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Environmental impact on *Ononis natrix*: Phytochemical profiling, heavy metal bioaccumulation, and multifunctional bioactivities

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Ononis natrix L., a Mediterranean medicinal species traditionally used for its diuretic and anti-inflammatory properties, has recently attracted global scientific interest for its rich profile of bioactive metabolites with potential pharmacological and ecological significance. This study aimed to evaluate the comparative impact of environmental contamination on phytochemical composition, biological activities and heavy metal bioaccumulation in *Ononis natrix* L. Samples were collected from a polluted site and a non-polluted site in Berezguel (Tebessa, Algeria). The aerial parts were analyzed for heavy metal content: lead (Pb), cadmium (Cd), zinc (Zn), and copper (Cu). Methanolic extracts were prepared from the same plant parts and were analyzed for total phenolic (TPC), flavonoid (TFC), and condensed tannin (TTC) contents. Antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), phenanthroline, and reducing power. In addition, enzyme inhibitory potential was examined against α-amylase, acetylcholinesterase, and butyrylcholinesterase, while antimicrobial activity was also assessed. Plants from the polluted site bioaccumulated higher levels of Pb, Cd, Zn and Cu. Phytochemical content was also higher in these samples with significantly elevated TPC and TFC. Plants collected from the polluted site exhibited superior antioxidant activity across all assays, with lower IC50 values for ABTS (15.49 ± 1.89 µg/mL) and DPPH (26.1 ± 0.9 µg/mL) compared to those from the non-polluted site (23.8 ± 1.9 and 33.7 ± 1.9 µg/mL, respectively). Extracts from the polluted site also showed stronger enzyme inhibition, particularly against α-amylase (IC_{50}: 268.1 ± 9.6 µg/mL) compared to acarbose (IC_{50}: 3177.3 ± 11.7 µg/mL). Both extracts exhibited high antibacterial activity with the polluted-site extract producing larger inhibition zones for Gram-positive and Gram-negative bacteria. These findings suggest that pollution induced environmental stress enhanced bioactive compound production and therapeutic potential in *O. natrix*, though metal accumulation in the raw plant raises safety concerns, underscoring the need for standardization and sustainable sourcing strategies.

Keywords: environmental pollution; heavy metal bioaccumulation; UPLC-ESI-MS/MS; phytochemical composition.

Introduction

Medicinal plants have long been recognized as a vital source of bioactive compounds with therapeutic potential serving as the foundation for numerous modern pharmaceuticals. Their secondary metabolites including phenolics, flavonoids, alkaloids, and terpenoids are known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer activities making them valuable both in traditional medicine and scientific research (Altaf et al., 2019; Ahmed et al., 2025). These phytoconstituents not only play a protective role in human health but also in plants themselves, where they act as defense agents against various biotic and abiotic stress factors (Khare et al., 2020). Understanding how environmental conditions influence the biosynthesis of these metabolites is therefore essential to elucidate the adaptive mechanisms of plants exposed to ecological stress.

Environmental pollution particularly heavy metal contamination is one of the most serious ecological problems affecting plant life and ecosystem balance (Aithani & Kushawaha, 2024). Heavy metals such as lead, cadmium, zinc and copper are persistent in the soil and can be absorbed by plants through roots and accumulated in aerial parts altering their physiological, biochemical, and molecular responses (Cîrţînă et al., 2022). Though some are essential micronutrients at trace levels, these elements become toxic when their concentrations exceed the physiological threshold by disrupting metabolic functions and inducing oxidative stress. The exposure to such pollutants can lead to an imbalance between the production of reactive oxygen species and the antioxidant defense systems resulting in lipid peroxidation, DNA damage, and protein oxidation (Kanso et al., 2022). Plants have developed various tolerance mechanisms such as chelation, compartmentalization, and the activation of enzymatic and non-enzymatic antioxidants to mitigate metal toxicity.

The biosynthesis of secondary metabolites is highly influenced by environmental conditions and stress exposure can trigger the overproduction of these compounds as part of the plant's adaptive strategy (Pradhan et al., 2024). Stress factors such as drought, salinity, ultraviolet radiation, and heavy metal pollution have been shown to enhance the accumulation of phenolic compounds, flavonoids and other antioxidants (Rao & Zheng, 2025). This phenomenon reflects the ability of plants to use oxidative stress as a biochemical signal to stimulite defense-related metabolic pathways. Thus, the study of plants growing under polluted conditions provides a valuable opportunity to understand how stress modulates the phytochemical composition and biological activities of medicinal species.

Among the Fabaceae, *O. natrix* commonly known as yellow restharrow represents an interesting perennial species widely distributed in the Mediterranean region including Algeria. Taxonomically, *Ononis* species have been well characterized within the Fabaceae family by Devesa Alcaraz & López González (1997) who described their morphological diversity and ecological adaptation. *O. natrix* has been traditionally used for its diuretic, anti-inflammatory and wound-healing properties and previous studies have revealed that its extracts contain diverse bioactive compounds with strong antioxidant and antimicrobial potential (Ahmed et al., 2025). These biological effects are mainly attributed to the presence of flavonoids, phenolic acids and

other secondary metabolites, whose concentrations may vary significantly depending on environmental conditions and stress exposure.

Heavy metal accumulation in medicinal plants raises significant concerns not only regarding their ecological roles but also their safety for human consumption (Dahiru et al., 2022). Several reports have shown that medicinal species growing near urban, industrial or roadside areas often accumulate metals beyond permissible limits (Donald et al., 2022). Such contamination may alter the biosynthetic capacity of plants and compromise the purity and standardization of herbal products (Bhairam et al., 2013). Therefore, evaluating both the phytochemical composition and the level of heavy metals in plants from different environments is crucial to assess their medicinal quality and ecological adaptation (Ewubare et al., 2024).

