Effects of heavy metals stress on phenolic contents and antioxidant activity of *Pistacia lentiscus* affected plant by acid mine drainage (AMD)

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Abstract

Pistacia lentiscus (PL) has traditionally been used in medicine for its beneficial properties for human health, such as reducing diseases caused by oxidative stress. This research reports the physiological responses of *Pistacia lentiscus* subjected to heavy metal stress from an abandoned Pb/Zn mine. The study investigates the concentrations of heavy metals present in soil impacted by acid mine drainage (AMD), their uptake by various parts of *Pistacia lentiscus*, as well as the phenolic compounds and the antioxidant activity. Standard techniques were utilized to quantify the total amount of phenolic and flavonoid compounds. The DPPH free radical scavenging activity was employed to analyze the antioxidant capacity, and the heavy metal analysis was done using a flame atomic absorption spectrophotometer (SAAF). The results show a high concentration of heavy metals in the soil, and *Pistacia lentiscus* appears to be well tolerant to these high metal concentrations by using an exclusion strategy, except in the case of Mn and Cr metals. The greatest amount of flavonoids and phenolic compounds was found in the dry leaf extracts using a methanol/water (80/20) solvent. However, DPPH testing revealed that non-affected *Pistacia lentiscus* leaves had higher antioxidant activity than those of affected plants. The results also demonstrated that the methanol/water solvent extracted the highest quantity of phenolic compounds and exhibited the best antioxidant activity. The polyphenol and flavonoid content showed a nonlinear connection with the antioxidant activity of *Pistacia lentiscus* leaves. Therefore, to address heavy metal stress situations, *Pistacia lentiscus* has developed several phytochemical defense mechanisms, and phenolic compounds play a crucial role in the plant's environmental adaptation.

Keywords: soil pollution, plant, metal stress, total phenolic, antioxidant activity

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Introduction

Worldwide, acid mine drainage (AMD) contamination of water, soil, and vegetation with harmful metals poses a major environmental threat (Mohammadi et al., 2021). AMD is highly acidic wastewater that contains significant amounts of salts and dissolved ferrous and non-ferrous metal sulfates (Simate and Ndlovu, 2014). Usually, tailings and waste from abandoned mines create unfavorable conditions for most plant species growing on them, such as low pH, toxic metal concentrations, low water retention capacity, and low levels of plant nutrients (Obrador-Pons, 2007; Wong, 2003). Mine locations typically see a slow natural colonization process as a result. However, some tolerant plant species have strong tolerance mechanisms and little competition, which allow them to spread effectively in these conditions (Spence and Tingley, 2020).

Therefore, the establishment of vegetation requires plant species adapted to pollution. These plants develop specific physiological and biochemical mechanisms that allow them to function normally on soils polluted with heavy metals, forming heavy metal-resistant populations (Shu et al., 2002). Metal-tolerant plants are not only of scientific interest but can also be used as phytoremediation technology for removing metals in soil remediation. However, there is still little information about their strategies against metal pollution and toxicity (Boojar and Tavakkoli, 2011).

In Algeria, there are several abandoned mining sites. These areas produce large amounts of waste tailings and mine waste, which are highly concentrated in heavy metals. One such site is the Sidi Kamber mine, which is 60 kilometers from Skikda town in northeastern Algeria (Medjram and Malika, 2014). Few plant species usually form the plant communities observed on mine tailings (Wu et al., 2021). *Pistacia lentiscus* has naturally colonized all the land around the abandoned Sidi Kamber mine, where Pb and Zn were extracted. The evergreen *Pistacia lentiscus* plant, which belongs to the Anacardiaceae family, bears globose berries that have a vivid red color. It is utilized as a food ingredient and is mostly found in the Mediterranean basin's "extreme" environments. Its antimicrobial and antiulcer properties are also well-known. In traditional medicine, the leaves of *Pistacia lentiscus* are widely used to treat eczema, diarrhea, and throat infections(Milia et al., 2021). Additionally, the plant's aerial portions have a hypotensive effect (Sebti et al., 2020).

Numerous studies have demonstrated the abundance of secondary metabolites, such as tannins, flavonoids, and phenolic compounds, in this plant. This is why it is used in the treatment of many diseases, as many diseases are typically linked to an increase in the formation of free radicals and a decrease in antioxidant potential (Chanwitheesuk et al., 2005; Cherbal et al., 2012); . Phenolic compounds are secondary metabolites present in plants that have been shown to have antioxidant activity and protect against stress. They also have a high propensity to chelate metals (Cervilla et al., 2012; Kısa et al., 2016; Tomás-Barberán and Espín, 2001).

In this study, we aim to determine whether certain physiological traits of plants alter in response to environmental variations, whether these changes have an impact on the useful applications of plants, and to what degree these changes occur. For this, we selected the *Pistacia lentiscus* plant, which is remarkably adaptable in a metal-polluted environment caused by the Sidi Kamber NE mine in Algeria. This study aims to assess the effects of soil heavy metal pollution on the physiology of plants growing in abandoned mining areas by: (1) measuring the mineral composition of the soil affected by acid mine drainage, (2) identifying the mechanism of metal accumulation and mobilization in the leaves, roots, and fruits of *Pistacia lentiscus* affected by AMD, and (3) assessing the concentration of polyphenols and investigating the non-enzymatic antioxidant defense mechanism of *Pistacia lentiscus* leaves exposed to heavy metal stress by measuring DPPH free radical scavenging.

