The toxic effect of Pb on polyphenolic, flavonoid content and antioxidant ability of Atriplex nummularia growing in galena mining area

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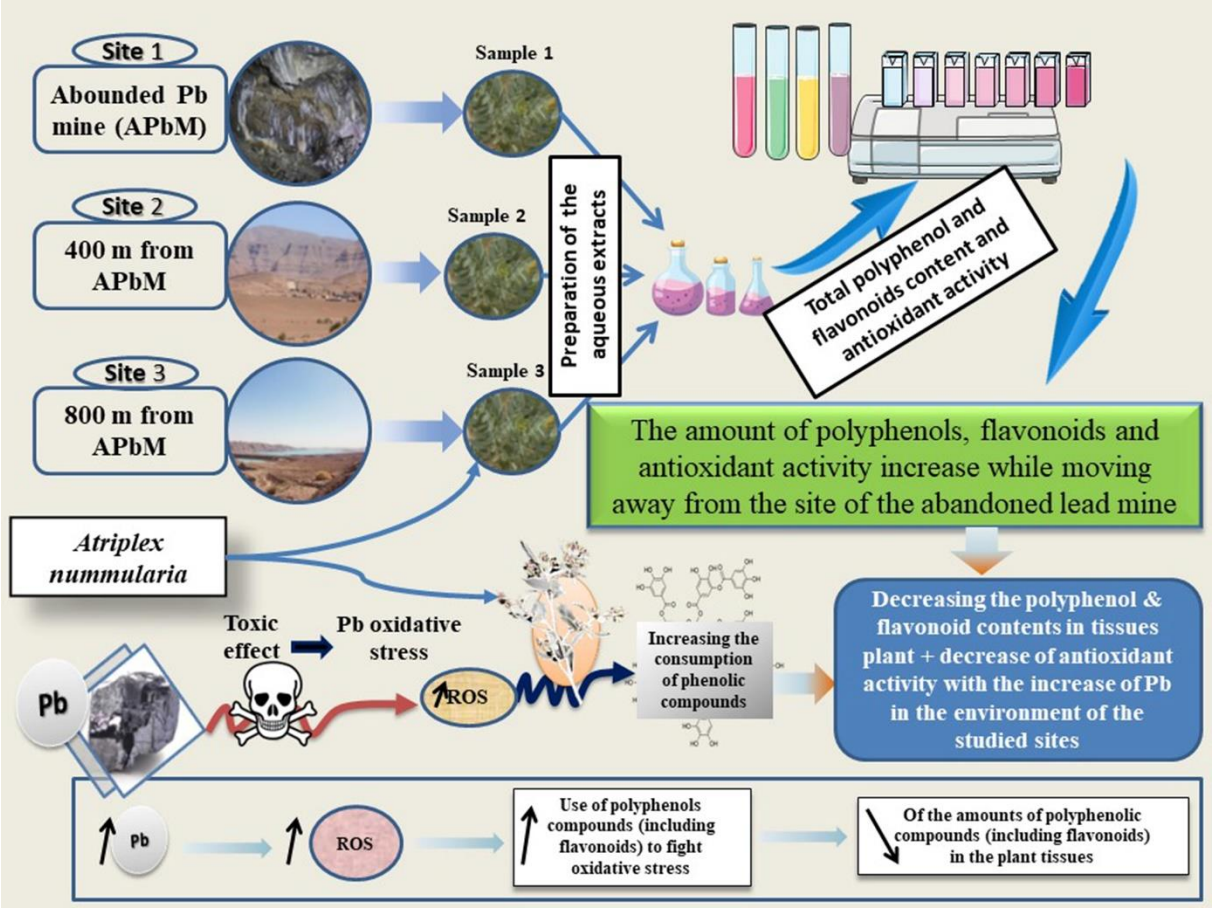
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Abstract

Pb has been reported to develop a wide range of toxic effects on physiology, morphology and biochemical performance of different living organisms. In plants, amongst other effects, it results in acceleration of growth, root length, transpiration, germination and seed development, chlorophyll development? and secondary metabolites content, along with cell division. The aim of this study is the assessment of the effect of an abandoned galena mining soil on the levels of phenolic compounds and flavonoids synthesized for the species *Atriplex nummularia*. In this study we analyzed the polyphenols, flavonoids and the antioxidant activity of three samples of the leaves of *Atriplex nummularia* collected from three different sites, one at the midst of an abandoned Pb mine, and the two others located at 400 and 800 m from this mining location. The findings of the present work indicated that total polyphenol content, flavonoid content and antioxidant activity of the methanolic extract of *Atriplex nummularia* significantly increase as the sample extracts were further away from the site of the abandoned lead mine. We suggest that Pb provokes potential oxidative stress for *A. nummularia*, leading to a potential use of phenolic compounds to deal with the toxic effect of Pb, which explains the significant decrease in the content of polyphenols, flavonoids and antioxidant activity at the heart and near the site of the abandoned lead mine.

Keywords: Lead, oxidative stress, *Atriplex nummularia*, polyphenols, flavonoids and antioxidant activity.



A graphical abstract resuming methodology, results and main conclusions of the current study.