Furthermore, the relationship between heavy metal exposure and the modulation of secondary metabolism is complex and species-dependent. While metal stress can cause toxicity and growth inhibition, it can also stimulate the biosynthesis of defensive compounds through the activation of specific transcriptional regulators and metabolic pathways (Khare et al., 2020). For instance, exposure to sublethal doses of metals such as copper and zinc can increase the production of phenolic compounds and enhance antioxidant enzyme activities as part of the plant's adaptive mechanism (Kanso et al., 2022; Pradhan et al., 2024). This adaptive plasticity may contribute to the ecological success of species like *O. natrix* in metal-impacted habitats where it could serve as a bioindicator of pollution and a potential phytoremediator.

Understanding the interplay between environmental metal stress and the phytochemical profile of *O. natrix*, in this context, is of both ecological and pharmacological importance. From an ecological perspective it provides insights into how this Fabaceae species tolerates and adapts to contaminated environments through metabolic regulation and metal sequestration mechanisms. From a pharmacological standpoint, it helps determine whether environmental stress enhances or diminishes the medicinal quality of its bioactive compounds. The dual consideration of phytochemistry and environmental toxicity thus enables a more holistic understanding of plant pollution interactions (Donald et al., 2022; Ewubare et al., 2024).

Previous reviews have emphasized that abiotic stress not only affects plant physiology but also serves as a biochemical signal to enhance metabolite production (Shri & Bansal, 2020), a concept that could be applied to *O. natrix*. Moreover, the environmental monitoring of medicinal plants from both polluted and non-polluted areas is increasingly recommended by researchers to ensure the safety and efficacy of herbal materials used in pharmacological preparations (Bhairam et al., 2013; Cîrţînă et al., 2022). Such comparative investigations are essential for establishing links between environmental pollution, phytochemical variability and biological activities particularly in species of high ethnobotanical and medicinal significance.

Therefore, the present study was designed to explore how environmental metal pollution influences the phytochemical profile and biological activities of *O. natrix* collected from contrasting habitats. By focusing on the accumulation of Pb, Cd, Zn, and Cu and analyzing the corresponding variations in total phenolic content, flavonoids, and biological activities this research aims to elucidate the adaptive responses of this species to metal-induced oxidative stress. The findings could contribute to understanding the ecological tolerance mechanisms of *O. natrix* and provide baseline information for the safe medicinal use and conservation of plants exposed to anthropogenic pollution.

Materials and methods

Plant material. The plants used in our work which belong to the species *Ononis natrix* L., were collected during the flowering period at the end of April 2022 in two different sites in the Berezguel region in the municipality of Elma Labiod, Province of Tebessa, Algeria. Samples from the contaminated habitat were collected in a location close to the discharge of domestic and industrial wastewater from the commune of Elma Labiod and at a distance of one to 3 meters from the shoulder of national road N°16. Samples of the non-polluted plant were collected in the same area in a location well away from any source of pollution including gas emissions from road traffic. The aerial parts (stem, leaves and flowers) of plants from both sampling sites were cleaned and dried in the shade for 21 days before being crushed and macerated in methanol to obtain methanolic extracts.

Bioaccumulated heavy metals concentration. The concentration of bioaccumulated heavy metals was determined by the method described by Awasthi (2023) with some modifications. The aerial parts (flowers, stems and leaves) of *O. natrix* from polluted and unpolluted environments were cleaned with running water to remove dust before rinsed with distilled water. After drying in an oven at 70 °C for 72 h, we proceeded to grinding to obtain a fine powder. In order to determine the concentration of bioaccumulated metals, 1 g of the obtained powder was subjected to digestion with HNO_3 (5:1) for several hours in order to achieve complete digestion. The liquid obtained was then filtered and made up to 100 mL with ultrapure water. Then, the concentration of Pb, Cd, Zn and Cu was determined using an Agilent 240 FS atomic absorption spectrometer.

UPLC-ESI-MS/MS analysis. The sample extracts were analyzed using a UPLC-ESI-MS/MS system (Shimadzu LCMS-8040) equipped with ultra-fast mass spectrometry (UFMS) technology and a Nexera XR LC-20AD binary pump. Detailed UPLC analytical parameters and electrospray ionization conditions are presented in Table 1. Data acquisition and processing were carried out using LabSolutions software. The identification of compounds was based on their mass-to-charge ratios, retention times, and fragmentation patterns, and confirmed by comparison with data from the literature and available mass spectral libraries.

Table 1
Analytical conditions of the UPLC-ESI-MS/MS

Conditions	Parameters	
Column	Restek Ultra C18 3 µm 150 x 4.6 mm	
Electro spray ionization (ESI)	CID gas, 230 KPs; conversion dynode, −6.00 Kv°C; DL temperature, 250 °C; nebulizing gas flow, 3.00 L/min; heat block, 400 °C; drying gas flow, 10 L/min.	
Mobile phase	Solvent A: water, 0.1% formic acid solvent B: methanol	
Flow rate	0.2 mL/min	
Volume injection	5 µL	
Gradient	0 min to 0.2 min	A 98%
	0.2 min to	A 25%
	7.5 min to 12.5 min	A 0%
	12.5 min to 17 min	A 0%
	17 min to 18 min	A 98%
	18 min to 21 min	A 98%

Total phenolic compounds determination. The total phenolic content was measured using the Folin-Ciocalteu method as described by Müller et al. (2010). A mixture was prepared by adding successively 20 µL of extract, 100 µL of diluted FCR (1:10) and 75 µL of 7.5% Na_2CO_3. The mixture was incubated for 2 hours in the dark. After incubation, absorbance was measured at 765 nm. A blank was also prepared following the same procedure but replacing the plant extract from the polluted or non-polluted site with methanol. The same procedure was repeated for the standard gallic acid and the calibration curve was constructed as the concentration of 25–100 µg/mL. All measurements were performed in triplicate.

