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Original Article

Interactive effects of toxic metals on the total phenolic and flavonoid in *Hydrocotyle umbellata* L.

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Abstract

Phenolic and flavonoid content in plants are important abiotic stress biomarkers. The individual and combined impacts of toxiferous metals Arsenic (As), Cadmium (Cd) and (Copper) Cu were employed in recent studies to investigate their effect on Total flavonoids content (TFC) and total phenolic content (TPC) in various parts of *Hydrocotyle umbellata* L., to explore the role of plant in abating metal contamination. Folin-Ciocalteu and $AlCl_3$ methods were used to study TPC and TFC, respectively. Two-way analysis of variance (ANOVA) and a classification and regression tree (CART) model was employed for statistical analysis. Highest TPC was observed in decreasing order as leaf > stem > root for all the metals stress. Whereas, highest TFC was found in all plant parts when subjected to As toxicity, and the lowest TFC was found in stem of the plant under Cu toxicity. There was significant effect on TPC when subjected to Cu and As stress; in addition, significant effects of Cd and combined metal stress were also evident. Treatment concentration had non-significant effect on TPC under single metal but had significant effect in case of combined metal stress. Similarly, in case of TFC no significant effect was recorded under all the stress types. Metal type had significant effect on TPC and TFC. Whereas plant part had significant impact on TPC but non-significant values were observed on TFC. This study epitomized TPC and TFC in *H. umbellata* L. as effective and viable tool to pertain its role in phytoremediation against contamination of Cd, Cu and As.

Keywords: Metal toxicity, Phytoremediation, Secondary plant metabolites, Water pennywort

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Introduction

Advancement in global industrialization and anthropogenic activities constantly add toxic metals in environment which results in environmental contamination and emerge as a wide-reaching persistent consequence (Alaboudi et al., 2018; Ghous et al., 2022). Some heavy metals (Cu, Zn, Mn, Ni, Co and Fe) act as micronutrients and are necessary for plants and animals in small amounts but if their concentration exceeds certain threshold value, then they can turn out to be ecotoxic. Other heavy metals such as Cd, Cr, Pb, As, Se, U, Au, and Hg are highly toxic even at low concentrations (Gupta et al., 2016; Dutta et al., 2018; Jan´czak-Pieniaz˙ek et al., 2023). This poses a serious threat since heavy metals are non-biodegradable, hazardous, and persistent, which causes them to accumulate in the soil (Zafar-ul-Hye et al., 2020). Additionally, the consumption of plant products from crops cultivated in heavy metal-contaminated areas could potentially have a detrimental effect on human health (Haque et al., 2021). Cd stress has a negative impact on the life cycle of plants by suppressing their growth (Khasanah and Rachmawati, 2020). When bioaccumulated Cd toxicity results in stomach ulcers, mutations, bone problems, infertility, neurological, immunological, and psychiatric diseases in humans. Similarly, Wilson's disease, genetic problems, liver damage, brain and nasal tumors, hepatic cirrhosis, renal and ocular impairments, and mortality are all examples of chronic Cu toxicity (Olafisoye et al., 2020). Arsenic exposure is also linked to non-communicable diseases like Diabetes mellitus and cardiovascular disease, skin diseases, neurological diseases, and various types of cancer (Rahaman et al., 2021).

In plants, exposure to heavy metals (HM) triggers physiological and biochemical responses (Kisa et al., 2016) and results in oxidative damage to plants (Anjitha et al., 2021). As plants are sessile in nature, they recognize these toxic metals stress signals and activate various defense responses to enable functional flexibility under the stressful constraints without disturbing cellular and developmental physiological processes (Yang et al., 2018; Arnold et al., 2019). Increased HM levels in plant tissues set off a chain reaction that results in the overproduction of reactive oxygen species (ROS). Enzymatic and non-enzymatic systems have both been created by plants to scavenge ROS (Das and Roychoudhury, 2014). Antioxidants remove, neutralize, and scavenge ROS in order to protect the cell from harm brought on by exposure of metal ions. ROS influence the biosynthesis and accumulation of plants' secondary metabolites and bring about structural and functional stabilization through signaling processes and pathways against HM stress (Anjitha et al., 2021). The secondary molecules are occasionally produced in living plant cells and do not significantly contribute to the primary lives of the plants that produce them (Ncube and Van, 2015). Enhanced production of secondary metabolites is an adaptive vibrant detoxification mechanism evolved in plant life to reduce the injurious effects triggered by toxic metals (Isah, 2019; Ghori et al., 2019). Of these secondary metabolites, phenolic (Kisa et al., 2016) and flavonoids (Izbiańska et al., 2014) are considered as stressful responses that help plants to live on metal contaminated soil. Phenolic and flavonoids work to eliminate ROS substances (Wu et al., 2011; Men et al., 2022).