1. Introduction

Lead (Pb) is present in solid form, regularly dense, moldable, and very soft to touch, it is characterized by a very low electrical conductivity. This heavy mineral universally exists in the environment, but its accessibility to plants is limited regarding its characteristic specificity of forming complexes with soil elements [1] [2]. Pb is considered as one of the most frequently occurring contaminant in the environment around the globe. When lead is present in the plant's environment, it is absorbed, especially in rural areas where the soil is contaminated by exhaust from cars and in areas contaminated by fertilizers containing heavy metals or in mining areas [3]. In fact, Pb is known to be absorbed from the soil solution into root system [4]. Remarkably, it is well reported that the main quantity of lead is accumulated in roots in insoluble form [5]. This chemical element can lead to a variety of biochemical and physiological defects in plant growth, seed germination, hydric situation, assimilation of different nutritional elements and secondary metabolites synthesis [6] [7]**.** Recently, researchers from different disciplines have been interested about the study of polyphenols for their essential functions in plant physiology (for growth, for reproduction, fighting pathogens, against predators), in addition to their antioxidant virtues and therapeutic utilization in the human body, their possible role in preventing ailments allied with oxidative stress [8].

Phenolic metabolites have taken great attention due to their antioxidant proprieties and their capacity to capture free radicals, and therefore affect positively the human health [9]. Obviously, heavy metals including lead significantly influence the growth and the development of different plant species, thus reducing the benefits expected. The average level of lead for natural and agricultural soils has been reported in the agricultural soils as 50 to 300 mg/kg [9], [10]. In this respect, it has been reported that if the level of lead element exceeds this critical limit, all physiological, morphological and biochemical parameters have serious impacts [10].

In fact, the contamination of this heavy metal in soil leads to a variety of damaging effects on plants such as the damage in nutrient uptake, adjustments in plant water absorption, enzyme activity and generation of reactive oxygen species (ROS); which leads to decreased photosynthesis and cell death causing substantial failure in crop yield. Lead toxicity causes inhibition of ATP production and DNA damage by over production of ROS. In addition, lead strongly inhibits seed germination, root elongation, seedling development, plant growth, transpiration, chlorophyll production, and water and protein content.

The objective of this work was to assess the effect of an abandoned lead mine soil on the quantities of phenolic compounds and flavonoids synthesized for the species *Atriplex nummularia*. This study will revolve around the analysis of the impact of lead concentration on polyphenols, flavonoids and the antioxidant activity of three samples of the leaves of *Atriplex nummularia* collected from three different sites. The first samples were collected in the midst of the abandoned mine, the second at 400 m from the mine and the third 800 m from this abandoned mine. A comparative analysis is suggested to determine how Pb affects the biosynthesis of phenolic compounds and flavonoids for this species as well as the antioxidant activity of its methanolic extract.

2. Material and methods

2.1. Description and location of the mining site

The “Kba” mine located near the Hassan Addakhil dam, located north of the Hassan Addakhil dam, 20 km from the town of Errachidia Morocco, the choice of this site was based on the presence of relatively sparse vegetation around the mine which was able to survive in the presence of relatively lead-rich mine tailings.

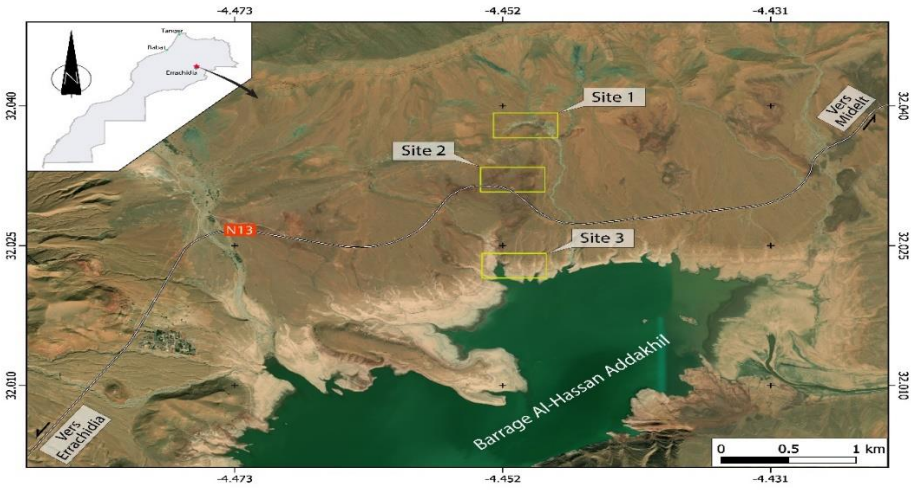


Figure 1: *distribution and location of the three sites from which the samples were collected.*

The samples of *Atriplex nummularia* were collected during the period from January to December 2021. These samples were taken monthly at three different sites from which the average result of three samples at each site was taken **(figure 1):** Site 1 near the mine activity, 400 meters from the mine towards the national road N13 for site 2 and 800 meters away from the mine near the Hassan Addakhil dam noted site 3. The distance between the samples from each site was 200 meters. In total a population of 108 samples were collected during the study period.