Total flavonoid content determination. The total flavonoid content of studied extracts was determined by the method of Topçu et al. (2007) for 96-well microplate analysis. The mixture reaction contains 50 µL of plant extract from the polluted or non-polluted site, 20 µL of $NaNO_2$ (05%), 20 µL of $AlCl_3$ (10%) and 100 µL of sodium hydroxide. The mixture was incubated for 40 minutes in the dark. TFC was expressed as µg quercetin equivalent (µg GAE/mL). The absorbance of the mixture was then measured at 510 nm.

Total condensed tannin content determination. Total condensed tannin content was measured using the protocol as described by Hagerman (2002) with some minor modifications. 80 µL of HCl was applied to 50 µL of plant extract from the polluted or non-polluted site and 160 µL of 4% vanilline. The mixture was incubated in ambient temperature for 20 minutes. The preparation's absorbance was measured at 500 nm. The concentrations of condensed tannins were calculated using a range of standard defined with the tannic acid and expressed in (µg TAE/mL).

Antioxidant activity determination. The DPPH antioxidant activity was determined according to the method as described by Blois (1958) with some modifications. The DPPH solution was prepared by dissolving 4 mg of DPPH in 100 mL of methanol. The solution was adjusted to an absorbance of 0.7 at 517 nm and protected from light. In a 96-microplate well, a volume of 40 µL of extract or standard antioxidants including α-tocopherol, BHT, and BHA at different concentrations, was added to 160 µL of the DPPH solution. After the incubation of 20 minutes in the dark, the absorbance was measured at 517 nm. The assay was carried out in triplicate.

The ABTS scavenging activity was determined using the method as described by Re et al. (1999). Initially, ABTS+ solution was prepared using 19.2 mg of ABTS (7 mM) powder dissolved in 5 mL of distilled water followed by dissolving 3.3 mg of $K_2S_2O_8$ (2.45 mM) in 5 mL of distilled water. The two solutions were mixed and incubated in the dark at room temperature for 16 hours. Before use, the mixture was diluted to adjust the absorbance at 0.700 ± 0.020 at 734 nm. In a 96 microplate well, 160 µL of the prepared solution was added to 40 µL of extract or reference standard (α-tocopherol, BHT, BHA) at various concentrations and then incubated in the dark for 10 minutes at room temperature before absorbance measuring at 734 nm. This activity was performed three times.

The activity of phenanthroline is determined using the Szydlowska-Czerniak et al. (2008) method with a 96-well microplate reader. Two solutions were prepared separately. The first solution was prepared by dissolving 0.05 g of 1,10-phenanthroline in 10 mL of methamol to obtain 0.5% phenanthroline solution. The second solution was prepared by dissolving 0.02 g of $FeCl_3$ in 10 mL of distilled water to obtain 0.2% ferric chloride solution. Then, 50 µL of 0.2% $FeCl_3$, 30 µL of 0.5% phenanthroline, and 110 µL of MeOH were added successively to 10 µL of extract or BHT standard compound at different concentrations. After that, the mixture was incubated at 30 °C for 20 minutes in the dark. Finally, the absorbance was measured at 510 nm. The result was calculated as $A_{0.5}$ (µg/mL) corresponding to the concentration indicating an absorbance of 0.5.

The assay of reducing power was conducted utilizing a methodology as described by Oyaizu (1986) with some modifications. In a 96-microplate well, 40 µL of phosphate buffer (pH 6.6) was put followed by 10 µL of extract, or reference compounds (ascorbic acid, BHA, BHT). After that, 50 µL of 1% [$K_3Fe(CN)_6$] was added and the mixture was incubated for 20 mn at 50°C. Then, 50 µL of 10% trichloroacetic acid, 40 µL of distilled water, 10 µL of a 0.1% $FeCl_3$ solution were added successively. The absorbance was measured at 700 nm.

Inhibition of enzymatic activity. The α-amylase inhibitory activity was performed using the iodine/potassium iodide (IKI) method as described by Zengin et al. (2014) with some modifications. In 96-micoplate well, 50 µL of α-amylase 1 U solution was added to 25 µL of extract solution or acarbose as a compound reference. After 10 min of incubation at 37 °C, 50 µL of 0.1% soluble starch was added to the mixture and returned to the incubation at the same conditions. To stop the enzymatic process, 25 µL of HCl (1M) and 100 µL IKI solution was added and the absorbance was measured at 630 nm. The percentage of inhibition was calculated using the following formula:

% INH=1-[(Ac-Ae)-(As-Ab)/(Ac-Ae)]

Ac=Absorbance [Starch + IKI + HCl + Methanol + Enzyme]

Ae=Absorbance [Enzyme + Starch + IKI + HCL + Methanol]

As=Absorbance [Enzyme + Extract + Starch + IKI + HCl]

Ab=Absorbance [Extract + IKI + 125µL Sodium Phosphate Buffer].

The inhibition of cholinesterase activity was evaluated according to Ellman's et al. (1961) microplate assay. In 96-microplate well, 150 µL of 100 mM sodium phosphate buffer (pH 8.0) was combined with 10 µL of extract solution or galantamine as standard compound dissolved in methanol at different concentrations, followed by 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) solution. After incubation at 25°C for 15 minutes, 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholine iodide (ACTI) (0.71 mM) or 10 µL of butyrylthiocholine chloride (0.2 mM) were added. The absorbance was then measured at 412 nm at T = 0 minutes. Second measurement was taken at T = 15 minutes after incubation at 37 °C.

