

Bioaccumulation of industrial heavy metals and interactive biochemical effects on two tropical medicinal plant species

KALICHARAN MANDAL (✉ kalicharanmndl.94@gmail.com)

CSIR-IMMT: Council of Scientific and Industrial Research Institute of Minerals and Materials Technology
<https://orcid.org/0000-0003-3648-2665>

Nabin Kumar Dhal

CSIR-IMMT: Council of Scientific and Industrial Research Institute of Minerals and Materials Technology

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Abstract

Concentrations of heavy metals (Cr, Cu, Fe, Mn, Ni, Pb, and Zn) accumulation were studied in the leaves of two medicinal plant species, namely *Holarrhena pubescens*, and *Wrightia tinctoria*, from two industrial areas and a control area. Our comparison study revealed that industrialization significantly increased the accumulation of heavy metals in both plant species. A comparison study in control and industrial areas exhibited that heavy metal accumulation was higher in the industrially affected area than in the control area. In the control area, both species accumulated the lowest heavy metal concentration. Where heavy metals concentration exceeded the permissible limit recommended by the WHO in both species of two industrial areas. Biochemical investigation specifies that in response to heavy metals accumulation, both species increased the activity of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) content, the activity of enzymatic [superoxide dismutase(SOD) and peroxidase (POD)] and nonenzymatic [ascorbic acid (AsA)] antioxidant, but decreased the primary (soluble carbohydrate and total protein), secondary metabolites (phenol and flavonoid) content and free radical scavenging (DPPH) activity. These study indicate that industrialization potentially harms medicinal plants by reducing the efficacy of their medicinal property.

1. Introduction

Rapid industrialization and urbanization have led to the extensive pollution of heavy metals with a persistent contaminant in the environment. Atmospheric particulate matter (PM_{10} and $PM_{2.5}$), which continuously increases the concentration in urban to rural areas, represents the primary airborne pollutants affecting air quality in most cities of India (Balakrishnan et al., 2019; Sharma et al., 2020). Due to the specific surface area of heavy metals, most atmospheric particulate matter generated from coal, fuel, and industrial pollution, such as iron (Fe), chromium (Cr), lead (Pb), cadmium (Cd), zinc (Zn), and copper (Cu). (Park et al. 2018 ;). Heavy metals deteriorate the natural ecosystem due to their bio-magnification ability at various trophic levels. It causes the ecosystem to break down, threatens biodiversity, and affects human health. These heavy metals also can have an adverse effect on plants (Ghori et al., 2019; Nargis et al., 2022). Plants accumulate atmospheric pollutants transported through air mass movements by dry and wet deposition; thus, they are the primary receptors for heavy metal pollution. (Sgrigna et al., 2020). Plants can directly accumulate heavy metal deposition on the leaves' surfaces via the stomata, which can disturb internal homeostasis and cause various morphological, physiological, and biochemical disturbances (Sun et al., 2010; Chaudhary and Rathore, 2019).

Heavy metals generate oxidative stress by producing high concentrations of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet-}$), singlet oxygen (1O_2), and hydrogen peroxide (H_2O_2) (Gill, M. 2014; Štolfa et al., 2015; Berni et al., 2019). These ROS react with lipids, nucleic acids, pigments, and proteins and cause lipid peroxidation, membrane damage, and inactivation of enzymes (Czarnocka and Karpiński, 2018; Silva et al., 2020). Plants withstand oxidative stress by applying enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and glutathione reductase (GR),

and the low molecular weight antioxidants such as cysteine, non-protein thiols, ascorbic acid, and glutathione (Mukherjee et al., 2020; Ibrahim et al., 2017). Some other processes involve the accumulation of plant secondary metabolites such as phenolic, flavonoids, alkaloids, and N-containing compounds. These secondary plant metabolites play an essential part in plant defense under various biotic and abiotic stress (Arbona et al., 2013; Houda et al., 2016; Kazan, 2018). Secondary metabolites have diverse pharmacological properties, and thus any environmental conditions that affect the amount or composition of secondary metabolites could potentially affect the effectiveness of medicinal plant products. (Yang et al., 2018; Gupta et al., 2019). There is limited information on specific physiological responses of medicinal plants to heavy metals in the atmosphere and the changes in efficacy of the plant. Uptake and accumulation of heavy metals in medicinal plants pose a serious safety threat to consumers and affect the plant growth, development, and quality of derived medicinal plant products (Lajayer et al., 2017; Kohzadi et al., 2019; Chen et al., 2021). Most of the studies in the literature are devoted to heavy metal concentrations in soils and plants of urban areas (Israr et al., 2011; Cui et al., 2022). The complex study on plants' biochemical condition in connection to heavy metal absorption in the environment remains urgent, as do determinations regarding the mechanisms that determine the effect of heavy metals on biochemical processes.

Holarrhena pubescens Wall. Ex S. Don and *Wrightia tinctoria* R. Br. were selected for this study. Both species belong to the Apocynaceae family and are widely acknowledged medicinal plants native to the Indian subcontinent. Both the plant species have extensive uses in indigenous medicinal systems. The leaves of those species are widely used for treating various diseases, viz. liver disorder, bowel syndrome, blood-related ailments, diabetes, urogenital disorders, anti-HIV, anti-diabetic (type 2 diabetic) properties, and many other diseases. The methanolic and hexane extracts of the aerial parts of *H. pubescens* and *W. tinctoria* show significant antimicrobial and free radical scavenging activity (Srivastava, R. 2014; Zahara et al., 2020). Previously, no study has been conducted to look into the impact of industry-generated heavy metal interactions with medicinal plants. This study investigated the detailed metabolic effects of heavy metals in industrial areas on medicinal plant species. In their natural habitat, we estimated physiological and biochemical changes in *H. pubescens* and *W. tinctoria*. In addition, we determined the secondary metabolite content and free radical scavenging activity to establish the impact of industrialization on the efficacy of medicinal plants.