2.2. Determination of lead concentration

The fresh leaves of *A. nummularia* were washed with distilled water to remove debris and dust particles. Special attention was made not to squeeze the leaves to. They were shade dried under 40°C for 24 hours, then pulverized with a manual blender. 1 g of the powder of the sample was balanced into a porcelain vessel, putted in a muffle furnace and heated at 450°C for 3h; a temperature ramp of 2°C/min was set to assure a better calcination [11]. The white residue was dissolved in 1 mL. HCl, 1M HNO_3 was added, after filtration into a 25 mL measuring flask, the volume was made up to the mark with 1M $HN0_3$ [12]. A calibration curve (0-100 mg/l) using lead nitrate to prepare different concentrations of Pb^{2+} [13] was used.

2.3. Preparation of the methanolic extract

The fresh leaves of *A. nummularia* were washed with distilled water without squeezing to remove debris and dust particles. They were shade dried under 40°C, and dried leaves pulverized with a manual blender. A portion (200 g) of the powdered leaves was cold macerated with methanol for 24 hours and filtered to obtain the *A. nummularia* methanolic extract (ANME) which was concentrated in a freeze dryer and stored for following tests.

2.4. Determination of total phenolic content

The total phenolic content of each sample was determined using the Folin-Ciocalteu method [14]. Gallic acid was used as standard. In summary, samples of GA were diluted appropriately in test tubes with the addition of Folin-Ciocalteu regent (100 L). The mixtures were allowed to stand for 6 min and then reacted with 1 mL of 7% sodium carbonate (w/w) for 90 min in the dark. The absorbance was measured at 760 nm. Results were expressed as mg GA equivalents (GAE)/g. All measurements? were performed in triplicate.

2.5. Determination of total flavonoid

A modified method [15] was used to determine the total flavonoid content, where, a diluted solution (1 mL) containing flavonoids, 5% (w/w) $NaNO_2$ (0.7 mL) and 30% (v/v) ethanol (10 mL) were mixed for 5 min, and then 10% $AlCl_3$ (w/w, 0.7 mL) was added and mixed altogether. Six minutes later, 1 mol/L NaOH (5 mL) was added. The solution was then diluted to 25 mL with 30% (v/v) ethanol. After standing for 10 min, the absorbance of the solution was measured at 430 nm with a spectrophotometer. A standard curve was plotted using quercetin as a standard. Different concentrations of quercetin were prepared in 80% ethanol and their absorbance was read at 430 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). The results were expressed in mg quercetin/g dry weight by comparison with the quercetin standard curve, which was made under the same condition.

2.6. Evaluation of the anti-free radical activity by trapping the free radical (2,2-Diphenyl-1-picrylhydrazyl: DPPH)

The scanning activity of the DPPH radical was measured according to the protocol described by Louli et al., A solution of DPPH was prepared by solubilizing 4 mg of DPPH in 100 ml of methanol. The solution was then placed in the dark for 3 hours. In glass tubes, a series of dilutions of the extract and the positive control butylhydroxytoluene (BHT) were prepared with a concentration of the stock solution of (5 mg / 10 ml) in order to obtain the following concentrations (31, 25, 62.5, 125, 250, 500 µg / ml). 5 ml of the DPPH solution was then introduced. After stirring, the tubes are placed in the dark at room temperature for 30 minutes. Regarding the negative control, it contained only the solution of DPPH and the methanol with an equal volume of 2.5 ml. The reading is carried out by measuring the absorbance at 515 nm using a UV / Vis spectrophotometer [16].

Antioxidant activity of TE was estimated according to the following equation:

$$\mathbf{(I\%) = [A_{control} - A_{sample} / A_{control}]}$$

Where:

- **(I%):** percentage of the anti-radical activity;
- $\mathbf{A_{control}}$: Absorbance of the control;
- $\mathbf{A_{sample}}$**:** Absorbance of the sample.

Result was expressed by mean (of three separated measures) ± SEM.

2.7. Statistical analysis

Data were expressed as mean ± SEM. Statistical differences among the means studied were assessed by two-way ANOVA followed by Bonferroni multiple comparisons test. GraphPad Prism 7 software was used to carry out statistical analysis. Differences were considered to be significant when $p < 0.05$.

3. Results

The results which concern the level of flavonoids and the concentration of lead in *Atriplex nummularia* are indeed our old work in an article published in 2022 in Mediterranean Journal of Chemistry. These results are added to new results of analyzes carried out on the same plant in order to better defend the hypothesis in the first articlecited below [11].

3.1. Lead accumulation in Atriplex nummularia

The lead accumulation was evaluated in three halophyte *Atriplex nummularia* growing at different points around a galena mining field. The results reported for **sites 1, 2 and 3** represent the mean value of three samples collected at each sampling site **(figure 2)**. The results are also reported **monthly** during 2021 from January to December. Only aerial parts (leaves) of plants were examined.