Determination of the antibacterial activity. The antibacterial activity of extracts from both sampling sites was evaluated using the disc diffusion method on Mueller-Hinton agar with reference laboratory strains "ATCC" according to the method of Hegstad et al. (2014). The two Gram positive strains used were *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 10876), while the two Gram negative strains used were *Escherichia coli* (ATCC 25923) and *Salmonella enteritidis* (ATCC 13076). The strains were revitalized, and turbidity was adjusted to 0.5 McFarland, corresponding to 1.5×10^8 CFU/mL with an optical density of 0.1 at 625 nm. 25 mL of MH was poured into Petri dishes and allowed to cool before inoculation. Discs (6 mm in diameter) impregnated with 20 µL of extract at various concentrations were then applied firmly to the surface of the inoculated agar plate. An ofloxacin disc (5 µg) was used as a positive control. Petri dishes were incubated at 37 °C for 18 hours. The diameter of the inhibition zone was measured using caliper including the disc diameter.

Statistical analysis. All data are presented as mean ± standard error (SE) from triplicate measurements. Given the small sample size (n = 3), non-parametric statistical tests were used throughout, as normality cannot be reliably assessed under these conditions. For comparisons between extracts from the two sampling sites (Tables 2 and 4), the Mann–Whitney U test was applied. Statistical significance is denoted as $P < 0.05$. For antioxidant and enzyme inhibition assays, IC_{50} values were determined using non-linear regression analysis. For antimicrobial data and all comparisons involving three or more groups, including reference standards (Tables 5, 6, and 7), the Kruskal–Wallis test followed by Dunn's post-hoc test with Bonferroni correction was performed. Groups sharing the same letter within a row or column do not differ significantly ($P \geq 0.05$).

Results

Extract yields. The extraction of phenolic compounds from the aerial parts (stem, leaves and flowers) of plants from polluted and unpolluted environments was carried out by the methanol maceration method and showed that plants from the polluted area gave a higher yield than plants growing in the unpolluted site with 6.39 ± 0.57% and 5.98 ± 0.32%, respectively.

Heavy metal bioaccumulation and stress indicators. Quantitative analysis of heavy metals in the aerial parts of *O. natrix* revealed a marked bioaccumulation pattern in plants from the polluted site (Table 2). Lead, absent in samples from the unpolluted area, exceeded 2.14 ± 0.01 mg/kg in contaminated plants. Zinc levels were more than fourfold higher than those measured at the unpolluted site. The resulting values describe clear differences in metal concentrations between polluted and non-polluted sampling sites during the field survey carried out in the studied region.

Table 2

Bioaccumulation of heavy metals in the aerial parts of *O. natrix* from polluted and unpolluted sites (mg/kg)

Samples	Pb	Cd	Zn	Cu
ONP	2.14 ± 0.01***	0.18 ± 0.01***	32.83 ± 0.01***	3.92 ± 0.01***
ONU	ND	ND	7.22 ± 0.01	0.88 ± 0.01

Note: ONP – *O. natrix* from polluted site, ONU – *O. natrix* from unpolluted site, Pb – lead, Cd – cadmium, Zn – zinc, Cu – copper, ND – not detected; statistical comparisons between ONP and ONU were performed using the Mann–Whitney U test; significance levels: * P < 0.05, ** P < 0.01, *** P < 0.001.

UPLC-ESI-MS/MS phytochemical profiling. The UPLC-ESI-MS/MS analysis revealed both qualitative and quantitative variations in secondary metabolites. Sixteen compounds were identified in extracts from both sites, many of which are known for their antioxidant and antimicrobial activities. Among these compounds (Table 3; Figures 1 and 2) were thymol, chrysin, resveratrol, riboflavin, ferulic acid, kaempferol, and caffeic acid. Additionally, 4-methoxybenzoic acid, chlorogenic acid, vanillic acid, folic acid, and naringenin were detected exclusively in extracts from plants collected at the polluted site, whereas gallic acid was found only in samples from the unpolluted site.

Table 3
UPLC-ESI-MS/MS phytochemical profiling of methanolic extracts of *O. natrix* from polluted and unpolluted environments

Peak	Compound	Molecular formula	Molecular weight	ESI charge (+/-)	m/z	RT, min	Area	
							ONP	ONU
1	Caffeic acid	$C_9H_8O_4$	180.16	(-)	179.1500>135.0000	1.861	8462	5555
2	Ferulic acid	$C_{10}H_{10}O_4$	194.18	(+)	194.8000>177.0500	2.817	142518	41633
3	Cinnamic acid	$C_9H_8O_2$	148.16	(+)	149.0500>131.0000	3.487	24868	36432
4	Oleuropein	$C_{25}H_{32}O_{13}$	540.5	(-)	541.3000>146.4000	4.016	14759	21238
5	4-Mythoxybenzoic acid	$C_8H_8O_3$	152.15	(+)	151.0500>107.0500	5.749	17022	0
6	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	(-)	353.0500>191.1000	6.283	7589	0
7	Vanillic acid	$C_8H_8O_4$	168.15	(-)	166.9500>151.9000	7.347	4987	0
8	Gallic acid	$C_4H_4O_4$	170.12	(-)	169.0000>124.9000	8.256	0	8904
9	p-Coumaric acid	$C_9H_8O_3$	164.16	(-)	163.0500>118.9500	8.696	99634	57894
10	Rutin	$C_{27}H_{30}O_{16}$	610.5	(+)	611.0000>465.2000	9.203	10005	18876
11	Sinapic acid	$C_{11}H_{12}O_5$	224.21	(+)	225.0000>91.1000	9.588	23725	22873
12	Kojic acid	$C_6H_6O_4$	142.11	(+)	143.0000>41.1000	9.722	9280	19595
13	Resveratrol	$C_{14}H_{12}O_3$	228.24	(+)	229.0500>107.1000	9.834	68207	56641
14	Luteolin	$C_{15}H_{10}O_6$	286.24	(+)	286.7500>153.0000	10.488	54981	46051
15	Kampferol	$C_{15}H_{10}O_6$	286.24	(+)	285.0000>93.0000	11.197	797	3195
16	Thymol	$C_{10}H_{14}O$	150.22	(+)	151.1000>109.0500	11.546	18865	21627
17	Folic acid	$C_{19}H_{19}N_7O_6$	441	(+)	442.9000>59.1000	12.479	2377	0
18	Chrysin	$C_{15}H_{10}O_4$	254.24	(+)	255.1000>68.8500	13.024	201239	292367
19	Naringenin	$C_{15}H_{12}O_5$	272.25	(+)	273.0500>153.0000	13.785	188190	0
20	Riboflavin	$C_{17}H_{20}N_4O_6$	376.4	(+)	377.9000>361.3500	14.712	443425	600512
21	Epicatechin	$C_{15}H_{14}O_6$	290.27	(+)	290.9000>139.0500	15.372	29969	8936
22	β-carotene	$C_{40}H_{56}$	536.87	(+)	537.2000>23.1000	15.504	25934	271783