2. Materials And Methods

2.1. Study sites and plant species

The Angul-Talcher industrial areas are situated in eastern India lie between latitudes 20° 41' 10" N and 21° 08' 37" N and longitudes 84° 55' 00" E to 85° 30' 00" E. The area comes under sub tropic monsoon climate with an average annual rainfall of 1370 mm. the temperature differs from 11.9° C to 44.4° C. In the early nineteenth century, heavy industries in the Angul-Talchar area began with 4106 industries ranging from small to large scale. It was listed among the 7th most polluted areas as evaluated by the Central Pollution Control Board. (CPCB) [CPCB, 2010]. Many industrial thermal power plants, steel plants,

and aluminium plants are in the area. In order to evaluate the industrial pollution impact on plants, two sampling areas were chosen, namely Angul and Talcher. The control area is situated 50 km away from the industrial zone, undisturbed by industrial or any other kind of pollution.

2.2. Sample collection

Leaf samples of five replicates of both species from three areas were selected for the collection. The replicate plants were considered as those individuals significantly distant from other individuals, having similar height and trunk diameter. Healthy, mature, and completely exposed to sunlight leaves were chosen. About 20 g of leaves were collected in a sealed bag from each plant, kept in a portable ice box, then transported to the lab, and stored at -20° C for biochemical analysis.

2.3. Analysis of heavy metals content

1 g of oven-dried leaves powder was digested with high-grade concentrated nitric acid (HNO₃) and hydrochloric acid (HCl) solution (3:1, V/V) in microwave digestion. Digested solutions were diluted with double distilled water, and the final volume was 50 ml. Then diluted solutions were analyzed for Cr, Cu, Fe, Mn, Ni, Pb, and Zn by PerkinElmer ICP-OES (Model: Optima 2100 DV).

2.4. Analysis of oxidative stress markers

2.4.1. Hydrogen peroxide (H₂O₂) content

H₂O₂ was evaluated with titanium reagent as Agarwal and Shaheen (2007) described. Leaf material (0.2 g) was homogenized in cold acetone. Titanium reagents were added to the extract, followed by a concentrated ammonium solution to precipitate the peroxide-titanium complex. The precipitate dissolved in 1M H₂SO₄, and absorbance was recorded at 415 nm. The concentration of H₂O₂ was calculated using a standard curve of known concentration of H₂O₂, prepared in the same method.

2.4.2. Malondialdehyde (MDA) content

MDA was estimated spectrophotometrically using thiobarbituric acid (TBA) assay to determine lipid peroxidation. The following formula estimated determination of MDA content:

$$\text{MDA level} = [\text{OD}_{532} \times 100 / 1.56] \times \text{TV} / [\text{d wt} \times 1000]$$

OD = optical density; TV = total volume of the extract (ml); d wt = weight of the dry leaf tissue (g)

2.5. Analysis of enzymatic and nonenzymatic antioxidants (SOD, POD, and ASA)

2.5.1 Superoxide dismutase (SOD)

The activity of SOD was analyzed according to Hu et al. (2011). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, two µM riboflavin, 0.1 mM EDTA, 75 µM NBT

and 50 µM of enzyme extract. The reaction mixture was positioned under a light intensity of 3000 Lux from a fluorescent lamp, and after 15 minutes, absorbance was recorded at 560 nm. The amount of enzyme that caused a 50% inhibition of photochemical reduction of NBT represented one unit of SOD activity.

2.5.2. Peroxidase (POD)

The activity of POD was determined according to Hu et al. (2011). The reaction cocktail contained 0.25% guaiacol that contained 50% ethanol, 0.75% H₂O₂, 0.1 M acetic acid, and 50 µl enzyme extract. Absorbance was recorded at 460 nm for every 3 min. One unit of POD activity was defined as an increase in absorbance at 460 nm for one min.

2.5.3 Ascorbic acid (ASA)

The ascorbic acid estimation was quantified using the colorimetric 2,6-dichlorophenol indophenol method. 0.2 g of the fresh leaf was homogenized in 4% oxalic acid and made up to 20 ml volume and centrifuge at 10000 rpm for 5 min. 5ml of the extract was used for titration against the dye. Titration was done till the pink colour appeared, which persisted for a few seconds.

$$AsA \left(\frac{mg}{g} \right) = \frac{0.5 (mg) * V2 (ml) * 30 (ml)}{V1 (ml) * 5 (ml) * weight of sample (g)}$$

Where,

V1 = Volume of dye titrated against the working standard

V2 = Volume of dye titrated against the sample

2.6. Determination of DPPH scavenging assay

The plant's free radical scavenging capacity was determined using DPPH (2, 2-diphenyl 1-picryl hydrazyl) according to the method Xie and Schaich (2014) with minor modification. Different concentrations of methanolic plant extract were mixed with freshly prepared 0.004% of DPPH solution. After 1 hrs of incubation in the dark, absorption of the solution was taken at 517 nm using a UV-vis spectrophotometer (Analytical UV-Vis 3090V). The radical scavenging activity (RSA) was calculated by using the following formula:

$$RSA\% = \left(\frac{Absorbance of control - Absorbance of test sample}{Absorbance of control} \right) \times 100$$

2.6. Determination soluble carbohydrates, and total protein content

2.6.1. Soluble carbohydrates

Soluble carbohydrate was estimated according to the phenol-sulphuric acid method described by Robyt and White (1987). 0.2 g of fresh leaf tissue was mixed with 2.5 N HCl and digested at 70°C for one hour. After cooling, one ml of the extract was mixed with 5% phenol and 5 ml of 95% sulfuric acid. Absorbance was recorded at 640 nm, and soluble carbohydrate concentration was determined with a standard curve prepared using a known glucose concentration.

2.6.2. Total protein content

The measurement of total protein content, 0.2g of fresh leaf tissue were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 6.8) and centrifuged at 10,000 rpm for 10 min. The supernatant was used for total protein content measurement according to Lowry et al. (1951) method using a standard curve of known concentration prepared by bovine serum albumin.