As expected Pb concentration decrease significantly as the plant is far away from ore storage area**.** A mean concentration of 252 mg Pb/Kg (DW) was measured at the mine site that decreased to 28 mg Pb/Kg (DW) at 800 m. this could be explained as aerial parts of the plants are exposed to dust produced by the exploitation work. Therefore, a foliar absorption mechanism of Pb could explain the results. This could explain the irregular Pb concentration observed in these plants since exploitation works are irregular in different sites. [11]

These results were compared to a control group (gratefully offered by the High Commission for Water and Forests and the Fight against Desertification of Errachidia) in which we found a mean value of 6.62 ± 0.82 mg Pb/Kg (DW). Differences in the levels of lead in *Atriplex nummularia* compared with the control variant are statistically significant across all sites.

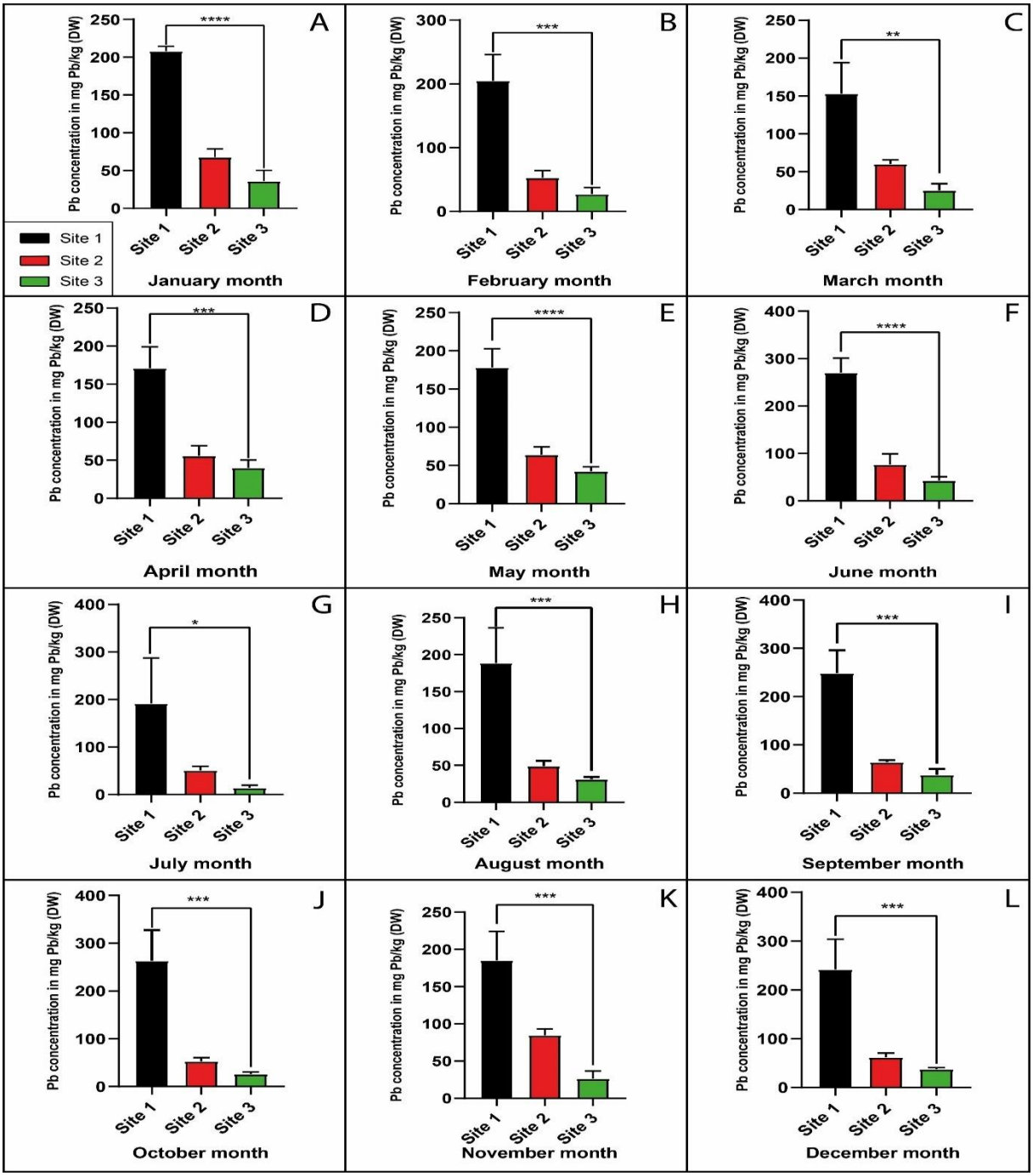


Figure 2: *Pb accumulation according to the site of sampling and the month of harvesting. All data are expressed as mean ± SEM (n=9). (*) p<0.05; (**) p<0.01; (***) p<0.001.* [11]

3.2. Polyphenols content

Figure 3 below demonstrates that while moving away from the abandoned lead mine (site 1), the quantity of polyphenols content in the methanolic extract of *A. nummularia* increases significantly, and this is valid for all

months of the year from January to December. Maximal amount of polyphenols content was measured for samples site 3 whereas, the minimal amount was reported for the samples in site 1.