Materials and Methods

Plant Material

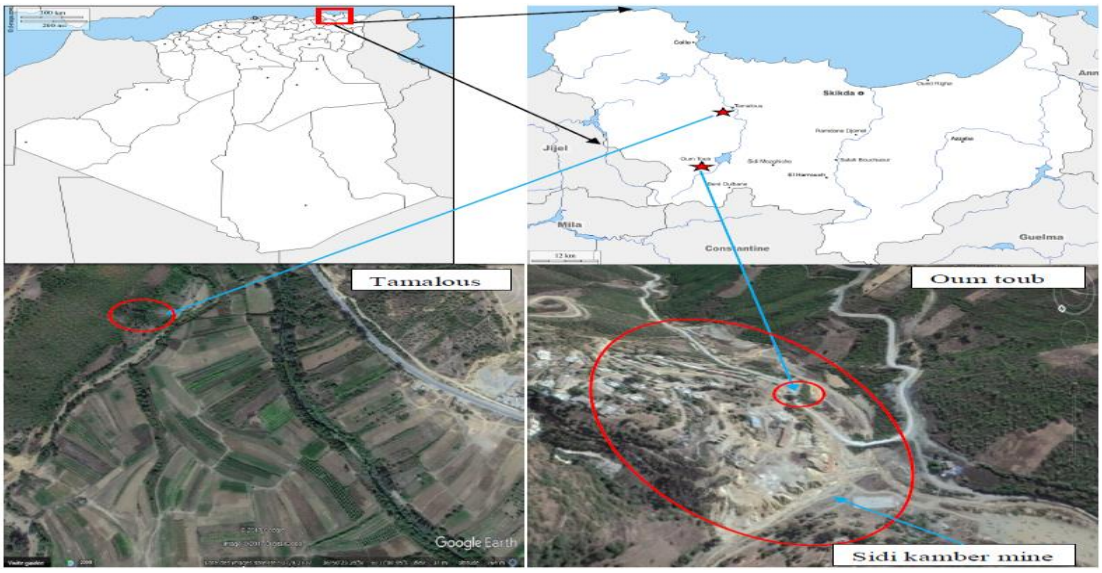

Fig. I. Map showing the location of the two area of plants sampling; metal-contaminated area (Z1) and non-contaminated area (Z2)

Fig. II. The medicinal Plant *Pistacia lentiscus* affected by acid mine drainage (AMD) from abandoned Sidi Kamber (pb/Zn) mine, Algeria

Pistacia lentiscus leaf samples were collected during the month of November in two different regions of Skikda, northeastern Algeria (Fig.I). The first location is the Sidi Kamber mining area, which is situated in Skikda town at 5 meters (202 meters) altitude and 37° 16′ 27″ N latitude and 9° 52′ 26″ E longitude. This region is highly contaminated (Fig. II), particularly with heavy metals from waste mines and tailings (Khelfaoui et al., 2020). The second region is located in Tamalous city, a distant area from the mining zone, at 37°16′ 27″ N latitude, 9° 52′ 26″ E longitude, and 500 meters above sea level. It is a natural area free from any kind of metal pollution.

The leaves of *Pistacia lentiscus* were thoroughly cleaned to eliminate any extraneous objects, including dust and broken leaves. For a month, the plants were dried at room temperature in a dry, well-ventilated space. After drying, the samples were chopped into extremely small pieces and stored in a dry, dark environment until further use.