2.7. Determination of secondary metabolites (Phenols and flavonoids)

2.7.1. Phenols content

The determination of total free phenols present in plants was estimated using a folin–ciocalteu reagent and according to the method described by Abdeltaif et al. (2018). 0.2 g of dried leaves were mixed with 10 ml of 80% methanol. Samples were vortexed and incubated at dark for 2 hrs. After centrifuging, one ml supernatant was added to 0.5 ml of folin-ciocalteu reagent in an acidic medium. The absorption was taken at 765 nm, and phenolic content was measured using a standard curve prepared by the known concentration of Gallic acid.

2.7.2. Flavonoids content

Flavonoids content from plant leaf extract was estimated according to the method described by Kim et al. (2003). 1 ml of leaf extract mix with 5% sodium nitrate solution and 5% aluminum chloride. The mixture was incubated in the dark for 30 min, and the absorbance was measured at 510 nm. Total flavonoids content was measured from a known concentration of standard curve made by catechin.

2.8. Statistical analysis

All tables and figures present the mean value $\pm$ standard error of five independent replicates. The results were compared using ANOVA and Duncan tests to determine the significant difference at a significant level of $P \leq 0.05$ by SPSS Statistics Software 22.0 (IBM, SPSS Statistics). Correlation between metal-metal and metal-biochemical results was carried out using bivariate correlation test with Pearson's correlation coefficient test at a significance level of $P \leq 0.05$ and ≤ 0.001

3. Results

3.1. Accumulation of heavy metal

The concentration of Cr, Cu, Fe, Mn, Ni, Pb, and Zn in the leaves of *H. pubescens* and *W. tinctoria* from three sites are represented in Table 1. The concentration of heavy metals differed among the three sites and both species. The heavy metal concentrations were significantly higher ($p < 0.05$) at the Angul and Talcher than in the control area. While, *W. tinctoria* accumulated more Fe, Mn, Zn, Cu, and Pb. *H. pubescens* accumulated more Cr and Ni. There were significant differences in Fe, Cu, and Pb concentrations in *W. tinctoria* in both Angul and Talcher areas, which were 1185% and 1476%, 6328% and 6720%, and 1156% 2159%, respectively higher than in the control area. *H. pubescens* also accumulated significantly more Cr in both Angul and Talcher areas which were 4675% and 3129%, respectively, and significantly more Ni accumulated from Angul, which is 1063%.

Table 1

– Heavy metal concentrations (mg/kg) in *Holarrhena pubescens* and *Wrightia tinctoria* from Angul, Talcher and control areas

Sampling area	Species	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Angul	<i>H. pubescens</i>	70.67 ± 8.11	50 ± 3.06	1183.67 ± 133.44	288 ± 30.79	17.33 ± 1.76	6 ± 1	63.67 ± 4.84
	<i>W. tinctoria</i>	45.33 ± 18.52	54 ± 14.47	1241.67 ± 212.51	337.33 ± 31.76	11.67 ± 7.17	8.67 ± 1.76	86.67 ± 21.46
Talcher	<i>H. pubescens</i>	47.8 ± 10.05	48.9 ± 6.81	783.45 ± 22.60	311.22 ± 14.43	10.27 ± 2.49	13.05 ± 1.3	59.23 ± 4.75
	<i>W. tinctoria</i>	27.30 ± 7.78	55.93 ± 5.26	1523.17 ± 217.20	389.03 ± 10.52	3.03 ± 1.09	15.90 ± 2.86	79.33 ± 34.44
Control	<i>H. pubescens</i>	1.48 ± 0.28	1 ± 0.17	80.18 ± 9.21	82.87 ± 4.63	1.91 ± 0.33	0.63 ± 0.08	12.54 ± 0.95
	<i>W. tinctoria</i>	0.59 ± 0.12	0.82 ± 0.04	96.60 ± 14.56	87.72 ± 22.96	1.49 ± 0.21	0.69 ± 0.04	18.65 ± 1.66

3.2. Responses of stress markers and antioxidants

3.2.1. H₂O₂ and MDA content

H₂O₂ and MDA contents in *H. pubescens* and *W. tinctoria* are represented in Fig. 1a. The H₂O₂ and MDA content showed a significantly ($p < 0.05$) increasing trend in both Angul and Talcher areas in both plants. In all areas, H₂O₂ content in *W. tinctoria* plant was higher than in *H. pubescens* plant. The H₂O₂ content in the *H. pubescens* and *W. tinctoria* plants in the Angul area was 1900% and 1951% higher than in the control area. In the Talcher area, the H₂O₂ content in the plant of *H. pubescens* and *W. tinctoria* were 2079% and 2300% higher than in the control area, respectively.

MDA content showed a decreasing trend from industrial to control areas (Fig. 1b). The MDA content in *H. pubescens* and *W. tinctoria* in the Talcher area was 2271% and 2778% higher than in the control area. In

both industrial areas, the MDA content in the plant *W. tinctoria* was higher than *H. pubescens*, although the differences were not significant.

3.2.2. Enzymatic and nonenzymatic antioxidant activity

The enzymatic antioxidant activity in *H. pubescens* and *W. tinctoria* is represented in Fig. 2a and b. The SOD and POD activity was highest at Talcher, followed by Angul, and lowest at the control area. In *H. pubescens*, the activity of SOD in Angul and Talcher areas was 745% and 794% higher than control area, respectively. On the other hand, compared with the control area, the SOD activity in *W. tinctoria* at the Angul and Talcher industrial areas were 934% and 988% higher, respectively. The activity of POD is also significantly higher in both plants in industrial areas than control area. The POD content in *H. pubescens* in Angul and Talcher areas was 719% and 780% higher than in the control area. In *W. tinctoria*, the POD content in Angul and Talcher areas was 876% and 751% higher than in the control area.

AsA activity in all plants showed a decreasing trend and industrial areas to the control area (Fig. 2c). The Highest AsA content (129%) increase showed in *W. tinctoria* in Talcher industrial area compared to the control area.