Note: ESI – electrospray ionization, m/z – mass/charge, RT – retention time, ONP – *O. natrix* from polluted site, ONU – *O. natrix* from unpolluted site.

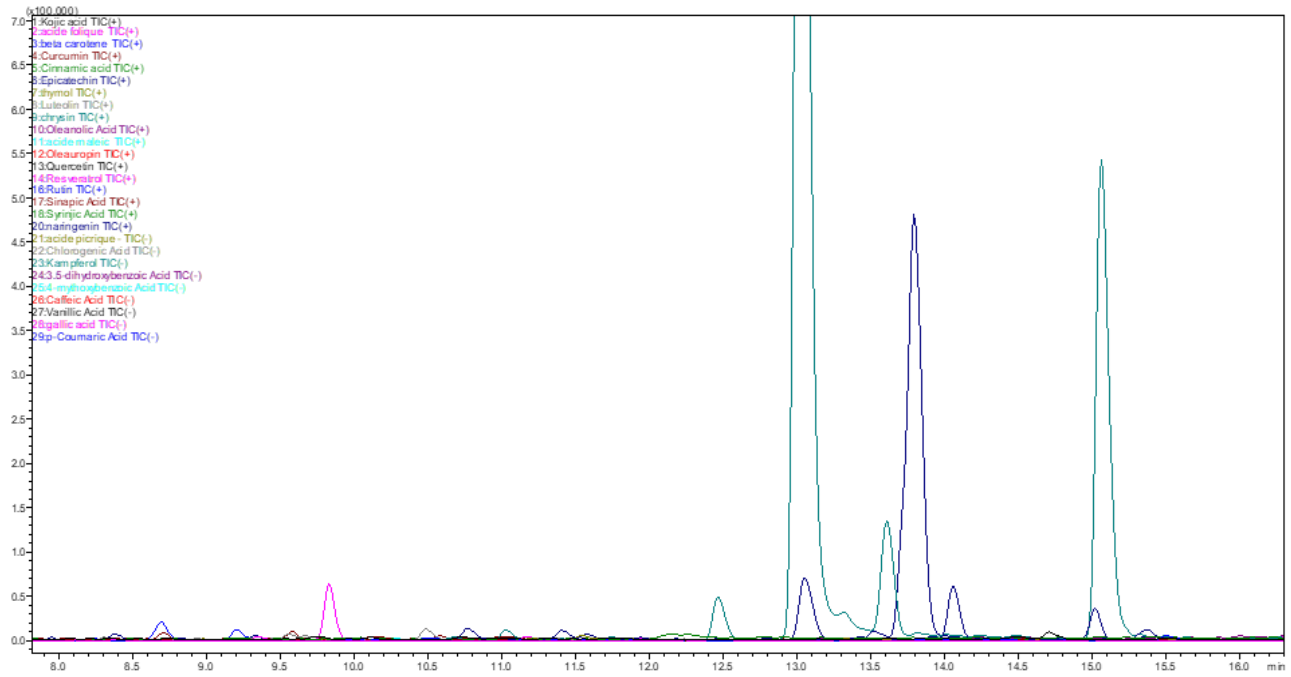

Fig. 1. Chromatogram of methanolic extract of *O. natrix* from unpolluted site

Polyphenol, flavonoid, and tannin contents. Extracts from plants collected in the polluted area showed higher total phenolics and flavonoids compared with those from the unpolluted site (Table 4). Total tannins were present at low concentrations in both sites, with only minor differences between polluted and unpolluted samples.

Antioxidant activity. The extract from plants collected in the polluted area outperformed that from the unpolluted site in all antioxidant assays (Table 5). It exhibited lower IC_{50} values, indicating a stronger free radical scavenging capacity. In the DPPH assay, the polluted-site extract had an IC_{50} values of 26.06 ± 0.91 μg/mL, compared with 33.66 ± 1.90 μg/mL in the unpolluted extract. In the ABTS test, the polluted-site extract also exhibited lower IC_{50} values. Additionally, it showed greater metal chelation activity and higher reducing power, indicating increased electron-donating capacity. These measurements illustrate the differences in free radical scavenging and overall antioxidant behaviour of extracts from plants grown in the two sites.

Table 4
Quantification of polyphenol, flavonoid, and tannin contents of *O. natrix* methanolic extracts (mean ± SE, triplicates)

Samples	Total phenolic content, μg GAE/mL	Flavonoids content, μg QE/mL	Total tannins content, μg TAE/mL
ONP	53.56 ± 1.19***	42.01 ± 0.32***	0.23 ± 0.05*
ONU	46.34 ± 0.39	33.23 ± 0.87	0.21 ± 0.04

Note: statistical comparisons between ONP and ONU were performed using the Mann–Whitney U test; significance levels: * P < 0.05, ** P < 0.01, *** P < 0.001.