Chemical Reagents

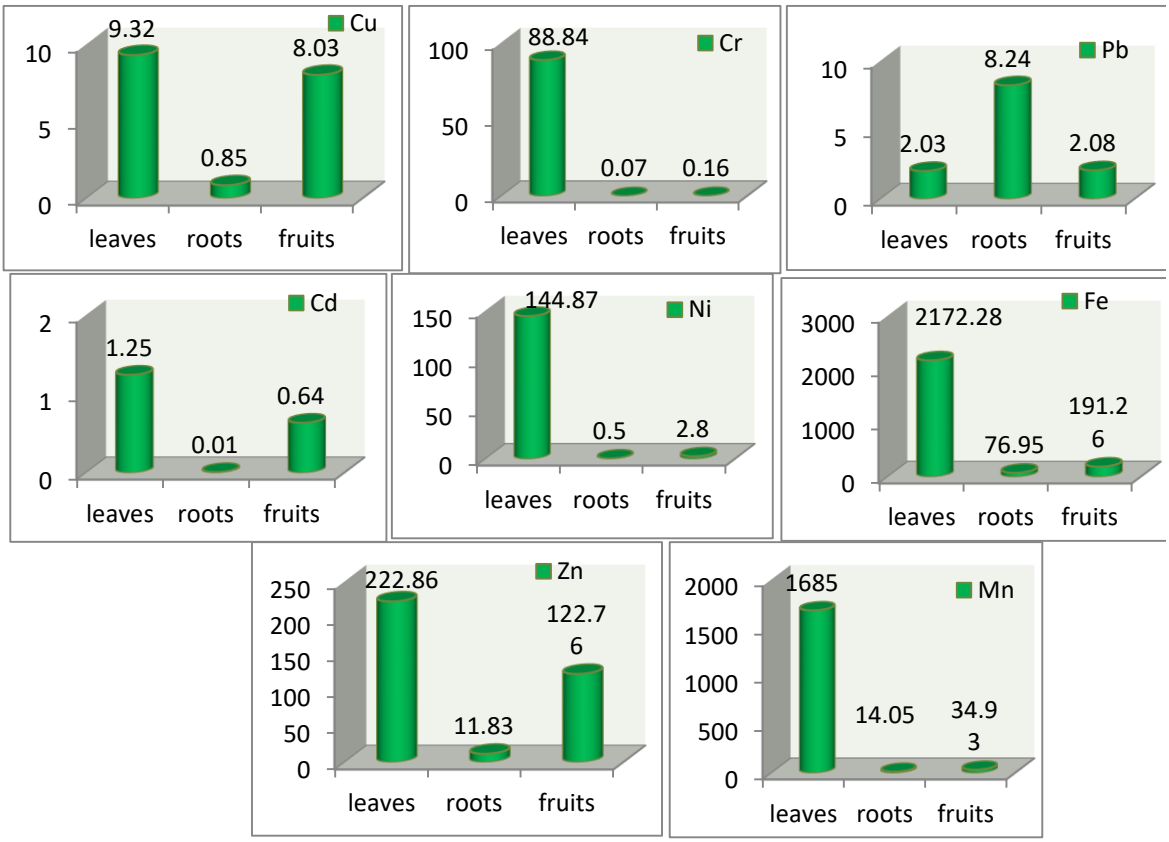

Fig. III. heavy metals Fe, Zn, Mn, Cu, Pb, Ni, Cd and Cr contents in leaves, roots and fruit of *Pistacia lentiscus* affected by acid mine drainage (AMD) from abandoned (Pb/Zn) mine, All values are in (mg/kg) DW, DW: Dry weight, (*N* = 3) contaminated area (Z2)

The chemical reagent DPPH (2,2-Diphenyl picrylhydrazyl) and Folin-Ciocalteu phenol were purchased from Sigma Co. Catechin and gallic acid (98%) were obtained from Sigma Aldrich (Steinheim, Germany). Concentrated hydrochloric acid (37%), concentrated HNO_3 acid (65%), and absolute methanol (≥99.8%) were purchased from Panreac Química, S.A. Aluminum chloride-6-hydrate ($AlCl_3 \cdot 6H_2O$) (>99%), sodium nitrite ($NaNO_2$), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), BHT, and all other chemical reagents used were obtained from Sigma Co.

Heavy Metals Content in Soil Affected by AMD

Using flame atomic absorption spectrometry (FAAS), equipped with deuterium background correction (Thermo-Scientific 3000), in the GL1K laboratory at Liquefied Natural Gas Installation, Algeria, heavy metal levels in soils affected by AMD were analyzed for concentrations of Pb, Zn, Fe, Ni, Mn, Cd, Cu, and Cr. The samples' pH was determined using a 2.5:1 water/soil ratio. The materials were pseudo-totally digested in aqua regia prior to chemical analysis. This was done in a closed tank in accordance with French norms (NF EN 13346 2000), with a few modifications.

Using this technique, 0.25 g of an accurately weighed sample was placed into Teflon bombs, followed by the addition of 6 ml of HCl and 2 ml of HNO_3. The medium-pressure vessels were then sealed and heated to 110 °C for two hours. After filtering, the samples were quantitatively transferred and filled to a capacity of 25 mL in a volumetric flask using ultrapure water. The metals were analyzed using direct calibration under the same conditions as the samples. Furthermore, to assess the reproducibility of the measurements, each sample was measured three times.

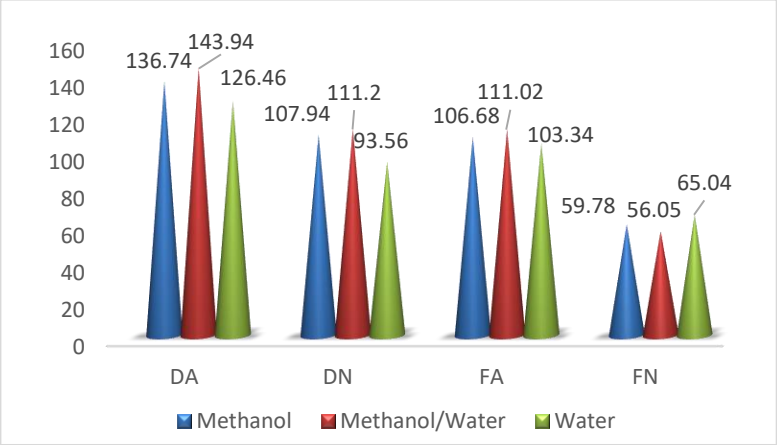

Fig IV: Total Phenolic contents of affected and non-affected *Pistacia lentiscus* plant using different solvents. Each value is expressed as mean ± SDs (*N*= 3)

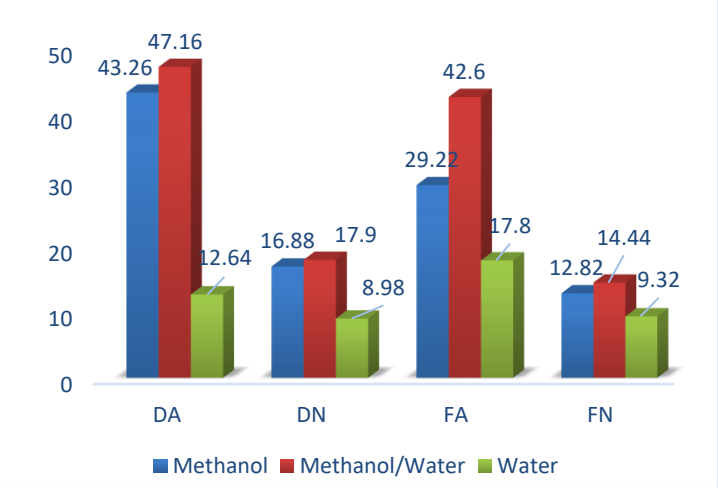

Fig.V. Total Flavonoid contents of affected and non-affected *Pistacia lentiscus* plant using different solvents. Each value is expressed as mean ± SDs (*N*= 3)