3.3. Primary metabolites content

Primary metabolite content differed among the areas and plant species (Fig. 3a and b). The soluble carbohydrate and total protein contents were highest in the control area, followed by Angul, and were lowest in Talcher industrial area. Compared to the control area, the soluble carbohydrate content at the Angul and Talcher areas was 47% and 49% lower than in *H. pubescens* and 60% and 67% lower in *W. tinctoria*, respectively. Total protein contents followed a similar trend as soluble carbohydrates. Compared to the control area, the total protein content at the Angul and Talcher areas was 65% and 68% lower than that in *H. pubescens* and 60% and 68% lower in *W. tinctoria*, respectively.

3.4. Secondary metabolites contents

The phenol and flavonoid content was highest in the control area, followed by Angul, and lowest in Talcher (Fig. 4a and b). Compared to the control area, the Angul and Talcher areas' phenol content was 48% and 52% lower than in *H. pubescens* and 31% and 45% lower in *W. tinctoria*, respectively. Like phenol, flavonoid content also decreased in both industrial areas compared to the control area. However, no significant differences were observed in flavonoid content.

3.5. DPPH radical scavenging activity

DPPH radical scavenging activity decreased, and the control to industrial areas (Fig. 5). The DPPH radical scavenging activity was highest in the control area, followed by Angul, and lowest in Talcher industrial area in both plants species. In the control area, 170 µg/mL leaves extract of *H. pubescens* showed the highest (97.79%) DPPH radical scavenging activity. In the Talcher area, the same species showed weak DPPH free radical scavenging activity (74.58%).

3.6. Correlation between heavy metal accumulation and biochemical parameter

A pairwise combinatorial correlation analysis was performed to determine the potential relationship between metal-metal and metal-biochemical responses (Tables 2 and 3). The heavy metal concentrations in both industrial areas shown significantly positive correlation with almost all analyzed heavy metals. For *H. pubescens*, a significantly negative correlation was found between heavy metal and carbohydrate, protein, phenol, and flavonoid content. Significant positive correlations were found between H₂O₂ and MDA content with Cr, Cu, Fe, Mn, Pb and Zn. At the same time, a significant positive correlation was found between SOD, POD, and AsA activity with heavy metal concentrations (Cu, Mn, and Pb). In *W. tinctoria* significantly negative correlations were observed between phenol and flavonoid content with Cu, Mn, and Zn. Additionally, there was a significant correlation between soluble carbohydrates and total protein content with Zn and Mn. Stress marker MDA significantly correlated with Cu, Mn, and Zn, and H₂O₂ correlated with Cu and Mn. All the antioxidants (SOD, POD, and AsA) were significantly correlated with Cu, Mn, and Pb.

Table 2

– The correlation coefficients between heavy metals concentrations and biochemical parameters in *Holarrhena pubescens*.

	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Cr	1						
Cu	0.894**	1					
Fe	0.952**	0.922**	1				
Mn	0.744	0.874*	0.794	1			
Ni	0.964**	0.793	0.935**	0.701	1		
Pb	0.560	0.821*	0.571	0.758	0.408	1	
Zn	0.865**	0.928*	0.914**	0.881*	0.850*	0.727	1
H ₂ O ₂	0.873*	0.954**	0.865*	0.953**	0.799	0.803	0.933**
MDA	0.865*	0.901**	0.801	0.902**	0.782	0.758	0.873*
SOD	0.876*	0.947**	0.895*	0.883*	0.832	0.818	0.980**
POD	0.787	0.908**	0.841*	0.893**	0.759	0.792	0.986**
AsA	0.753	0.710	0.638	0.520	0.684	0.724	0.704
Carbohydrate	-0.909**	-0.916**	-0.856*	-0.838*	-0.850*	-0.771	-0.917**
Protein	-0.790	-0.870*	-0.807	-0.759	-0.718	-0.734	-0.861*
Phenols	-0.869*	-0.953**	-0.844*	-0.869*	-0.765	-0.811	-0.910**
Flavonoids	-0.814	-0.836*	-0.694	-0.655	-0.671	-0.812	-0.717
DPPH	-0.808	-0.948**	-0.801	-0.935**	-0.703	-0.882*	-0.906**
The symbols ** and * represent significant differences at $P \leq 0.01$ and $P \leq 0.05$, respectively.							

Table 3

– The correlation coefficients between heavy metals concentrations and biochemical parameters in *Wrightia tinctoria*

	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Cr	1						
Cu	0.603	1					
Fe	0.664	0.858*	1				
Mn	0.698	0.929**	0.957**	1			
Ni	0.778	0.135	0.360	0.327	1		
Pb	0.519	0.799	0.894**	0.876*	0.083	1	
Zn	0.645	0.855*	0.580	0.718	0.084	0.664	1
H ₂ O ₂	0.711	0.877*	0.941*	0.961*	0.470	0.806	0.634
MDA	0.639	0.885*	0.890**	0.943**	0.390	0.781	0.656
SOD	0.626	0.957**	0.928**	0.929**	0.192	0.821*	0.742
POD	0.695	0.854*	0.906**	0.945**	0.425	0.850*	0.674
AsA	0.739	0.752	0.792	0.868*	0.420	0.845*	0.725
Carbohydrate	-0.624	-0.867*	-0.880*	-0.930**	-0.334	-0.784	-0.646
Protein	-0.709	-0.870*	-0.950**	-0.972**	-0.457	-0.832*	-0.628
Phenol	-0.497	-0.753	-0.794	-0.796	-0.168	-0.842	-0.623
Flavonoid	-0.423	-0.728	-0.494	-0.670	-0.066	-0.492	-0.707
DPPH	-0.632	-0.942**	-0.911**	-0.949**	-0.301	-0.838*	-0.745
The symbols ** and * represent significant differences at $P \leq 0.01$ and $P \leq 0.05$, respectively.							