Flavonoids (promising abiotic stress markers) perform a protective role under stressful circumstances by neutralizing radical changes before they cause adverse effects in the cell. They are among the most vital secondary metabolites produced in almost every plant parts. Flavonoids increase plants' tolerance to abiotic stress at physiological and biochemical levels by increasing antioxidant capacity, controlling cellular redox, activating stress-responsive transcription factors (TFs), regulating osmoregulation, and participating in the stress response signaling network as a signaling molecule (Shomali et al., 2022). Phenolic compounds have manifold roles regarding plant defense responses to heavy metal stress by mitigating the toxic effects of HM. In order to estimate the range of tolerance to stress factors that occur in plants, researchers can use the concentration of phenols in plant tissues as an excellent indicator. Increased phenolic compound production in heavy metal-stressed plants helps shield them from oxidative stress (Sharma et al., 2019).

Phytoremediation of this metal contaminated environment is crucial in order to tackle this environmental issue (Lamine and Saunders, 2022). *H. umbellata* L. (the invasive perennial herb) belongs to the family of Araliaceae possess filiform roots, stoloniferous reproduction, and quick growth in marshy areas as well as marginal lands, its phytoremediation potential against different heavy

metals (Bokhari et al., 2022; Taufikurahman et al., 2019; Rashid et al., 2020) has been studied earlier but total phenolic content (TPC) and total flavonoids content (TFC) under the influence of Cu, Cd & As either alone or in combination has been scarcely evaluated in *H. umbellata* L. Therefore, the aim of present study is to evaluate TPC and TFC in *H. umbellata* L. against the stress of Cu, Cd and As, individually as well as in combination, in different parts of the plant to examine their role in phytoremediation of these HM. Moreover, CART model had been employed for first time in such studies.

Material and Methods

Collection of plant material

Plant material of *H. umbellata* L. was collected from a marshy area (34.1916°N, 73.2426° E) of Jinnah Abad in District Abbottabad, Pakistan and then propagated through rhizome cuttings. As soon as the rhizome developed fresh meristematic buds, the plants were dug out, properly cleaned with distilled water and then transplanted to pots containing Hoagland solution. After attaining a consistent size, plants from each pot having uniform fresh weight (0.50±10 g) were carefully chosen. We used Randomized Block Design (RBD). The control group was set without giving any metal treatment, but the experimental group were treated with various metal concentrations. After every three days, nutrient solution was constantly examined and add-on to stable transpiration loss.

Metal treatments

Metal treatments were given on the base of the literature survey. A molar strength stock solution of copper sulphate ($CuSO_4.5H_2O$) was prepared and the following 5 different molar concentrations i.e., 5, 10, 15, 20, 25 mg/L were prepared. For cadmium a solution of cadmium chloride ($CdCl_2$. H_2O) was synthesized by using 200,400,600,800 and 1000 µg/L. For Arsenic toxicity a solution of Arsenic trioxide was prepared (As_2O_3) for following 5 different concentrations: 0.5, 0.75, 1.0, 1.25 and 1.5 mg/L. And for combined metals stress the above concentrations were combined for each treatment of As, Cd and Ch. On 10th day of metal treatment, plants were harvested and separated into roots, stolon and leaves. Plant parts were bathed with distilled water to get rid of any adsorbent particles and air-dried on blotting paper.

Methanol extract preparation for TPC and TFC

By following the protocol of Martínez-Villaluenga et al. (2009), 80% aqueous methanol (1g/10 ml) extracts were prepared from fresh samples of roots, stem and leaves of *H. umbellata* L. and placed at shaker incubator at 37℃ for 2 hours. Then the mixture was centrifuged at 6000 rpm for 15 minutes. Fresh supernatants were collected in a sterile container; this procedure was repeated thrice. The solid residue was re-extracted three times in methanol (1/10w/v) and supernatants were pooled for determination of TPC and TFC as shown in Fig 1.

