chlorophyll *b* ratio which reduction

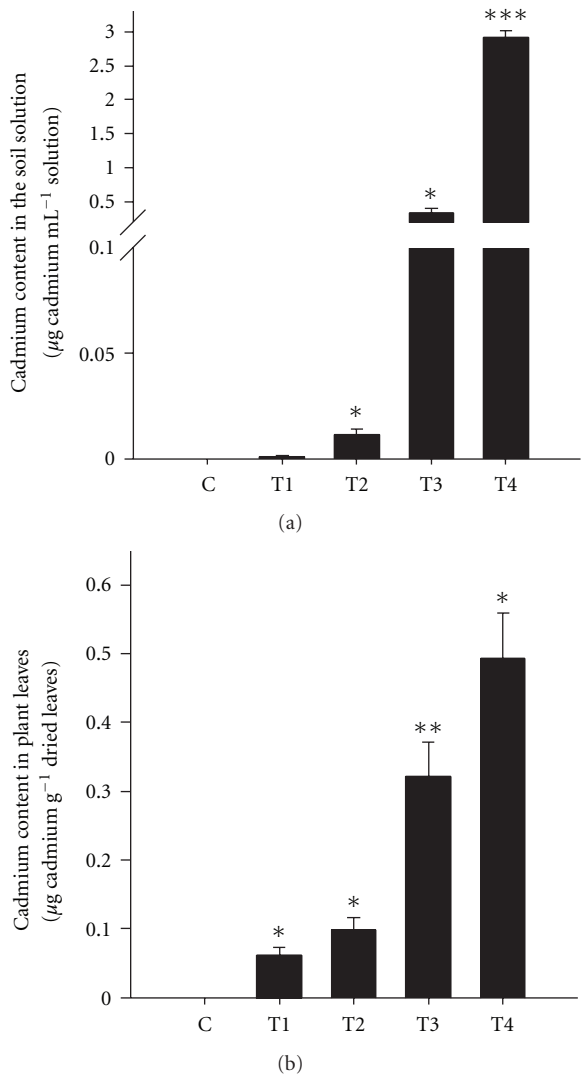

Figure 1: Cadmium content in the water extracts from the treated soils (a) and cadmium accumulated in leaves extracts of *E. andevalensis* after five days of treatment period (b), exposed to 0, 0.05, 0.5, 5, or 50 μg cadmium g^{-1} soil (C, T1, T2, T3, and T4). All the values are the mean of three different samples ± standard error. The asterisks denoted significant differences compared to the control group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

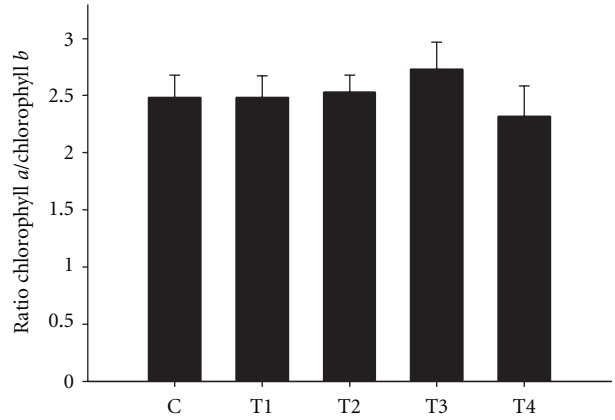

Figure 2: Ratio between chlorophylls *a/b* after five days of treatment period, exposed to 0, 0.05, 0.5, 5, or 50 μg cadmium g^{-1} soil (C, T1, T2, T3, and T4). All the values are the mean of three different samples ± standard error. The asterisks denoted significant differences compared to the control group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

can be used as an indicator of metal-induced stress [26, 27] (Figure 3).

Cadmium is a highly toxic metal to plants, and it is present in the soils where *E. andevalensis* grows naturally [5], but little attention has been paid to the effects of this metal in plant studies in the area. Cadmium tolerant species must be able to control the metal uptake or to possess mechanisms to detoxify the metal and avoid internal damages [28]. *E. andevalensis* must possess internal mechanisms to counteract the effects of cadmium due to its naturally polluted habitat and the fact that the metal is reaching the aerial organs. Despite this, this species is only found growing in polluted soils, but our previous studies showed that the species is also able to grow under controlled conditions in the absence of pollution [15].

The levels of total phenolic compounds and total flavonoids presented a similar pattern (Figures 3(a) and 3(b)). Although in the case of total phenolics T3 plants presented the highest significant phenolic content, while in the case of the flavonoids significantly higher levels than the control were observed from T2 to T4. When the individual phenolic compounds were analysed by HPLC, the highest values were found in T3 plants (Table 1). A more detailed analysis showed that a derivative of cinnamic acid reached a value higher than 300% in T1 plants than observed in control plants. This high concentration was slightly diminished in T2 and T3 plants but decreases to a no-significant value of 168% of the control in T4 plants. However, the behaviour of other phenolics and flavonoids compounds was different; the highest variation was observed for epicatechin in T3 plants with an increase of 81% versus the value measured in control plants (Table 1). The total antioxidant capacity (TAC) of the leaves extracts is shown in Figure 3(c). All the treatments exhibited a significant increase compared to the control group although T4 presented a smaller level than T3. However, the DPPH radical scavenging activity only showed a significant increase in the T3 extracts (Figure 3(b)).