4. Discussion

4.1. Industrialization effects on the heavy metal accumulation of plant

Comparison study of the areas showed that industrialization considerably affected the heavy metal concentration in the medicinal plants. In both industrial areas, the concentration of analyzed seven heavy metals in two species surpassed the permissible limit of WHO (1996) standards. The industrial areas restrain more pollutants, and heavy metals in plants exhibit anthropogenic emission inclined. Fe and Mn were found in great quantity in the leaves of both plants and showed a significantly positive correlation

between these two heavy metal concentrations. Fe and Mn are considered mobile elements and the plants accumulate a large portion of Fe and Mn. In both industrial areas, *W. tinctoria* accumulates the highest heavy metal concentration except Cr and Ni, indicating that heavy metal accumulation differs between plant species. Thus the accumulation of heavy metals in plants depends on species and can be varied by different environmental factors (Karmakar and Padhy 2019; Mandal and Dhal 2022). The predominant source of heavy metals is coal burning, oil dust, chemical industry, and the motor vehicle exhaust in these two industrial areas. Zn and Pb are commonly from anthropogenic sources, but Cu and Mn were apart from crustal and anthropogenic sources. Thus, the accumulation concentration of these heavy metals increased from control areas to industrial areas, showing the industrialization and pollution gradients.

4.2. Changes in biochemical parameters

Under the steady-state circumstance, reactive oxygen species (ROS) production and scavenging are in a dynamic equilibrium. This dynamic equilibrium can be disrupted by various abiotic stresses, like heavy metals, drought, heat, and air pollution, which can cause oxidative stress in the plant (Chan et al., 2016; Berni et al., 2019). Under heavy metals exposure, plants accumulate more free radicals such as superoxide anion ($\text{O}_2^{\cdot-}$), hydroxyl ($\text{OH}^{\cdot}$), and hydrogen peroxide (H_2O_2). An increase in free radicals causes lipid peroxidation and produces malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) (Syta et al., 2013). The increase in H_2O_2 and MDA levels in both plants in the industrial areas may be due to a positive correlation between H_2O_2 and MDA with heavy metals concentration. Our result suggested that the equilibrium process of ROS production in these two plant species is disrupted due to heavy metals accumulation. Similar results were also found by Petukhov et al. (2021) and Cui et al. (2022); they mentioned that MDA content was increased in plants upon exposure to heavy metals. ROS can react with biomolecules such as amino acid, protein, nucleic acid, and lipids, leading to metabolic abnormality and cell death. Hence, plants enhance the production of enzymatic and nonenzymatic antioxidants, like SOD, POD, and AsA, to protect biomolecules from oxidative damage (Caverzan et al., 2016). SOD is one of the essential enzymes in the antioxidant defense system, which actively catalyze the $\text{O}_2^{\cdot-}$ and modifies it into H_2O_2 (Ighodaro and Akinloye 2018). Both the plant species showed enhanced SOD activity in industrial areas. The correlation study showed a significantly positive correlation between SOD activity and the concentration of heavy metals. Therefore our results suggested that the accumulation of heavy metals induced the SOD 1 gene, which is responsible for synthesizing the SOD enzyme (Sharma et al., 2016). The outcome of SOD catalyzed activity is H_2O_2 , which is still toxic and eliminated to H_2O by the number of enzymes. POD is considered one of the most important in regulating H_2O_2 at the intracellular level (Smirnoff and Arnaud, 2019). Our study revealed increased POD activity in the *H. pubescens* and *W. tinctoria* collected from industrial areas with higher heavy metals content. The activity of POD in both plants positively correlated with heavy metals concentration. Several acidic and basic POD genes were strongly expressed under all metal concentrations (Kosakivska et al., 2021). This study indicates that increased H_2O_2 levels in response to heavy metal stress are closely linked to an improved antioxidant

defense system mediated by POD. These study results specify that increased H_2O_2 levels in response to heavy metal stress are closely linked to an improved antioxidant defense capability mediated by POD.

AsA is another nonenzymatic antioxidant for plant growth and development that responds against oxidative stress. AsA can directly react with ROS like $\cdot\text{O}_2^-$, $\text{OH}\cdot$, and $^1\text{O}_2$ and reduce H_2O_2 and H_2O (Njus et al., 2020). In industrial areas with higher heavy metal loads, we observed increased AsA activity in *H. pubescens* and *W. tinctoria*. AsA antioxidant activity positively correlated with heavy metals concentration. Karmakar and Padhy (2019) and Sharma et al. (2019) reported higher AsA activity in higher air and heavy metal pollution levels.

Carbon and nitrogen metabolism are primary processes for plants' growth and development and play an essential role in stress tolerance. High concentrations of toxic heavy metals disrupt the plant's physiological and biochemical processes by altering carbohydrate and protein contents (Hossain and Komatsu, 2013; Geng et al., 2021). Our study revealed the altered carbon-related compound, which is soluble carbohydrates. Lower levels of soluble carbohydrates were verified in the industrial area in both species. It has been reported that higher heavy metal concentration tends to lead to longer-distance transport, therefore, affecting the leaf carbohydrate concentration (Stobrawa and Plucińska, 2007). Thus, the carbohydrate reduction probably reflects the high levels of metals found in these two industrial areas and the costs associated with survival and tolerance to metal pollution. Thus, the reduction of carbohydrates probably reflects the higher levels of metals found in these two industrial areas and maybe the due to costs associated with survival and acclimation to metal contamination.

In plants, the protein content is susceptible to stress, and in response to stress, it may decrease or increase depending on the species and its underlying resistance (Rai, 2014). Here, we observed reductions in total protein content in *H. pubescens* and *W. tinctoria*. Positive correlations between protein content and several metals such as Cu, Fe, and Zn were observed in *H. pubescens* and *W. tinctoria*. A decrease in protein content might be due to the increased protein denaturation rate, which is also supported by the investigation of Senthil et al. (2020) and Sharma et al. (2008). The heavy metal toxicity overreached the limitation and enhanced the expression of hydrolytic enzymes (e.g., proteases), resulting in the denaturation of protein structure.