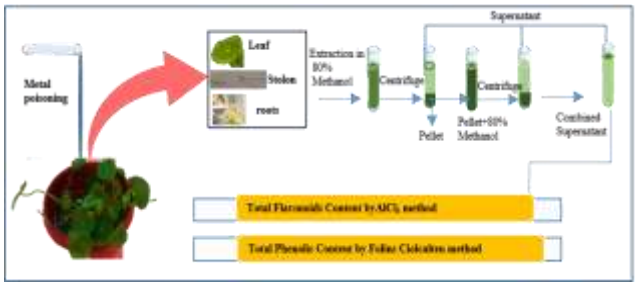

Figure-1. Preparation of samples for TPC and TFC

Determination of total phenolic and flavonoids content (TPC & TFC)

The Folin and Ciocalteu reagent was used to calculate the total phenolics in the extracts, by following the procedure (Chan et al., 2014; Basit et al., 2023) with slight modifications. Using a spectrophotometer, measurements of the sample and the standard were taken at 760 nm in comparison to the reagent blank. Folin-Ciocalteu's phenol reagent (1:1) and water (0.6 mL) were mixed with the test sample (0.2 mL). After 5 minutes, the mixture was added 1 mL of saturated sodium carbonate solution (8% w/v in water), and the volume was then increased to 3 mL with distilled water. After centrifuging the reaction for 30 minutes in the dark, the absorbance of blue color in various samples was measured at 760 nm. On the basis of a gallic acid standard curve, the phenolic content was estimated as gallic acid equivalents GAE/g of plant material

Determination of TFC

TFC was determined using the aluminum chloride colorimetric method as described by Chandra et al. (2014). Quercetin was utilized to create the standard calibration curve for the measurement of total flavonoid concentration.

The standard quercetin solutions were made by serially diluting quercetin with methanol (5-200 g/mL) after the stock quercetin solution was made by dissolving 5.0 mg of quercetin in 1.0 mL of methanol. Standard quercetin extracts or solutions in a volume of 0.6 mL were separately combined with 0.6 mL of 2% Aluminum chloride. The mixture was then left at room temperature for 60 minutes. The absorbance of the reaction mixtures was measured using a spectrophotometer against a blank at 510nm. The total flavonoid content of the test was determined using the calibration plot, and the result was expressed as mg quercetin equivalent (QE)/g of plant material.

Statistical analyses

Analysis of Variance (ANOVA) and Classification and Regression Trees (CART) were used to analyze the statistical significance of the data. In the present study, two-way ANOVA was used to determine the effects of plant part, treatment type and metal type on TPC and TFC (Larson, 2008). CART models were used for a tree-building algorithm using if-then split conditions. At each node of the tree, the best split of data is determined by using Gini (G) index (Razi and Athappilly, 2005). In the end, the model selected the variables having the lowest index value. The process is repeated for each node until further splitting of the data is no longer possible (Daniya et al., 2020).

Results

In order to compare the levels of metals in various plant sections across five different treatments, a two-way ANOVA was used in this study. The numerical outcomes of this analysis are shown in tables below. Basic descriptive data, such as the count, total, mean, and variance, are provided for each plant part and treatment in the tables 1-9. Plant component and treatment were examined as potential drivers of variance.

Total phenolic content (TPC)

The two-way ANOVA for Cd exposure revealed non-significant interactions between plant part and treatments, on TPC proving that the effect of treatments of Cd concentration was independent of the plant part and TPC was consistent. The study revealed that neither the treatments nor the plant parts had any discernible effects on the concentration of TPC ($F(4, 18) = 0.491$, $p=0.74$) or ($F(2, 18) = 1.549$, $p=0.27$, respectively). These findings imply that none of the Cd treatment significantly affected the TPC concentration which was stable throughout the various plant components as shown in Table 1.

Table-1. Effect of Cd toxicity on TPC at 760 in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	3.47	0.694	0.097
Stem	5	4.174	0.835	0.076
Leaf	5	5.091	1.0182	0.039
Treatment 1	3	2.387	0.796	0.040
Treatment 2	3	2.958	0.986	0.004
Treatment 3	3	2.775	0.925	0.027
Treatment 4	3	2.041	0.680	0.204
Treatment 5	3	2.574	0.858	0.198

The descriptive data for plant parts and treatments concentration for Cu metal in *H. umbellata* L. are enlisted in Table 2. Two-way ANOVA for Cu exposure revealed that there was a significant effect of plant parts on TPC when exposed to Cu stress. It implied that different plant parts may accumulate Cu to different extent but treatment concentration employed in recent studies had no impact on TPC.