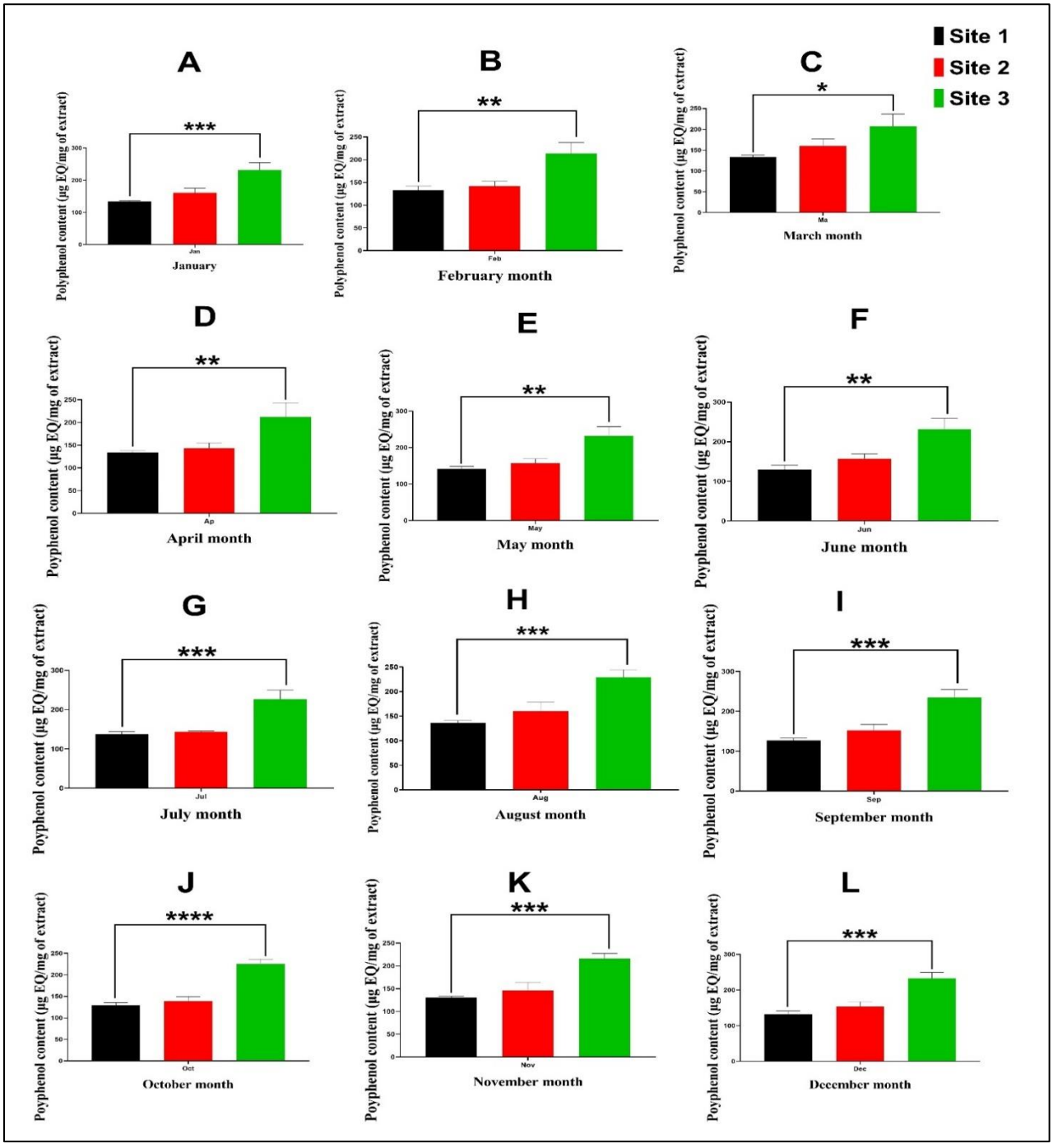


Figure 3: *development of the amounts of polyphenol content in ANME according to the site of sampling and the month of harvesting. All data are expressed as mean ± SEM (n=9). (*) p<0.05; (**) p<0.01; (***) p<0.001.*

3.3. Flavonoids content

Figure 4 below shows that while moving away from site 1, the quantity of flavonoids content in the methanolic extract of *A. nummularia* increases significantly, for all months of the year. Maximal amount of flavonoids content was revealed for the sample of the plant harvested from site 3, whereas, the minimal amount was

reported at site 1. Significant differences in the amounts of flavonoids (P<0,05 to P<0,001) are reported depending on the month of harvesting [11].