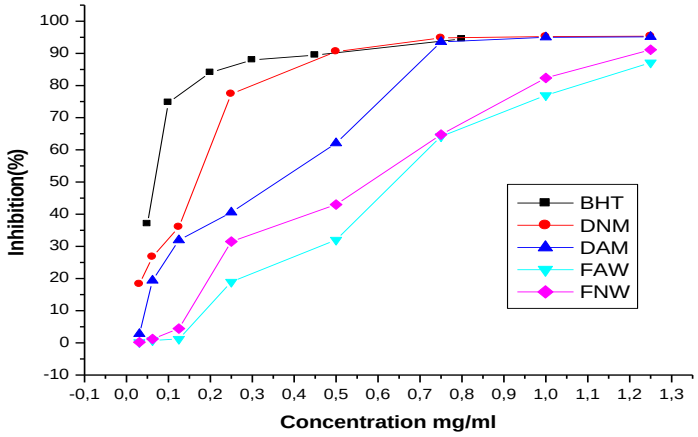

Fig VI: Scavenging ability of BHT, dried methanol of non- affected (DNM) and affected (DAM) leaves *of Pistacia lentiscus* extracts and fresh water of non- affected (FNW) and affected (FAW) leaves of *Pistacia lentiscus* extracts on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Each value is expressed as mean ± SDs (*N*= 3).

Heavy Metals Content in *Pistacia lentiscus* Leaves

Heavy metal levels of Pb, Zn, Fe, Ni, Mn, Cd, Cu, and Cr in *Pistacia lentiscus* affected by AMD were

analyzed. A total of 1000 mg of previously dried plant material was introduced into a capsule. The capsule was placed in an oven, where the temperature was gradually increased to 500 °C and maintained for 2 hours. A plateau was held at around 200 °C until the smoke emission ceased. After cooling, the ash obtained from the calcination of the dried plants was treated with 2 ml of aqua region (a mixture of HCl and HNO_3 in a 3:1 ratio) as the starting solution for heavy metal determination. This mixture was boiled for three hours on a hot plate. The appropriate wavelength for each element was measured directly for the Cu, Cr, Pb, Cd, Ni, Fe, Mn, and Zn metals. Standard solutions were used for calibration. After cooling, the solution was filtered into a 25 ml volumetric flask and topped up with deionized water to the appropriate level.

All analyses were performed in triplicate at the GL1K Skikda laboratory (liquefied natural gas facility) using a flame atomic absorption spectrophotometer (SAAF) with Thermo-Scientific 3000 deuterium background correction. The results are expressed in mg/kg relative to dry matter (MS). To evaluate the metal absorption capacity of plants, the following biological coefficients were calculated:

a) **Biological accumulation coefficient (BAC)**

BAC = [Mep] / [Ms] (Marchiol et al., 2013) Where [Mep] and [Ms] represent the metal concentration in the epigeal parts and metal concentration in the soil, respectively.

b) **Biological concentration factor (BCF)**

BCF = [Mr] / [Ms] (Fellet et al., 2007) Where [Mr] and [Ms] represent the metal concentration in the roots and metal concentration in the soil, respectively.

c) **Translocation factor (TF)**

TF = [Mep] / [Mr] (Brunetti et al., 2009) Where [Mep] and [Mr] represent the metal concentration in the epigeal parts and the metal concentration in the roots, respectively.

Antioxidant Content

Preparation of Extracts

Different solvent systems were used to evaluate the best extraction solvent for phenolic and flavonoid compounds: M/E: methanol/water (80:20, V/V), M: methanol (100%), and E: water (100%). Two milligrams of fresh and dry samples were extracted with 40 mL of different solvents for 24 hours. The supernatant was filtered through Whatman No. 40 (Whatman International, England).

Total Phenolic Content

The modified Folin-Ciocalteu method, as described by some researches (Blainski et al., 2013; Vl, 1999), was used to calculate the total phenol content of each extract. Briefly, 1 mL of each *Pistacia lentiscus* extract solution was mixed with 1 mL of the distilled water-diluted Folin-Ciocalteu reagent (1:9). The mixtures were allowed to develop color for five minutes at room temperature. After that, 1 mL of 7 % Na_2CO_3 was added to stop the process. Following homogenization and vortexing, the resulting solution of each extract was heated to 40 °C for 10 minutes before being left in the dark for 30 minutes. Utilizing a UV spectrophotometer (Shimadzu UV-Vis spectrophotometer T60U), the absorbance was determined at 765 nm. The total phenol concentration in all samples was calculated from the regression equation of the calibration curve established using different concentrations from a stock solution of gallic acid (12-152 µg/mL). The results were expressed as milligrams of gallic acid equivalents (GAEs) per gram of sample (S = fresh/dry weight), (mg Eq. GA/g SW for fresh weight or mg Eq. GA/g DW for dry weight).