Anti-enzymatic activities. The inhibitory effects of *O. natrix* extracts on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and α-amylase were assessed and expressed as IC_{50} values (μg/mL) (Table 6). Extracts from plants collected in the polluted area generally exhibited stronger inhibition across all tested enzymes compared with

those from the unpolluted site. The polluted-site extract showed IC_{50} values of approximately 60.73 ± 1.49 µg/mL for AChE and 108.22 ± 5.79 µg/mL for BChE, whereas the unpolluted-site extract recorded higher values. Galantamine, used as a reference inhibitor, exhibited much lower IC_{50} values for both AChE and BChE. Both plant extracts also demonstrated notable α-amylase inhibition relative to acarbose. This allows direct comparison of the extracts' inhibitory effects with a standard therapeutic inhibitor under the two environmental conditions.

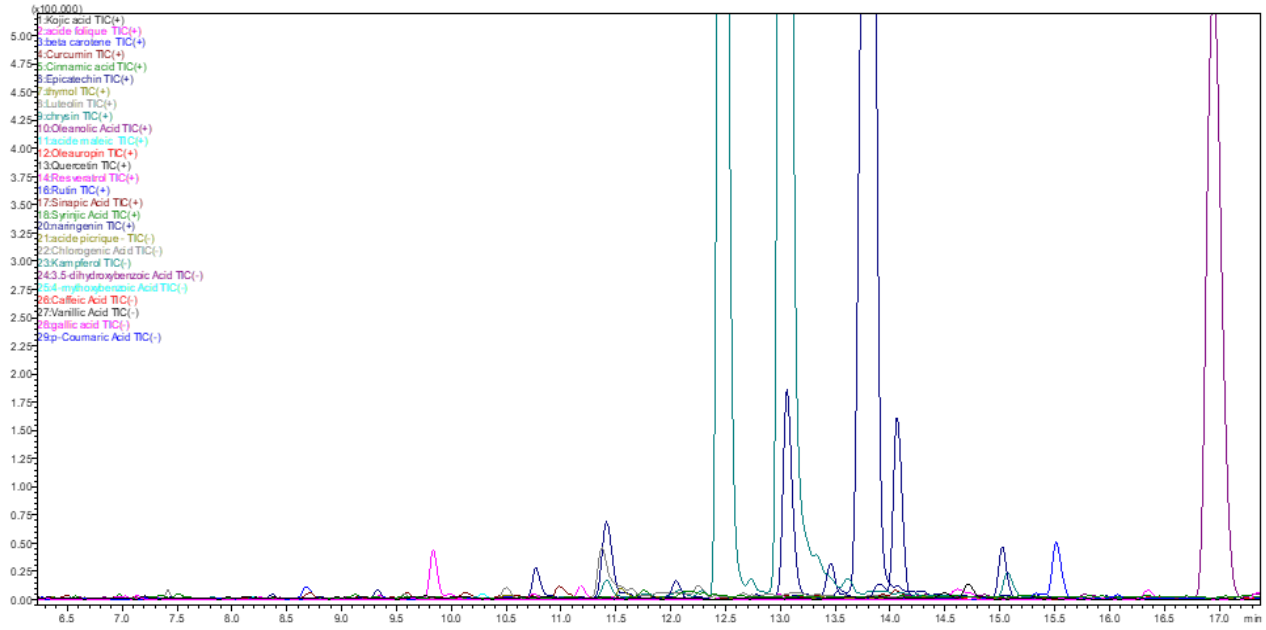

Fig. 2. Chromatogram of methanolic extract of *O. natrix* from polluted site

Table 5
Antioxidant activities of *O. natrix* methanolic extracts (mean ± SE, triplicates)

Samples	IC_{50}, µg/mL		$A_{0.5}$, µg/mL	
	ABTS	DPPH	phenanthroline	reducing power
ONU	23.75 ± 1.86^{c}	33.66 ± 1.90^{d}	10.09 ± 0.37^{c}	52.15 ± 1.81^{c}
ONP	15.49 ± 1.89^{b}	26.06 ± 0.91^{c}	6.18 ± 0.66^{b}	33.03 ± 2.89^{b}
α-Tocopherol *	NT	12.72 ± 0.25^{b}	NT	34.97 ± 2.55^{b}
Ascorbic acid *	NT	NT	NT	6.88 ± 0.69^{a}
BHA *	1.84 ± 0.16^{a}	6.03 ± 0.34^{a}	0.89 ± 0.04^{a}	NT
BHT *	1.31 ± 0.09^{a}	13.03 ± 0.64^{b}	2.21 ± 0.12^{a}	NT

Note: ONP – *O. natrix* from polluted site, ONU – *O. natrix* from unpolluted site, BHA – butylated hydroxyanisole, BHT – butylated hydroxytoluene, NT – not tested, * – reference compounds, IC_{50} – concentrations causing 50% inhibition, $A_{0.5}$ – concentration at 0.50 absorbance; values within the same column followed by different lowercase letters (a, b, c, d) are significantly different from each other ($P < 0.05$); statistical significance was determined using Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction.

Table 6
IC_{50} (µg/mL) values for enzymatic inhibition activities by methanolic extracts (mean ± SE, triplicates)

Extracts	AchE	BchE	α-Amylase
ONU	70.5 ± 1.7^{c}	127.6 ± 4.4^{b}	335.6 ± 8.3^{b}
ONP	60.7 ± 1.5^{b}	108.2 ± 5.8^{b}	268.1 ± 9.6^{a}
Galantamine*	6.8 ± 1.3^{a}	33.9 ± 1.9^{a}	NT
Acarbose*	NT	NT	3177.3 ± 11.7^{c}

Note: ONP – *O. natrix* from polluted site, ONU – *O. natrix* from unpolluted site, NT – not tested, AChE – acetylcholinesterase, BChE – butyrylcholinesterase, * – reference compounds, IC_{50} – concentrations causing 50% inhibition; values within the same column followed by different lowercase letters (^{a}, ^{b}, ^{c}) are significantly different from each other ($P < 0.05$); statistical significance was determined using Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction.