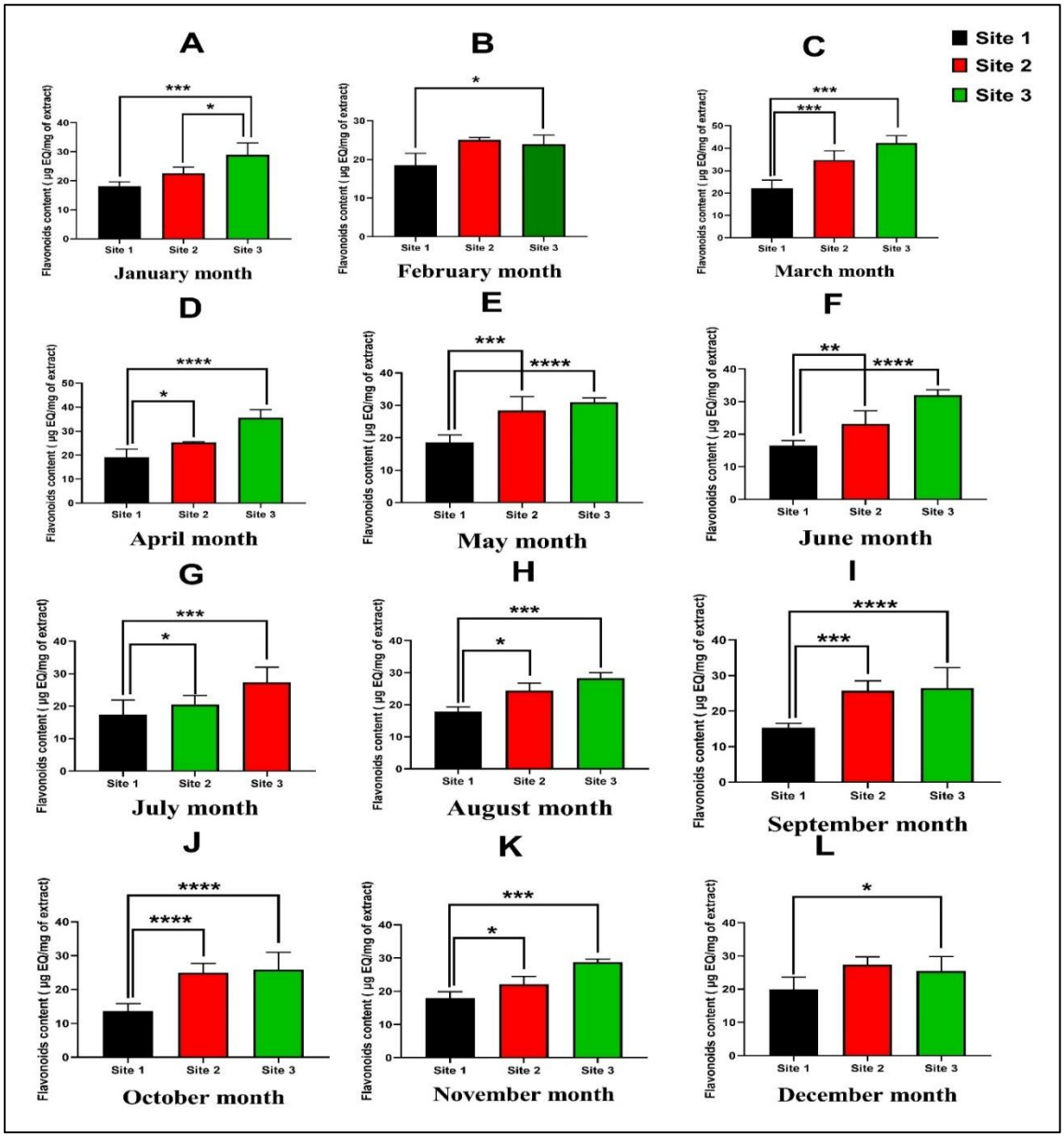


Figure 4: *development the amounts of flavonoids content in ANME according to the site of sampling and the month of harvesting. All data are expressed as mean ± SEM (n=9). (*) p<0.05; (**) p<0.01; (***) p<0.001. [11]*

3.4. Antioxidant activity

Figure 5 below shows that while moving away from the abandoned lead mine (site 1), the DPPH scavenging activity of the aqueous methanolic of *A. nummularia* increases significantly, across all months of the year. Minimal IC50 was revealed for the sample of the plant harvested from the site 3. While, the maximal IC50 was reported at site 1. Significant differences in the IC50 (P<0,05 to P<0,001) are reported depending on the month of harvest.