The total level of phenolic compounds in control plants was similar to the levels previously described in wild plants [14]. However, the levels of phenolics compounds were not increased as expected by the presence of cadmium, taking into account that the control plants did not contain any cadmium and that the metal content in plants from T1 to T4 plants was higher than eightfold. Interestingly, the highest phenolic concentration was observed in T3 plants as well as the highest flavonoid content even though that T4 plants contain ca. 40% more cadmium than T3 plants. Similarly, individual phenolic and flavonoid compounds were also mostly increased in T3 plants. The fact that no

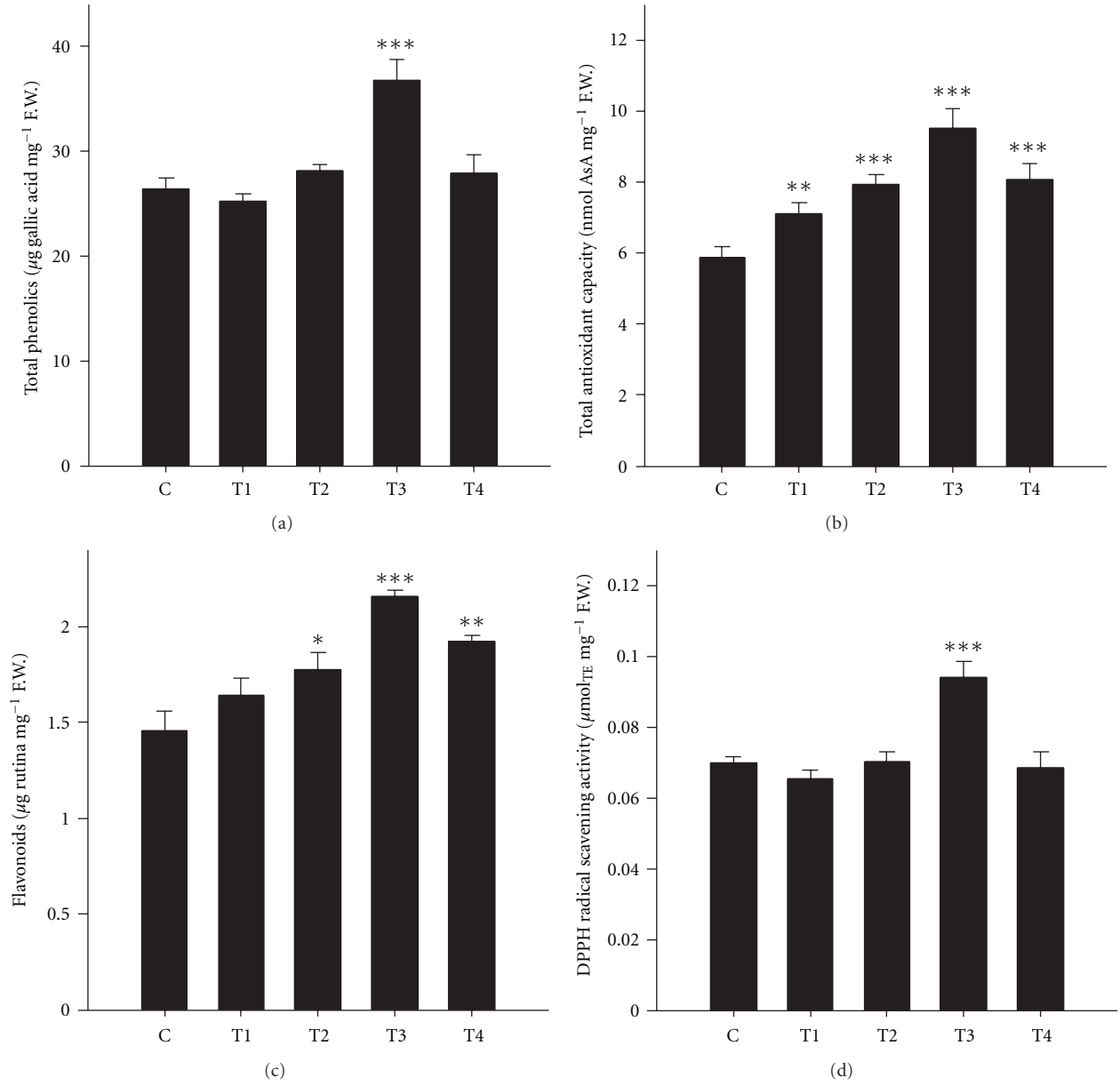

Figure 3: Content of the total phenolic compounds (a), flavonoids (b), total antioxidant capacity (c), and DPPH radical scavenging activity (d) in leaves extracts of *E. andevalensis* after five days of treatment period, exposed to 0, 0.05, 0.5, 5, or 50 μg cadmium g^{-1} soil (C, T1, T2, T3, and T4). All the values are the mean of three different samples ± standard error. The asterisks denoted significant differences compared to the control group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