Antibacterial activity. Both extracts inhibited Gram-positive and Gram-negative strains, with the extract from plants collected in the polluted area showing slightly broader activity. The inhibition zone against *Staphylococcus aureus* was about 14.67 ± 0.33 mm for the polluted-site extract compared with 13.83 ± 0.17 mm for the unpolluted-site extract. *Bacillus cereus* and *Salmonella enteritidis* were also more strongly inhibited by the polluted-site extract. Both extracts showed similar activity against *Escherichia coli.* In comparison, the reference antibiotic ofloxacin produced larger inhibition zones, indicating stronger antibacterial activity than both plant extracts (Table 7).

Table 7
Inhibition zones (mm) produced by plant aerial parts extracts and the control antibiotic (mean ± SE, triplicates)

Strains	Plant extract (300 mg/mL)		OFX (5 µg)
	ONU	ONP	
Staphylococcus aureus ATCC 25923	13.83 ± 0.17^{a,A}	14.67 ± 0.33^{a,A}	23.03 ± 0.41^{a,B}
Escherichia coli ATTC 25922	17.50 ± 0.58^{b,A}	17.50 ± 0.29^{b,A}	31.17 ± 0.28^{c,B}
Bacillus cereus ATCC 10876	12.67 ± 0.33^{a,A}	14.00 ± 0.58^{a,A}	28.37 ± 0.43^{b,B}
Salmonella enteritidis ATCC 13076	14.00 ± 0.12^{a,A}	14.33 ± 0.33^{a,A}	32.20 ± 0.47^{c,B}

Note: ONP – *O. natrix* from polluted site, ONU – *O. natrix* from unpolluted site, OFX – ofloxacin; values within the same row followed by different uppercase letters (^{A}, ^{B}) are significantly different between groups ($P < 0.05$); statistical significance was determined using Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction.

Discussion

According to World Health Organization guidelines (2007), the maximum permissible levels in medicinal plants are 10 mg/kg for lead (Pb) and 0.3 mg/kg for cadmium (Cd). In plants collected from the polluted site, both Pb and Cd levels are below these thresholds, but clearly exceed the concentrations found in plants from the unpol-

luted area. A study by Pandey & Singh (2012) has shown that cadmium concentrations above 0.1 mg/kg can disrupt enzymatic activity, photosynthesis, and secondary metabolism in Fabaceae. Another study conducted by Raza et al. (2020) reported that even low concentrations of cadmium are capable of inducing sublethal biochemical responses including the activation of antioxidant enzymes and stimulation of phenolic compound biosynthesis. Similarly, Dias et al. (2019) reported that Pb concentrations of 10 mg/kg may also stimulate stress-related defense mechanisms. The observed bioaccumulation patterns confirm the ability of *O. natrix* to tolerate and sequester metals likely due to physiological adaptations such as the activation of antioxidant enzymes, enhanced phenolic biosynthesis, and metal-binding peptides.

The results of UPLC-ESI-MS/MS phytochemical profiling showed a similarity to what Erturk et al. (2018) found, who identified quercetin, taxifolin, hyperoside, and eriodyctiol as the main chemicals in methanolic extracts of *O. natrix*. Another study of Bhiri et al. (2025) showed that extracts from other species of *Ononis* had more secondary metabolites but fewer of these chemicals showing the differences between species. Mhamdi et al. (2015) found a lot of quercetin (24.5%) and kaempferol (10.5%) in wild *O. natrix* in Tunisia, which matches what we found. Also, Sayari et al. (2016) saw that there was a strong connection between phenolic content and biological activities, which agrees with our results. Notably, folic acid, naringenin, chlorogenic acid, vanillic acid, and 4-methoxybenzoic acid were detected exclusively in plants from the polluted site. These metabolites have documented roles in oxidative stress defense particularly folic acid and chlorogenic acid, which are involved in scavenging free radicals and protecting nucleic acids from metal-induced genotoxicity (Kováčik & Klejdus, 2008). The presence of these molecules only in the polluted plants may represent a specific metabolic response to metal stress. In contrast, the absence of gallic acid in plants under heavy metal stress may indicate a metabolic reprogramming in favour of more specific and effective stress-responsive metabolites (Tripathi & Tripathi, 2019).

When compared to the available literature, the levels of polyphenols, flavonoids, and tannins measured in this study show noticeable variation. Ertaş et al. (2025) reported a total phenolic content of 74.60 mg GAE/g DW and a flavonoid content of 27.88 mg RE/g DW in methanolic extracts of *O. natrix* subsp. *natrix*, whereas Mhamdi et al. (2015) found lower values of 51 mg GAE/g DW and 14.76 mg CE/g DW, respectively. Interestingly, total tannin contents were similar between both samples (polluted site: 0.23 ± 0.05 µg TAE/mL; unpolluted site: 0.21 ± 0.04 µg TAE/mL), suggesting that tannin biosynthesis may be less affected by metal stress or may reach a steady metabolic threshold (Molnar et al., 2024). The elevated phenolic and flavonoid levels in plants from the polluted area relative to the unpolluted area likely indicate an adaptive metabolic adjustment to abiotic stress, consistent with the principle that plants under environmental pressure intensify secondary metabolite synthesis via the phenylpropanoid pathway (Goncharuk & Zagoskina, 2023).