Table-2. Effect of Cu toxicity on TPC at 760 nm in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	1.805	0.361	0.004
Stem	5	3.064	0.613	0.050
Leaf	5	5.134	1.027	0.136
Treatment 1	3	1.949	0.650	0.045
Treatment 2	3	2.373	0.791	0.175
Treatment 3	3	1.394	0.465	0.022
Treatment 4	3	2.457	0.819	0.381
Treatment 5	3	1.83	0.61	0.201

Data on As toxicity in this study are provided in Table 3. There was a significant effect of plant part on TPC when exposed to As toxicity. But there was non-significant effect of treatment concentration on TPC when exposed to As ($p= 0.430971$). With a computed F-value of 5.077 and a p-value of 0.038, the results demonstrated that plant component was a substantial source of variation in TPC and that there were considerable variations in As levels throughout

the root, stem, and leaf. With a calculated F-value of 1.07 and a p-value of (0.4309) >0.05, concentration was not discovered to be a significant source of variance. These results collectively imply that different plant sections may accumulate As to varying degrees.

Table-3. Effect of As toxicity on TPC at 760 nm in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	3.669	0.734	0.122
Stem	5	6.085	1.217	0.017
Leaf	5	6.115	1.223	0.099
Treatment 1	3	3.589	1.196	0.231
Treatment 2	3	3.607	1.202	0.003
Treatment 3	3	3.205	1.068	0.075
Treatment 4	3	2.381	0.794	0.220
Treatment 5	3	3.087	1.029	0.176

Numerical data for TPC for treatments, plant parts, sum and variance are described in Table 4 for combined metals (Cu, Cd and As). The root, stem, and leaf were all present in five counts and their corresponding sums and averages of TPC values were observed in the two-way ANOVA for combined metal treatment. TPC observations for various metal treatments, with their sums, averages, and variances shown in Table 4.

Table-4. Effect of combined metals (Cd, Cu &As) on TPC at 760 nm in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	2.271	0.454	0.063
Stem	5	3.393	0.679	0.105
Leaf	5	3.401	0.680	0.020
Treatment 1	3	2.591	0.864	0.001
Treatment 2	3	0.974	0.325	0.025
Treatment 3	3	1.98	0.66	0.005
Treatment 4	3	1.515	0.505	0.048
Treatment 5	3	2.005	0.668	0.137

ANOVA results for investigating the effects of plant portion on TPC exhibited the following characteristics: Sum of Squares (SS) = 0.169, two degrees of freedom, Mean Sum of Squares (MS) = 0.084, F = 2.548, p = 0.139, and F critical = 4.458. Similarly, the treatments had an MS of 0.122 and an SS of 0.487, both with four degrees of freedom. Overall, the types of plant parts and employed treatments had non-significant impact on the TPC.

For Cu accumulation in stem, the value was 0.64; for Cd alone or combined accumulation in stem, the value was 0.83; when it is a root and the metal is Cu, the value is 0.41; when it is a root and the metal is combined, the value is 0.71; when it is a leaf, the value is 0.68; and when it is a leaf and the metal is Cd or Cu, the value is 1.02. Average TPC under different metals decreases in following order: As > Cd > Cu > combined metals.

Table-5. Effect of Metal type and Plant Part TPC in *H. umbellata* L.

Cd				
	Root	**Stem**	**Leaf**	**Total**
Count	5	5	5	15
Sum	3.47	4.174	5.091	12.735
Average	0.694	0.8348	1.0182	0.849
Variance	0.096739	0.076319	0.039529	0.079
Cu				
Count	5	5	5	15
Sum	1.805	3.064	5.134	10.003
Average	0.361	0.613	1.027	0.667
Variance	0.004377	0.050	0.136	0.135
As				
Count	5	5	5	15
Sum	3.669	6.085	6.115	15.869
Average	0.734	1.217	1.223	1.058
Variance	0.122	0.017	0.099	0.124
Comb				
Count	5	5	5	15
Sum	2.271	3.393	3.401	9.065
Average	0.4542	0.679	0.680	0.604
Variance	0.063	0.105	0.020	0.066
Total				
Count	20	20	20	
Sum	11.215	16.716	19.741	
Average	0.561	0.836	0.987	
Variance	0.086	0.110	0.102	

TPC's sum, average, and variance for each metal-part combination are shown in Table 5 along with their corresponding totals. Based to our ANOVA findings, the TPC was significantly influenced by both the metal type and the plant component. The F-values for the metal type and plant component were 9.022 and 13.504, respectively, while the mean square values were 0.624 for the metal type and 0.934 for the plant component. There was a statistically significant difference in averages across various metal types and

plant sections, as shown by the P-values for both variables being less than 0.05. With a P-value of 0.341, the interaction term was not statistically significant. This shows that the plant component is not a factor in the effect of metal type on the response variable. The results indicated that both the plant component and the type of metal employed had a sizable impact on the outcomes. Significant results were also obtained from the two components' interaction. This implies that the results were more significantly influenced by the combination of metal type and plant component than by either condition alone. These findings can be useful in understanding how various metals affect various plant parts and interact with one another. There was a substantial difference in the sum and average of TPC for each metal and component combination, as well as their individual totals, indicating a considerable impact of the plant part on the outcomes. According to the relatively low variance for each metal-part combination, the findings were uniform across all plant components and metal kinds.