Total Flavonoid Content

The method described by Zhishen and coworkers (Zhishen et al., 1999), based on the formation of flavonoid-aluminum complexes, was used to determine the total flavonoid content in *Pistacia*

lentiscus leaves. To 75 µL of a 5% W/V $NaNO_2$ solution, 125 µL of the extract solution was added. After letting the mixture stand for six minutes, 500 µL of a freshly prepared 10% $AlCl_3 \cdot 6H_2O$ solution was added. After five minutes of rest, 500 µL of a 1M NaOH solution was added. The solution's final volume was adjusted to 2500 µL with distilled water (Igueld et al., 2015). Using a Shimadzu UV-Vis spectrophotometer T60U, the absorbance was measured at 510 nm. The total flavonoid content of *Pistacia lentiscus* was expressed in terms of catechin equivalents (CE) per gram of sample (S = fresh/dry weight), (mg Eq. C/g SW for fresh weight or mg Eq. C/g DW for dry weight).

Determination of Antioxidant Activity

DPPH Radical Scavenging Assay

Pistacia lentiscus leaf extracts were tested using spectrophotometry to examine their ability to scavenge radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The assay's basic principle is that when the antioxidant quenches the radical, the DPPH solution's color changes from purple to yellow. In short, methanol was used as the solvent to generate a 50 $mg \cdot mL^{-1}$ stock solution for each extract of *Pistacia lentiscus* leaves, and if necessary, serial dilutions were made (Sokmen et al., 2004). Subsequently, 50 µL of methanolic *Pistacia lentiscus* leaf extract was added to 1950 µL of freshly prepared 4.10^{-3} mM DPPH in methanol, bringing the total volume to 2 mL. The mixture was left to stand for half an hour at room temperature in the dark (Fadhil et al., 2019). A UV-Visible Spectrophotometer was used to measure the mixture's absorbance at λ = 517 nm to assess the free radical scavenging capacity of the DPPH radical. Further dilution was required if the measured DPPH value exceeded the standard curve's linear range. The formula for calculating the inhibitory activity I (%) was as follows:

$$I\ (\%) = ((A_o - A_t) / A_o) \times 100$$

where A_t represents the absorbance of the test sample, and A_o represents the absorbance of the control sample. The extract concentration that provides 50% inhibition (IC_{50}) was determined based on the graph showing the inhibition percentage against extract concentration. The same procedure was repeated with the same concentration of BHT. After the incubation time, the mixtures' absorbance at 490 nm was measured. The antioxidant activities of the samples, BHT, and the blank were compared. The experiment was conducted in triplicate.

Statistical Analysis

The results presented are the mean obtained from three replicates. IBM SPSS Statistics software version 19 was used for statistical analysis, and graphs were plotted using Microsoft Excel 2007.

Results

Contents of Metals in Soil

The main chemical characteristics of the soils affected by AMD are reported in Table 1. Referring to this table, the pH of all soil samples is classified as acidic, with an average value of 2.75, and conductivity is also very high, with an average value of 1860 µS/cm. The metal content analysis shows a significant variation in the concentration values of the same metal at the same location. The heterogeneity of AMD composition and the variety of mining waste dumped on the abandoned mining site account for the significant range in total concentrations found in the soil samples. According to the metal analysis results of soils affected by acid mine drainage, it is observed that the soil contains the highest concentrations of Fe, Mn, Zn, and Pb, with moderate amounts of Cd and Ni, and minimal concentrations of Cr and Cu.

The average concentration of Fe detected in all samples is very high, with a maximum average value of 58,411.66 mg/kg, followed by Zn with a very high concentration of 4,269.73 mg/kg, and Mn, which reaches a value of 887.20 mg/kg. Pb, Ni, and Cd are highly toxic metals, with significant concentrations of 574.09 mg/kg, 222.78 mg/kg, and 168.24 mg/kg, respectively. The levels of Cu and Cr found in the samples are relatively low, with an average value of 35.56 mg/kg for Cr and 15.89 mg/kg for Cu.

Furthermore, we found that the concentrations of Pb, Cd, Fe, Zn, and Mn far exceed Level A, and Zn and Cd exceed Level B. These extremely high metal levels found in the soil samples are well above the contamination thresholds set by Italian law for soils (Level A* and B*). Level A represents the upper limits for residential zones, and Level B represents the upper limits for industrial zones.

Contents of Metals in the Three Parts of the Plant *Pistacia lentiscus*

Fig.(III) shows the average values of heavy metal analyses (Fe, Cd, Zn, Cr, Pb, Cu, Mn, and Ni) in the three parts of the *Pistacia lentiscus* plant: leaves, roots, and fruits. The results show that metal content in all studied parts of *Pistacia lentiscus* varies considerably from one part to another, generally following this order of abundance:

Fe > Mn > Zn > Ni > Cr > Cu > Pb > Cd

In addition, the leaves of the plant accumulate very high levels of Fe, Mn, Zn, Ni, Cr, Cu, and Cd in their tissues, with average values of 2172.28 mg/kg (DW), 1685 mg/kg (DW), 222.86 mg/kg (DW), 144.87 mg/kg (DW), 88.84 mg/kg (DW), 9.32 mg/kg (DW), and 1.25 mg/kg (DW), respectively. After the leaves, the fruits also contain considerable concentrations of metals. On the other hand, the roots present low concentrations of all the studied metals, except for Pb, whose maximum average value is 8.24 mg/kg (DW), which exceeds the maximum average value in the leaves, at 2.03 mg/kg (DW). Several studies have confirmed this result.