Secondary metabolites contain an array of beneficial natural products that make up essential components of the plant's defense system to counteract the harmful effects of environmental stress (Ravi et al., 2020; Endara et al., 2022). These metabolites have significant biological functions and are often used as medicinal and food ingredients for therapeutic and aromatic purposes (Kumar et al., 2022). Many studies (Okem et al., 2015; Berni et al., 2018; González-Mendoza et al., 2018) demonstrated that applying single or double heavy metals increased the accumulation of phenols and flavonoids content. Our study revealed that secondary metabolite (phenols and flavonoids) accumulations significantly decreased in both industrial areas, which indicates that combined elevated heavy metal stress harmed these two secondary metabolites.

The decrease in primary metabolite that is carbohydrate would not provide the necessary carbon for the synthesis of secondary metabolites.

Also, higher heavy metal concentration in the plant likely suppressed enzymes related to the synthesis of secondary metabolites by reacting with the sulfhydryl groups in enzymes to form metal sulfides. Sulfhydryl groups in enzymes function as fundamental constituents associated with maintaining the right structural relationships of the enzyme (Knowles1991). Therefore, as enzyme activity is suppressed under heavy metal stress, the production of secondary metabolites would also decrease.

Phenolic and flavonoid compounds with antioxidant characteristics can scavenge free radicals by providing hydrogen atoms in plants (Dangles 2012). Thus, the reduced content of these compounds may increase the sensitivity of plants to injury and damage in polluted environments and bring down the potentiality of plants to adapt to stresses.

DPPH radical scavenging activity negatively correlated with heavy metals concentration in both industrial areas. Decreased antioxidant capacity of plants under heavy metal stress may be associated with decreased phytochemicals, such as phenolics and flavonoid content. Reduced DPPH activity was also reported by Ibrahim et al. (2017) in a medicinal plant (*Gynura procumbens*) under the combined stress of Cd and Cu.

5. Conclusions

In conclusion our study revealed the accumulation order of heavy metals ($\text{Fe} > \text{Mn} > \text{Zn} > \text{Cu} > \text{Cr} > \text{Ni} > \text{Pb}$) in two species along with industrial-control gradient. Both the species undergo heavy metal-derived oxidative stress, which increases the concentration of enzymatic and nonenzymatic antioxidants. A synchronized elevation in enzymatic antioxidants (SOD and POD) and nonenzymatic antioxidants (AsA) was observed in response to heavy metals that induce oxidative stress. Elevated activity of enzymatic and nonenzymatic anti-oxidants suggested that *H. pubescens* and *W. tinctoria* may have a detoxification process to overcome the stress of the heavy metal. Also noticed that in industrial areas, primary (soluble carbohydrate and protein) and secondary (phenol and flavonoid) metabolites decreased in both species. The decreasing concentration of secondary metabolites contents from industrial areas lowers the medicinal plant's free radical scavenging activity. Furthermore, heavy metal stress on the biosynthesis of different primary and secondary metabolites and the role of enzymatic and nonenzymatic antioxidants in detoxification of heavy metals for the medicinal plant is not fully understood, need to be explored.

Declarations

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1. Kalicharan Mandal: (First and Corresponding author)

Contribution: Conception and design, analysis and data interpretation, drafting of the article, and final approval.

2. Nabin Kumar Dhal: (Co-author)

Contribution: Conceptualization of the work, critical revision of the article for important intellectual content, final approval of the article.

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Figures

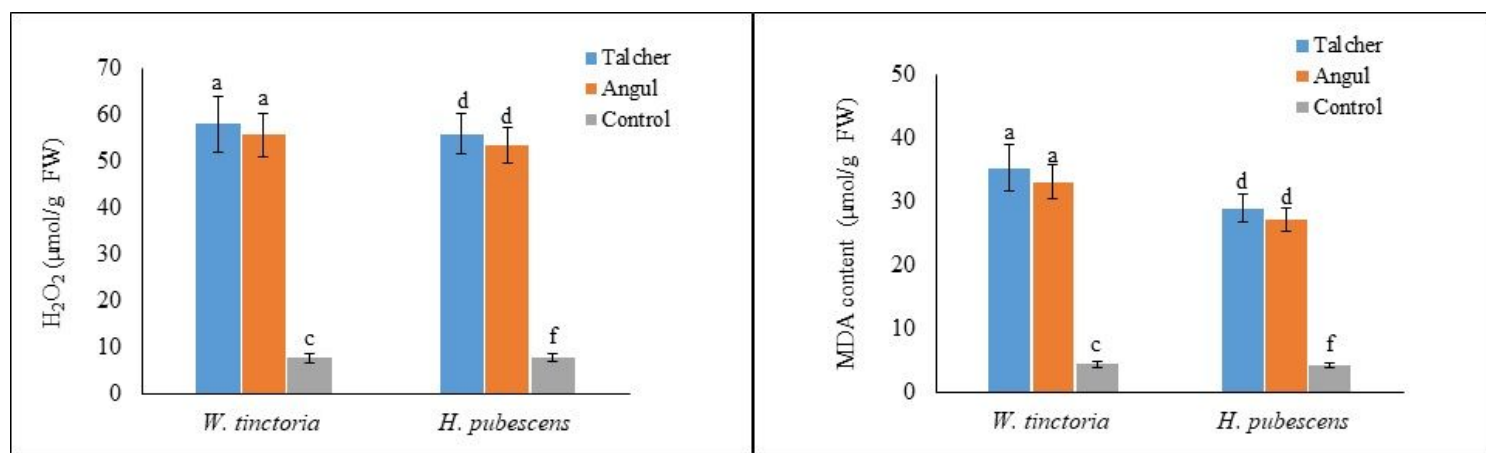


Figure 1

(a) H_2O_2 and (b) MDA content in *H. pubescens*, and *W. tinctoria* from three different areas. Different lowercases letters represent significant differences ($P < 0.05$).