increase in the phenolic compounds was observed in T4 might be explained by taking into account that the phenoxyl radicals resulting from the antioxidative reaction could act as prooxidants [29]. Therefore, plants exposed to the highest concentration of cadmium may reduce synthesis or release of phenolics by an unknown mechanism to avoid a deleterious effect caused by the phenoxyl radicals produced. Another hypothesis to explain the lack of increase of phenolic compounds in T4 plants could be the excess of cadmium may have impaired the antioxidative system responses based on phenolics and other compounds in such a way that plants are not able to synthesize new phenols. A similar situation has been observed in *Spartina densiflora* which is not able to counteract high cadmium exposition, but it successfully responds by synthesising antioxidante metabolites such as ascorbic acid or glutathione when exposed to moderate concentrations of cadmium [30].

The increase of total phenolic compounds in T3 plants is accompanied by an increase in the flavonoid content. Also, T3 plants contain higher significant levels of epicatechin, rutin, and rutin derivative-1 than control plants. However, both quantitative and qualitative differences have been observed between the plants cultured under controlled conditions and those obtained in its natural soils in the

Table 1: Concentration of individual phenolic compounds identified by HPLC in *E. andevalensis* plants exposed to cadmium treatments[a].

Compound	Treatments control	T1	T2	T3	T4
Phenolic acids					
Cinnamic acid derivate	4.80 ± 2.05	17.48 ± 3.12[b]***	13.03 ± 2.51***	14.92 ± 4.17***	8.08 ± 5.27
m-Coumaric acid derivate	56.37 ± 5.20	57.47 ± 5.82	74.08 ± 3.81***	61.45 ± 15.99	86.10 ± 12.08***
p-Coumaric acid derivate	488.10 ± 16.90	518.98 ± 58.96	543.69 ± 36.62*	510.42 ± 60.65	527.23 ± 78.41
Flavonoids					
Epicatechin	187.21 ± 11.10	154.38 ± 43.48	188.43 ± 4.37	340.32 ± 70.33***	195.8 ± 52.69
Rutin	2145.95 ± 108.27	2362.49 ± 204.20*	2203.64 ± 45.19	2839.56 ± 203.73***	2359.39 ± 419.8
Rutin derivate 1	83.04 ± 21.83	79.83 ± 13.06	91.98 ± 3.92	113.82 ± 4.55**	82.15 ± 17.49
Rutin derívate 2	182.50 ± 32.98	183.19 ± 2.45	159.78 ± 17.28	193.14 ± 23.9	181.86 ± 34.49

[a]Results are the mean (±SD) of two replicates of three different samples. All data are expressed as $\mu g\, g^{-1}$ F.W.
[b]Asterisks show the significant differences between treated plants (T1, T2, T3, and T4) and control plants (Control) at the significance levels of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Pyritic Belt [14]. For instance, ellagic and vanillic acids, catechim, kaempferol, and myricetin were detected in wild plants and not in lab-cultivated plants, while a derivate of m-coumaric acid was only detected in lab-cultivated plants and not in the field. Interestingly, the concentration of cinnamic acid derivative in cultivated plants is much smaller than the level detected in wild plants [14], and this concentration increased in the presence of cadmium (T1 to T3). This data points out the importance of this compound in the metal tolerance of *E. andevalensis*. The accumulation of cinnamate derivatives in the presence of cadmium has been also described for *Matricaria chamomilla* [31] supporting the hypothesis of the importance of this group of compounds in heavy metal tolerance in plants. Also rutin and rutin derivative compounds are increased in the presence of cadmium pointing out the importance of metal defence in the plant.

The overall results presented in this study showed that the phenolic compounds play an important role in the cadmium defence of *E. andevalensis*. The variations observed between control and cadmium-treated plants were parallel to an increase in antioxidant defences measured as ascorbate-reductant and free-radical scavenging capacities. Surprisingly the highest response was observed at 5 μg cadmium g^{-1} soil added to the soils, while with 50 μg cadmium g^{-1} soil plants responded similarly to those exposed to low concentrations, such as 0.5 μg cadmium g^{-1} soil, indicating a possible selective mechanism which render plants as efficient metal tolerators at a defined range of cadmium concentrations. Since *E. andevalensis* lives under harsh acidic and metal polluted soils, more investigations are needed to analyse the interaction-synergies and antagonisms between metals at controlled acid pH and phenolic compounds, but phenolic compounds could be used as indicators of metal presence in the leaves of plant species.

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