The results show a great capacity for accumulation, tolerance, and adaptation to unfavorable conditions by *Pistacia lentiscus*. Therefore, this plant can be used for the rehabilitation of soils polluted by metals in this mining area. The values of the three biological accumulation parameters (BAC, BCF, and TF)(Table 3) suggest that the tolerance of *Pistacia lentiscus* to these heavy metals is based on exclusion, with the exception of Mn and Cr, where BAC values were greater than 1 (BACMn = 1.899 and BACCr = 2.498). Translocation to leaves, expressed by TF values, was very high (TF > 1), with the exception of Pb, which reflects the uptake and bioaccumulation of this metal in roots.

Total Phenolic Contents

All types of dried and fresh leaves of *Pistacia lentiscus*, whether grown in a metal-contaminated area (Zone 1) or a non-contaminated area (Zone 2), were significant sources of polyphenols. However, the total amount in dry plants varied significantly between 93.56 and 143.94 mg gallic acid/g DW, while the total amount in fresh leaves varied significantly between 56.05 and 111.02 mg gallic acid/g FW.

For the three types of solvents (E, M, M/E), the levels of total phenolic compounds in plants affected by acid mine drainage (DA, FA) were very high compared to the values of unaffected plants (DN, FN) for both dry and fresh leaves (Fig. IV). In addition, dry plants had the highest phenolic content compared to fresh plants in both cases: plants affected and non-affected by AMD. The analysis showed that the highest phenolic concentration was observed in the DA plant for the ME solvent, with a value of 143.94 mg gallic acid/g DW, while the lowest content was recorded in the FN plants for the M solvent, which contained approximately 56.05 mg gallic acid/g FW.

It is interesting to note that the best phenolic concentration was observed with the M/E (methanol/water 80/20) solvent, followed by methanol, and then water, for all cases of plants.

Total Flavonoid Contents

The three solvents, M/W (80:20), M, and W, showed varying total flavonoid contents. The M/W (80:20) extract had the highest values, with 47.16 mg EC/g DW for DA and 42.6 mg EC/g FW for FA. Similarly, methanolic extracts from DA and FA showed significantly larger quantities of flavonoids than those from DN and FN, which showed lower values of 8.98 mg EC/g DW. Consequently, the total flavonoid content of the AMD-affected plants was higher than that of the

Table 1
Average heavy metal concentration of soil affected by acid mine drainage (SMR) in the abandoned mine of Sidi Kamber.

Sample	metals	Mean ± standard deviation	ILA	ILB
	Pb	574,09 ± 202,05	100	1000
Soil (SMR)	Cd	168,2367± 62,82	2	5
pH=2.70	Fe	58411,66 ± 5985,69	-	-
	Zn	4269,73 ± 614,15	150	1500
	Mn	887,20 ±178,28	-	-
	Ni	222,78±143,35	120	500
	Cr	35,56± 5,63	150	800
	Cu	15,89 ± 6,46	120	600

All values are in mg/kg, (N = 3), IL: Italian laws for soils (level A* and B*). Level A: upper limits for residential areas. Level B: upper limits for industrial areas. SD standard deviation

Table 2
Common ranges for the metals Fe, Cd, Zn, Cr, Pb, Cu, Mn and Ni in plants.

Elément	Normal	Phytotoxique
Pb a	5-100	30-300
Cd a	0.05-0.2	5-30
Cu a	5-30	20-100
Fe b	30-300	>500
Zn a	27-150	100-400
Mn a	30-300	400-1000
Ni a	0.1-0.5	10-100
Cr a	0.1-0.5	5-30

All values are in (mg/kg) DW, DW : Dry weight
[a] Kabata-Pendias, A., 2001, [b] Pugh, R.E., et al., 2002

Table 3
Biological coefficients of *Pistacia lentiscus* plant

Biological coefficients	BAC		BCF	TF	
	leaf/soil	fruit/soil	root/soil	Leaf/root	Fruit/root
Pb	0.003	0.0036	0.014	0.2463	0.252
Cd	0.0070	0.0038	0.00005	125	64
Fe	0.037	0.0032	0.0013	154.61	2.485
Zn	0.052	0.0287	0.0027	18.838	10.377
Mn	1.899	0.0393	0.0158	119.928	2.486
Ni	0.650	0.0001	0.0022	289.74	5.6
Cr	2.498	0.0045	0.0019	1269.142	2.2857
Cu	0.586	0.5050	0.0535	10.964	9.447

Table 4
IC50 of BHT, dried methanol of non- affected (DNM) and affected (DAM) leaves of *Pistacia lentiscus* extracts and fresh water of non- affected (FNW) and affected (FAW) leaves of *Pistacia lentiscus* extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

Simaples extracts	IC50 (μg/mL)
DN(Methanol)	160
DA(Methanol)	350
FNW(water)	590
FAW (water)	630
BHT	60

unaffected plants, and it was higher in the dried leaves than in the fresh ones.

Evaluation of Antioxidant Activity

In this present study, the free radical scavenging activity (DPPH) assay method was used to measure the antioxidant activities of *Pistacia lentiscus* leaves that were affected and non-affected by AMD. The results showed a steady increase in the scavenging activity of free radicals in all extracts, with a standard range of 160 to 630 μg/mL (Fig.VI). It was observed that the ability of test materials (pure antioxidants BHT and leaf extracts) to scavenge DPPH was assessed based on their IC50 values, defined as the concentration of test material required to decrease the absorbance at 517 nm (or concentration) of DPPH solution to half of its initial value. The IC50 values of *Pistacia lentiscus* leaf extracts are given in Table 4.