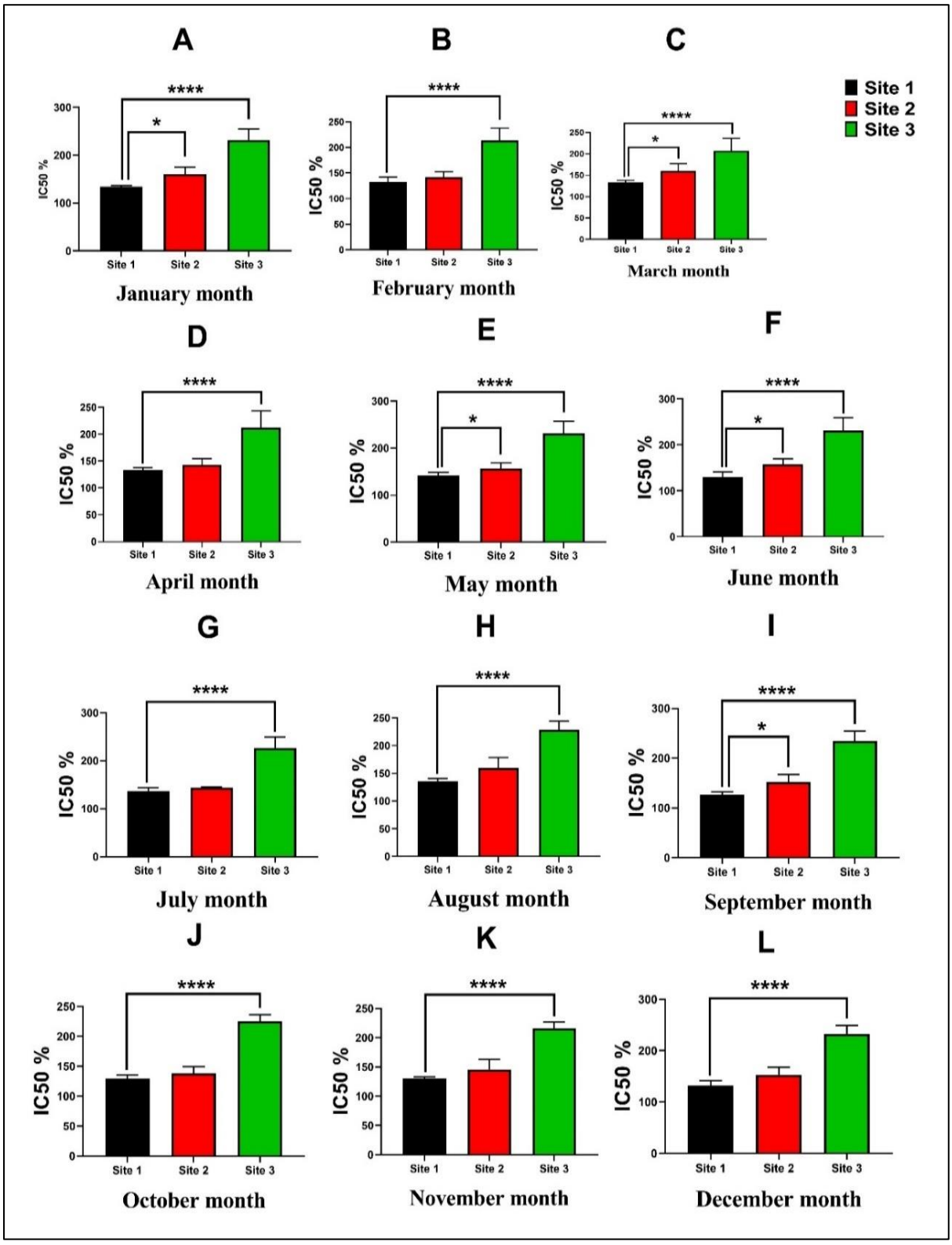


Figure 5: *development the IC50 in ANME according to the site of sampling and the month of harvesting. All data are expressed as mean ± SEM (n=9). (*) p<0.05; (**) p<0.01; (***) p<0.001.*

4. Discussion

The findings of the present work indicate that total polyphenol (TP) and flavonoid content of the methanolic extract of *Atriplex nummularia* significantly increase while moving away from the site of the abandoned lead mine, corresponding to an increase in Pb concentration of the soil **(figure 6)**. The antioxidant activity of the studied methanolic extract of this species follows the same trend as that of polyphenol and flavonoid content. This is to be expected as the antioxidant activity of a plant extract is strongly correlated to the polyphenolic amount in this plant. Contrasting to the results we have measured in this study , increasing amounts of total phenolics and flavonoids were reported under Pb stress [17].