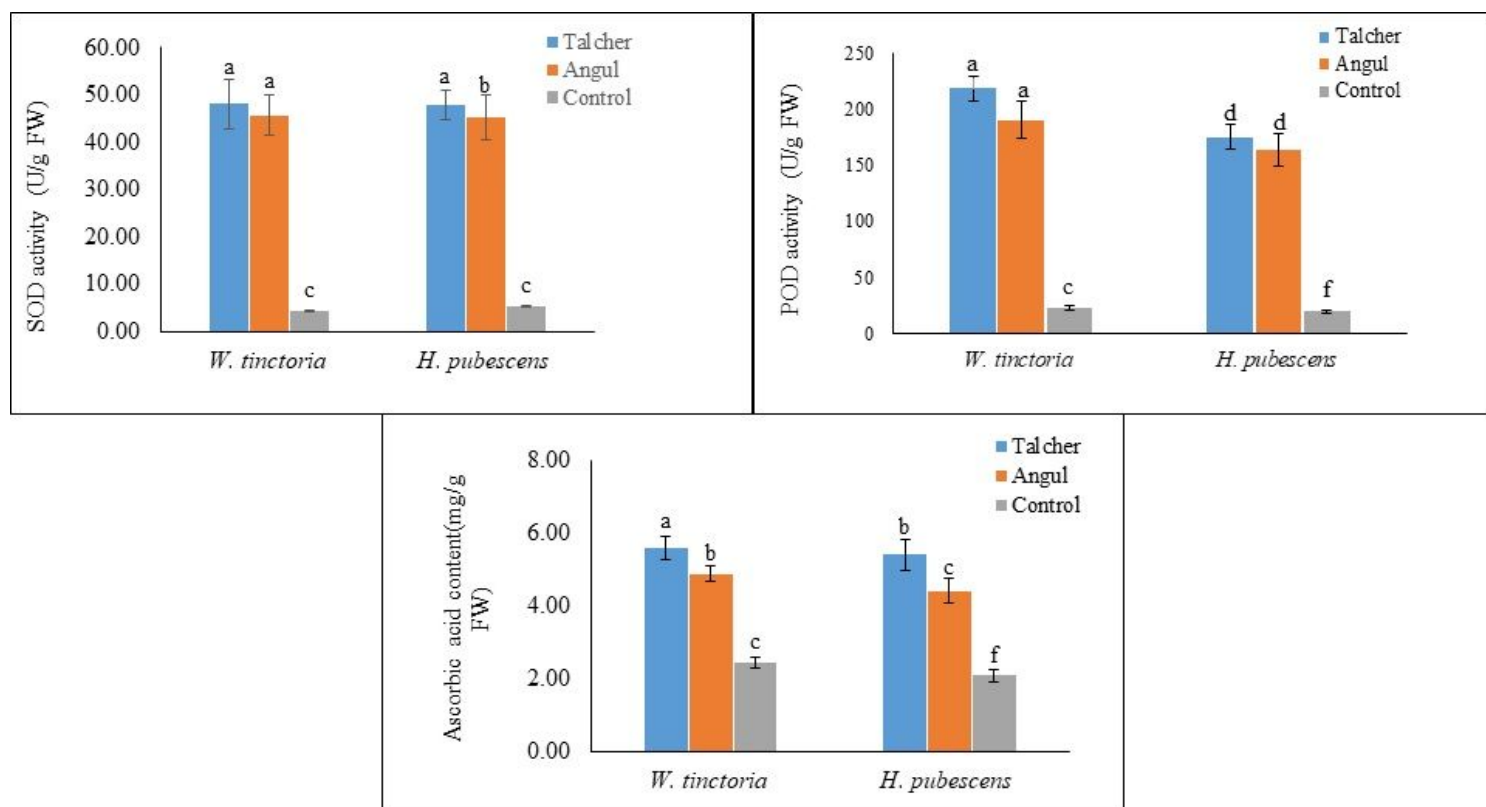


Figure 2

(a) SOD, (b) POD activity and (c) AsA content in *H. pubescens*, and *W. tinctoria* from three different areas. Different lowercases letters represent significant differences ($P < 0.05$).

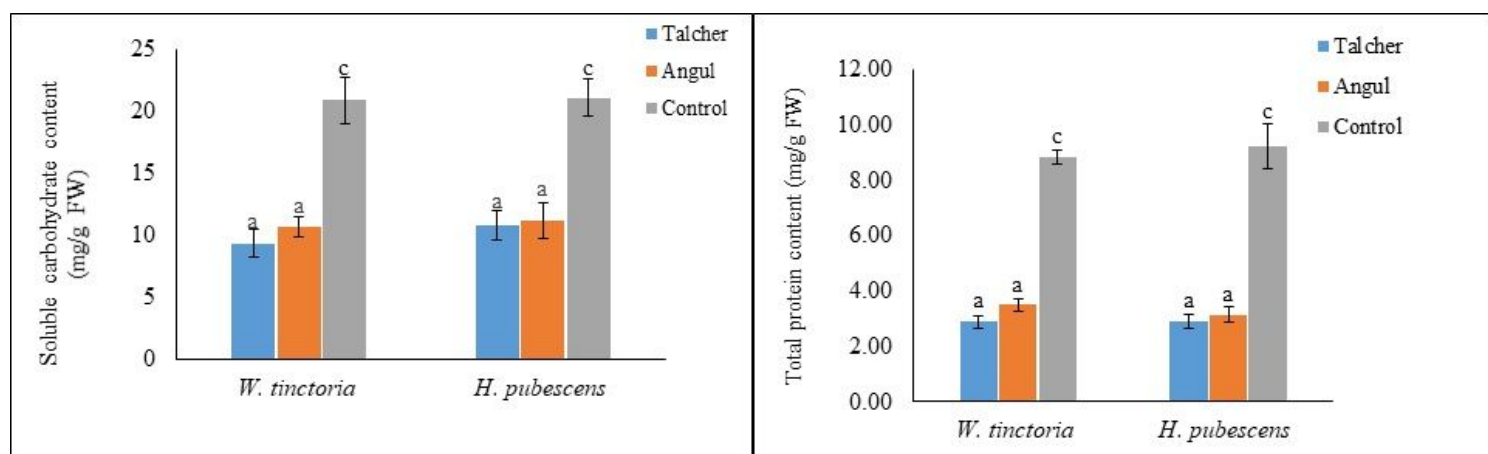


Figure 3

(a) soluble carbohydrates and (b) total protein content in *H. pubescens*, and *W. tinctoria* from three different areas. Different lowercases letters represent significant differences ($P < 0.05$).