It can be noted that the dry, non-affected leaves of the methanol solvent (IC50 = 160 μg/mL) inhibited DPPH radical more rapidly than the dry, affected plant (350 μg/mL), and even the fresh, non-affected plant exhibited higher scavenging activity compared to the affected fresh leaves of *Pistacia lentiscus*. This result shows that the non-affected plant demonstrated higher antioxidant efficiency compared to the affected plant, despite the lower phenolic and flavonoid content. Therefore, even with low phenolic and flavonoid content, *Pistacia lentiscus* leaves exhibited high DPPH radical scavenging activity. The higher DPPH radical scavenging activity is associated with a lower IC50 value.

Discussion

These levels are much higher than the normal values reported by Pugh et al. (2002)and Pendias et al. (1992), (Table 2) and may show signs of phytotoxicity. This result can be attributed to the initial metal content of the soil, the bioavailability of the metal itself depending on the physicochemical properties of the soil, the biological role of each metal, and the bioaccumulation of the plant.

The high content of Zn, Fe, Mn, and Cu is attributed to their high mobility and availability in the soil and their biological function as essential micronutrients for plant development (Gherib et al., 2017). Furthermore, the high values of Fe, Mn, Cu, Pb, and Zn found in the tissues of the aerial parts of the plant confirm the results of the study by Abreu et al. (2012), who demonstrated the high tolerance of *Pistacia atlantica* to heavy metals and its suitability to be used in the phyto-extraction process.

Plants commonly contain phenolic compounds as secondary metabolites. There are already more than 8,000 identified phenolic structures. They can vary from highly polymerized substances (melanins, lignins, lignans, and tannins) to simple low molecular weight molecules (flavonoids, phenolic acids, phenylpropanoids) (Kumar et al., 2020). Phenols are substances that have an aromatic ring directly linked to one or more hydroxyl groups. They resemble aliphatic alcohols, which have a carbon chain linked to the hydroxyl group. However, because phenols are labile and their hydrogen is affected by the presence of an aromatic ring, they are weak acids (Balasundram et al., 2006).

According to Márquez-García et al. (2012), cadmium raised the overall amounts of flavonoids and phenolics. Since phenols are reactive oxygen species scavengers and metal chelators, Malčovská et al. (2014) proposed that when plants are under heavy metal stress, the production of phenolic compounds rises in plant cells. Kisa et al. (2016) found that when plants were exposed to lead and cadmium, rutin and chlorogenic acid levels in their leaves increased, whereas ferulic acid and caffeic acid levels decreased.

The findings of Vidal et al. (2020) provide evidence that *Imperata cylindrica* may withstand exposure to elevated Cu levels by enhancing phenolic compound synthesis in shoots. The protective function of plant phenolic compounds may help to explain how their levels fluctuate in response to environmental stressors. The primary source of phenolic compounds' antioxidant activity is their redox properties, which can be crucial in

absorbing and neutralizing free radicals (Márquez-García et al., 2012).

Phenolic compounds are distinguished by their ability to act as antioxidants under various stress conditions, including exposure to heavy metals, and by their multiple roles in plants. According to Kisa et al. (2016) and Vidal et al. (2020), heavy metals produce reactive oxygen species (ROS), which encourage plants to produce phenolic compounds as a means of defense and survival. Through their hydroxyl and carboxyl groups, phenols function as metal chelators. They also prevent lipid peroxidation by scavenging alkoxyl radicals. The chemical structure, type, and quantity of functional groups in phenolic compounds affect their ability to scavenge ROS (Manquián-Cerda et al., 2018; Michalak, 2006).

Numerous studies have shown that when plants are exposed to heavy metal stress, phenolic synthesis is boosted. For example, when exposed to copper sulfate spray, *Phyllanthus tenellus* exhibits a greater build-up of phenolics in its leaves (Díaz et al., 2001). Similarly, *Phaseolus vulgaris* accumulates both soluble and insoluble phenolics when exposed to cadmium (Winkel-Shirley, 2002).

Flavonoids are the largest family of natural products; more than nine thousand of these phenolic substances have been found in various plants. The primary subclasses of flavonoids are flavonols, flavones, flavanones, flavanonols, anthocyanins, isoflavonoids, and chalcones (Shah and Smith, 2020).

Because flavonoids can chelate transition metals like Fe, they can prevent the Fenton reaction, which turns H_2O_2 into the harmful OH• radical, and therefore provide plants with a strong antioxidative environment (Leopoldini et al., 2006; Yeshi et al., 2022). In addition, flavonoids have gained interest as a possible source of bioactive compounds. The structure of their flavan nucleus is related to their antioxidant potential. These compounds have the ability to neutralize free radicals such as hydroxyl, peroxyl, alkoxyl, and superoxide. According to Villalpando-Rodriguez and Gibson (2021), the reduction happens through a hydrogen transfer process to reactive oxygen species (ROS). Aside from that direct scavenging effect, other possible modes of action include chelating trace metals and/or inhibiting the enzymes that catalyze the production of reactive oxygen species (ROS) (Mansoor et al., 2023).

Thus, it is evident that the affected dry leaves of *Pistacia lentiscus* are the primary source of both total phenolics and total flavonoids, with corresponding amounts of roughly 143.94 mg gallic acid/g DW and 47.16 mg EC/g DW. Since flavonoids and phenolics can contribute electrons or hydrogen atoms, they can scavenge active oxygen species directly (Ghori et al., 2019). Plants' tolerance and capacity to adapt to stressful conditions can be increased by manipulating the synthesis, activity, and genes involved in the production of secondary metabolites (Anjitha et al., 2021; Jan et al., 2021).