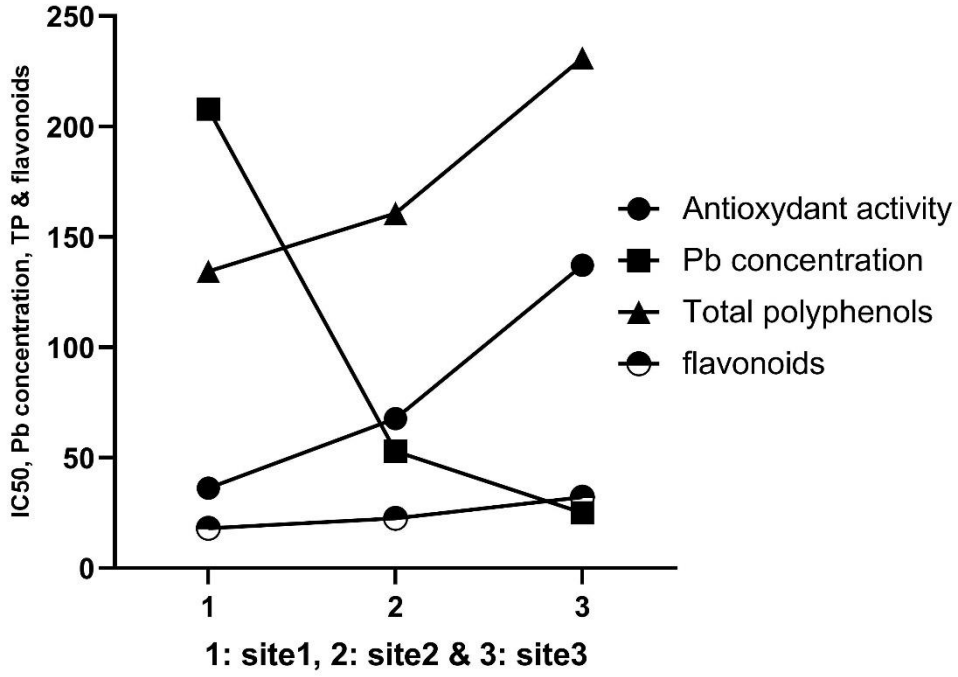


Figure 6: *impact of Pb concentration on antioxidant activity, total polyphenols & flavonoids on three sampling sites during January month in A. nummularia*

Likewise, Ali et al. reported an increase of total phenolics and flavonoids content in root suspension cultures of *Panax ginseng* when it was exposed to 50 µM Cu [18]. Clearly, several studies revealed increased phenolics and flavonoids under heavy metals exposure such as Pb and Cd. Actually, the rise of phenolic content is related to the growth of enzymatic activity in secondary metabolites pathways [17]. However, several other studies are in agreement with our results. Maslennikov reported that total contents of aqueous-soluble antioxidants in plant tissues was found to reduce with a rise in Pb levels in the soil [19]. The same researchers indicated that in shrub species, a reduction in polyphenol content under the effect of Pb pollution was detected in the leaves of *Syringa vulgaris*, *Viburnum opulus*, *Ribes alpinum*, *Symphoricarpos rivularis* and *Philadelphus coronarius* [19]. Likewise, in a pot experiment, *Chenopodium murale* was tested for the phytoextraction of Pb from the polluted soils. The experiment was controlled for 8 weeks to assess the impact of diverse Pb regimes (300, 400 and 500 mg/kg) on the bioaccumulation and physiological parameters of *Chenopodium murale* species. Findings of this experimental study showed a slight reduction in chlorophyll content, biomass yield and protein amounts [20]. Saleem et al. demonstrated that lead contamination decreased the plants growth,

physiology (including chlorophyll ‘a’, ‘b’ and carotenoids content) and yield at all levels of Pb stress for *Helianthus annuus* [21]. Similarly, it has been demonstrated that common basil cultivation in lead-contaminated soil could lead to negative impacts on the growth process, germination indices, and morphological characters, and physiological traits, while, may have a favorable effect under low level (25 µM) on the essential oil composition [22].

Interestingly, Maslennikov et al. by studying the antioxidant activity of urban plants showed different strategies for plant species resistance. It was reported that six species accumulated phenolic compounds in response to anthropogenic stress. A decrease in water-soluble antioxidants was described as a consequence of the involvement of the polyphenol compounds in neutralizing ROS. These species showcased an adaptive benefit, which lies in activating the biosynthesis of the phenolic constituent of the antioxidant defense. Thus, they became more resistant to anthropogenic contamination. On the other hand, in thirteen species, a decrease in the polyphenol content and water-soluble antioxidants showed a suppression of metabolic processes as a result of a lower adaptive power and a higher vulnerability to environmental stress. As conclusion of this study, the findings highlight the importance of polyphenols to the vitality of plants and their potential role to the functioning of the antioxidant system of plant cells. The mechanism of such a system was associated to the environmental conditions and the effect of different stress factors, including Pb pollution [19].

Finally, based on the previously discussed data, we suggest that the high level of Pb leads to physiologic perturbations in *Atriplex nummularia*. The toxic effect of Pb was translated by a high oxidative stress leading to a great use of phenolic compounds including flavonoids by the plant in order to limit this stress. Therefore, we could conclude that for *Atriplex nummularia as correlation between* increasing Pb level and increasing levels of oxidative stress that drives the plant to use more phenolic compounds as a defense mechanism. This leads to a significant decrease of these secondary metabolites in the tissues of *A. nummularia* associated decrease of phenolic compounds in the plant’s tissue.

5. Conclusion

During their life, plants are constantly facing different types of stress that influence their growth and development. Consequently, the more a plant is able to develop rapid and adaptive defense mechanisms, the less stress can affect its health. This was reported in this study, and although we did not follow all stress biomarkers, we were able to formulate fairly clear conclusions. Indeed, Atriplex species are able to cope with any ROS excess by activating the antioxidant defense mechanisms allowing to restore the homeostasis of the cell and to tolerate the different treatments.

The contents of total polyphenols and flavonoids were found to decrease under Pb stress in the present study. The antioxidant activity was also reduced following Pb stress. Similar effect of Pb toxicity was reported for several species in published studies. It was found that the impact of Pb was translated at different physiologic levels. In the current study, we suggest that it provokes potential oxidative stress for *A. nummularia*, leading to a potential use of phenolic compounds to deal with the toxic effect of Pb. This explains the significant decrease in the content of polyphenols, flavonoids and antioxidant activity in the middle and near the site of the abandoned lead mine, showcasing that plant secondary metabolites play an important role in plant development and protection against various stresses.

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