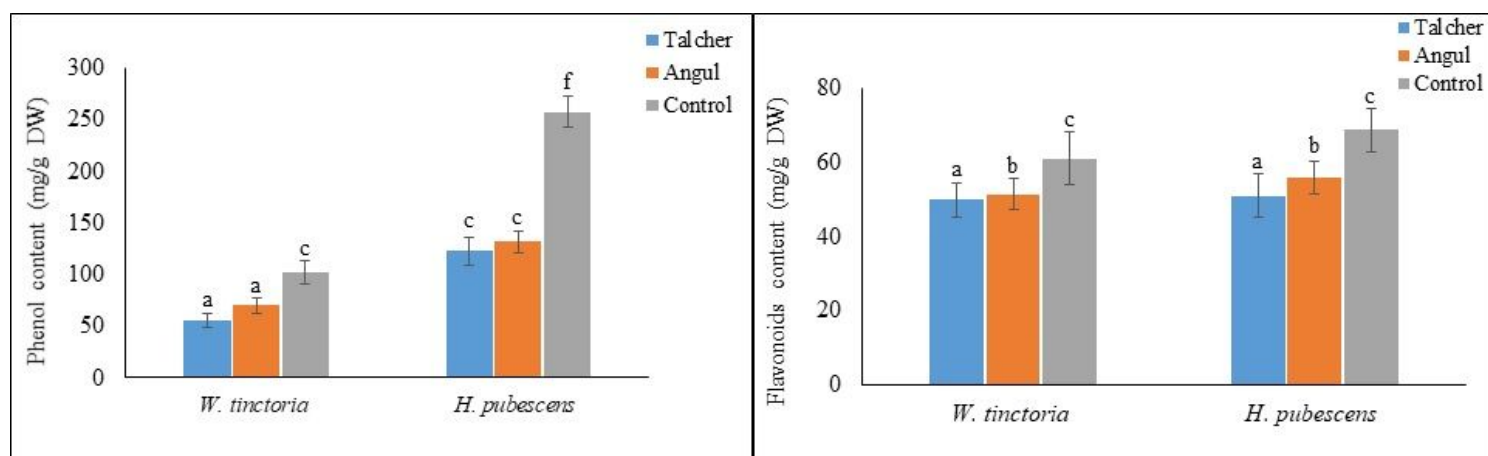


Figure 4

(a) Phenols and (b) flavonoids content in *H. pubescens*, and *W. tinctoria* from three different areas. Different lowercases letters represent significant differences ($P < 0.05$).

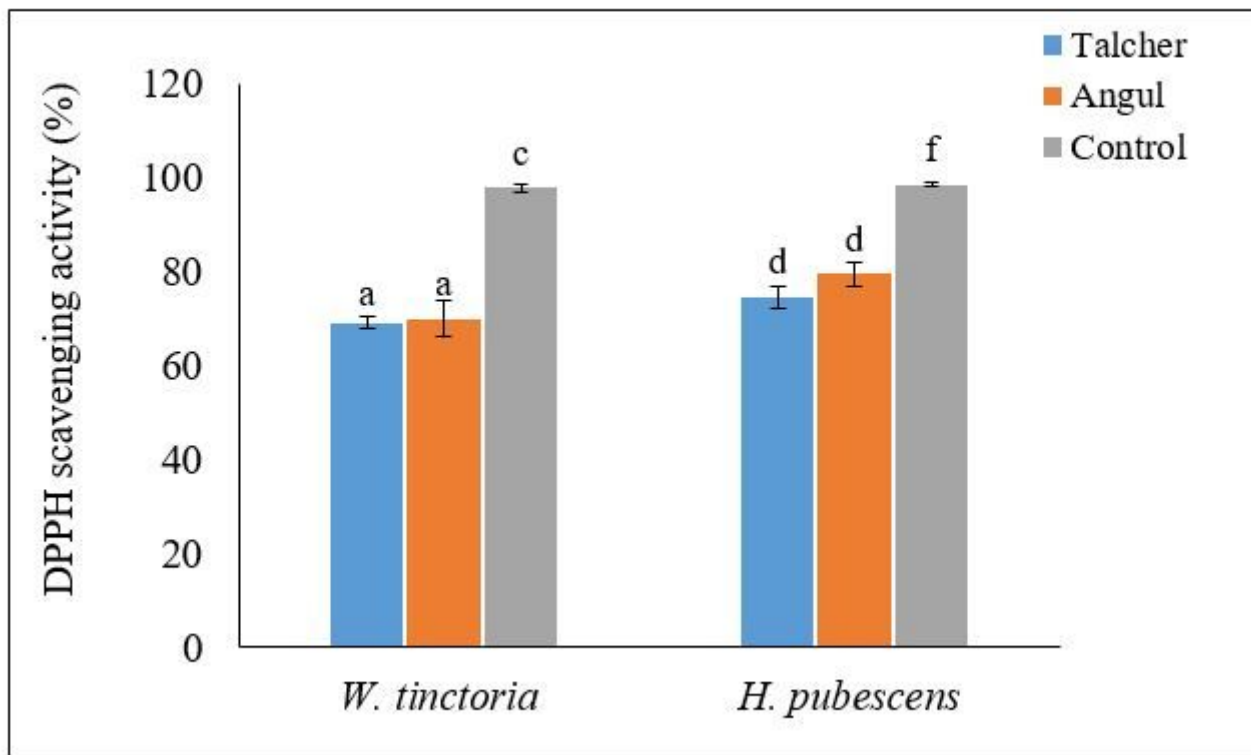


Figure 5

DPPH free radical scavenging activity in *H. pubescens*, and *W. tinctoria* from three different areas. Different lowercases letters represent significant differences ($P < 0.05$).