Exposure to heavy metals leads plants to generate excessive reactive oxygen species which can damage vital cellular components such as DNA, lipids, and proteins (Kaur & Goyal, 2022). To counteract this oxidative stress, plants activate antioxidant defense mechanisms by stimulating the synthesis of secondary metabolites through the phenylpropanoid and flavonoid pathways (Kumar et al., 2023). In plants from the polluted site, the higher levels of polyphenols and flavonoids indicate the presence of specific bioactive compounds that strengthen their defense against metal-induced stress. The detection of chlorogenic acid and rutin further supports this adaptive response as these molecules act as powerful antioxidants that protect plant tissues from oxidative damage (Sharma et al., 2021). Likewise, phenolic acids such as vanillic acid and 4-methoxybenzoic acid contribute to stress tolerance by chelating toxic metals like Pb^{2+} and Cd^{2+} and stabilizing cell membranes (Ejaz et al., 2023). Another notable finding is the presence of folic acid exclusively in plants from polluted sites. Although folic acid is mainly associated with fundamental physiological roles such as DNA repair, it can also enhance stress protection by supporting redox balance and cellular stability (Gorelova et al., 2017). The presence of this compound in polluted plants indicates its participation in the plant's defense mechanism against metal-induced stress as these plants also displayed elevated concentrations of phenolic acids and flavonoids which are well known for their dual function in enhancing stress tolerance and providing antimicrobial protection (Khare et al., 2020). The reduced level of gallic acid might reflect its transformation into other derivatives under stress conditions, a metabolic adjustment commonly observed in plants, and altogether these results demonstrate that *O. natrix* activates a complex biochemical network under metal exposure, leading to the accumulation of protective compounds that enhance its survival in harsh environments, a response that may hold potential for developing stress-tolerant crops or plant-based bioactive products (Sanal, 2019).

Regarding the anti-enzymatic activities, the stronger enzyme inhibition of plants from the polluted site may be attributed to the presence of specific flavonoids and phenolic acids known to target cholinesterase. Compounds such as naringenin, chrysin, kaempferol, and caffeic acid, detected in higher abundance or exclusively in polluted plants have been reported to exert neuroprotective effects through cholinesterase inhibition mechanisms (Cichon et al., 2025). These molecules can interact with the active site or peripheral anionic site of the enzyme, preventing substrate hydrolysis (Karunakaran et al., 2022). Similarly, extracts from the polluted site demonstrated a stronger inhibition of α-amylase, which is relevant for antidiabetic potential. This effect is also consistent with the presence of chlorogenic acid, ferulic acid, and rutin compounds shown to bind to α-amylase via hydrogen bonding and hydrophobic interactions, leading to catalytic site obstruction (Ibrahim et al., 2024). Interestingly, this elevated enzymatic inhibition coincides with its higher levels of bioaccumulated metals especially Zn and Cu, which are known to stimulate oxidative stress and trigger adaptive metabolic pathways in plants (Son et al., 2024). Such stress often results in the overexpression of phenolic biosynthesis genes like phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) leading to the accumulation of bioactive compounds (Sharma et al., 2019). Thus, the enhanced anti-enzymatic activity observed in plants from the polluted site can be viewed as a downstream effect of both its unique phytochemical signature and its environmental stress adaptation.

The enhanced antibacterial activity in plants collected from the polluted environment correlates with its richer and more diverse phytochemical profile. These plants uniquely contained compounds such as naringenin, chlorogenic acid, folic acid, and vanillic acid, all known for their antimicrobial properties. For instance, naringenin is reported to disrupt bacterial cell walls and interfere with quorum sensing while chlorogenic acid has been shown to increase membrane permeability and inhibit bacterial enzymes (Tian et al., 2021). These actions may explain the superior inhibition observed against Gram (+) bacteria which possess simpler membrane structures (Yu et al., 2024). In addition, compounds present in both extracts such as thymol, resveratrol and epicatechin are established antimicrobial agents. Thymol in particular acts by disrupting membrane integrity and reducing bacterial ATP levels (Kowalczyk et al., 2020). The synergy between such molecules likely enhances the overall antibacterial activity, particularly in the more chemically diverse extracts from plants exposed to metal stress.

Conclusion

This study provides comprehensive evidence of the biochemical and physiological strategies developed by *Ononis natrix* L. in response to metal-contaminated environments. Plants collected from polluted areas exhibited distinct metabolic profiles characterized by a higher accumulation of phenolic acids, flavonoids, and other bioactive metabolites reflecting a clear adaptive mechanism to metal-induced oxidative stress. The elevated concentrations of these secondary metabolites are consistent with the activation of specific biosynthetic pathways particularly those regulated by enzymes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) which are key mediators in the phenylpropanoid route. This activation enhances the

synthesis of antioxidants and metal-chelating molecules helping the plant maintain redox homeostasis and mitigate cellular damage.

The exclusive detection of compounds such as folic acid, vanillic acid, chlorogenic acid, and naringenin in plants from polluted sites further supports their involvement in stress mitigation. These molecules play multiple roles including scavenging of reactive oxygen species, stabilization of cell membranes and chelation of toxic metals like lead and cadmium. Their accumulation suggests that *O. natrix* undergoes a sophisticated metabolic reprogramming to counteract abiotic stress while preserving essential physiological processes. The observed reduction in gallic acid content may also indicate a redirection of metabolic flux toward the synthesis of more efficient defense molecules.

Beyond oxidative stress tolerance, the extracts from polluted-site plants displayed stronger anti-enzymatic and antibacterial activities. Such enhanced bioactivities likely result from the synergistic effects of diverse secondary metabolites that interact to inhibit enzymes and disrupt microbial integrity.

Overall, these findings highlight the resilience of *O. natrix* and its capacity to thrive in contaminated soils through adaptive biochemical mechanisms. This species emerges as a promising candidate for phytoremediation, as well as a potential source of natural antioxidants, antimicrobial agents and enzyme inhibitors for pharmaceutical and biotechnological applications.

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