Comparing the limited information from the literature about polyphenol and flavonoid content seems challenging. For Portuguese *Pistacia lentiscus*, Mendes et al. (2011)reported a much lower DPPH radical scavenging potential (IC50 = 87 mg/L), consistent with Moroccan Amezouar et al. (2013), who measured a lower DPPH radical scavenging potential (IC50 = 10 mg/L) for a total flavonoid content of 54 mg QE/g. These data are contradictory and do not clearly indicate a correlation between the concentration of polyphenols and antioxidant activity. Studying variations in polyphenol forms and their antioxidant activity, both qualitatively and quantitatively, is therefore necessary.

Numerous investigations have demonstrated that the most crucial factor is the polyphenols' structure, with structural characteristics significantly influencing their redox power (Öztürk et al., 2007). High reduction power and the potential for oxidation to quinoid forms are indicated by the presence of 2,3-unsaturation in conjugation with a 4-oxo-function in the C-ring, as well as functional groups able to bind transition metal ions. Despite research efforts, a clear connection between polyphenol and flavonoid profiles with antioxidant capacity is still lacking due to the complexity of these profiles in plants.

Reactive oxygen species (ROS) production is the primary response of plants exposed to heavy metals. When the quantity of ROS exceeds the antioxidant capacity, plant damage occurs. Plants have evolved a variety of defense systems to counter oxidative stress caused by heavy metals, including both enzymatic and non-enzymatic systems that scavenge free radicals (Kısa et al., 2016; Michalak, 2006).

It is not always the case that an antioxidant's ability to scavenge free radicals corresponds with its ability to suppress lipid peroxidation or other forms of antioxidant activity. The degree of lipid peroxidation inhibition by antioxidants can be used to assess their antioxidant capacity. Free radical scavenging ability is only one factor that determines this; other factors include the antioxidant's mobility within the microenvironment, its location, and its interactions with other antioxidants (Niki, 2010). Understanding the function and potential of antioxidants requires taking these elements into account.

These results may also suggest that a plant's flavonoid and polyphenol content are not as significant as their quality. Vasco et al. (2008)claim that the correlation is mostly dependent on the sample, the hydrophilicity of the compounds, the extraction solvent, and the type of phenolic molecule. According to research by Bramorski et al. (2011), lipophilicity and metal chelation play a major role in the capacity of chemicals to quench oxidative damage and lipid peroxidation in vivo. Moreover, the test used here (DPPH) does not evaluate total antioxidant properties.

Conclusion

Medicinal plants are rich in secondary metabolites such as phenolic compounds, tannins, and flavonoids, which are used to prevent chronic and degenerative diseases by scavenging free radicals. Plant cells suffer oxidative damage due to heavy metal toxicity, which is one of the main abiotic stresses brought on by physiological and metabolic changes. Many plants can withstand and even thrive in the presence of heavy metals; despite their high concentration, the plant *Pistacia lentiscus* is one of them, growing in the abandoned Zn/Pb mine from Sidi Kamber, NS Algeria. This study focused on the impact of heavy metal stress in the contaminated area on phenolic compounds and the antioxidant activities in two types of *Pistacia lentiscus* leaves: one growing in a heavy metal-contaminated area (Sidi Kamber mining region) and the other in an uncontaminated area (Tamalous town). The obtained results demonstrate that *Pistacia lentiscus* has developed a variety of phytochemical defense mechanisms to withstand the stress of heavy metals. Furthermore, it has been shown that metal stress tolerance in plants can be enhanced through the regulation of secondary metabolite accumulation and production. One of the stress responses **is** phenolic substances, which play a variety of functions, especially in defensive mechanisms. Strong antioxidant qualities help them neutralize the consequences of oxidative stress, and some of them can even chelate heavy metal ions. They have at least one aromatic ring and one or more hydroxyl groups, which play a very important role in the defense against heavy metals. Therefore, the enhanced accumulation in affected *Pistacia lentiscus* by AMD is a response to adapt to metal stress. According to the results, there is a significant variation in the contents of polyphenols and flavonoids between affected and unaffected plants; their contents for the affected plant are higher than the content in the unaffected plant. In addition, this study focused solely on the stress responses of the *Pistacia lentiscus* plant to very high concentrations of metals and sulfate from acid mine drainage from an abandoned mine on the total quantity of polyphenols and the antioxidant activity of this plant without knowing the exact molecules that change under stress. The acquired results in this work offer additional experimental evidence about the effects of abiotic stress imposed by heavy metals, high sulfate concentration, and low pH on the phenolic compounds and the antioxidant capacity of exposed plants to the AMD. The affected *Pistacia lentiscus* plant presented high contents of phenolic compounds and low antioxidant capacity compared to the plant not affected by AMD. The many aspects of plant metabolism, including photosynthesis, mineral distribution, and

antioxidant defense, are impacted by environmental conditions, particularly the excess of heavy metals. As a result, plants have developed adaptive mechanisms to survive. *Pistacia lentiscus* exposure to heavy metals increases the production of phenolic and flavonoid compounds. Finally, high heavy metal content generally increases total phenolic compounds in *Pistacia lentiscus* leaves. For **these** reasons, we can use this medicinal plant for two purposes: firstly, to remedy soils polluted by metals, and secondly, to increase the quantity of phenolic compounds in order to use them in several beneficial fields for humans.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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