Total flavonoids content (TFC)

The data description for TFC under Cd metal in *H. umbellata* L. are enlisted in Table 6. The study's findings shown for Cd are such that the average count, total, and variance of the root, stem, and leaf samples did not differ significantly from one another. With respect to the root, stem, and leaf samples, the average counts were, respectively, 0.647, 0.581, and 0.663, with variance values of 0.269, 0.285, and 0.296. The five treatments did, however, show substantial variances from one another. With an average value of 1.187 and a variance value of 0.003, Treatment 4 had the highest sum value, and Treatment 5 had the lowest sum value of 0.383.

Table-6. Effect of Cd toxicity on Total Flavonoids content at 510nm *in H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	3.2362	0.647	0.269
Stem	5	2.904	0.581	0.285
Leaf	5	3.316	0.663	0.296
Treatment 1	3	0.894	0.298	0.003
Treatment 2	3	2.555	0.852	0.259
Treatment 3	3	2.062	0.687	0.344
Treatment 4	3	3.562	1.187	0.003
Treatment 5	3	0.383	0.128	0.012

Two way ANOVA results showed that the plant part factor had no discernible impact on the samples when exposed to Cd, because the F-value was 0.062 and the P-value was 0.939, both of which were higher than the significance level of 0.05. Similar for the treatment factor, TFC was not significantly affected by it, when exposed to Cd because the F-value was 3.557 and the P-value was 0.059, both of which are more than the significance level of 0.05.

Table-7. Effect of Cu toxicity on TFC at 510 nm in *H. umbellata L.*

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	0.594	0.119	0.005
Stem	5	2.596	0.519	0.361
Leaf	5	2.35	0.47	0.251
Treatment 1	3	0.348	0.116	0.0004
Treatment 2	3	0.646	0.215	0.015
Treatment 3	3	1.433	0.478	0.484
Treatment 4	3	1.876	0.625	0.312
Treatment 5	3	1.237	0.412	0.411

Table-8. Effect of As toxicity on TFC at 510nm in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	1.985	0.397	0.244
Stem	5	1.658	0.332	0.223
Leaf	5	1.08	0.216	0.012
Treatment 1	3	0.498	0.166	0.032
Treatment 2	3	0.436	0.145	0.011
Treatment 3	3	0.377	0.126	0.007
Treatment 4	3	1.954	0.651	0.251
Treatment 5	3	1.458	0.486	0.357

The data description for Cu exposure for TFC at 510 nm are described in Table 7. With an F-value of 0.509 and a P-value of 0.731, the results of the Two-Way ANOVA demonstrated that the Treatments factor had no discernible influence on the samples. Interestingly, the plant part factor had an F-value of 0.968 and a P-value of 0.420, indicating that it had no discernible impact on the samples. Overall, the results indicate that the samples were unaffected by Cu exposure, and there were no significant changes noted in TFC between the Plant Parts when exposed to Cu toxicity. With variance values of 0.244, 0.224, and 0.012, respectively, the average values for the root, stem, and leaf samples were 0.397, 0.332, and 0.216, respectively as shown in Table 8. The TFC values for different treatments did, however, differed

significantly from one another.

The results for the combined metals exposure showed that there were no significant differences among the plant parts as shown in Table 9. However, there were significant differences observed due to the treatments. Treatment 3 had the highest sum value of 1.342, with an average value of 0.447 and a variance value of 0.074, while Treatment 5 had the lowest sum value of 0.216, with an average value of 0.072 and a variance value of 0.001. This was further proven by Two-Way ANOVA, with an F-value of 5.148 and a P-value of 0.024, these findings demonstrated that the treatments factor significantly affected the TFC. The Plant Part factor had an F-value of 2.453 and a P-value of 0.148 but had no statistically significant impact on the TFC.

Table-9. Effect of Combined metals (Cd, Cu & As) toxicity on TFC at 510 nm in *H. umbellata* L.

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Root	5	0.521	0.104	0.008
Stem	5	0.903	0.181	0.007
Leaf	5	1.328	0.266	0.079
Treatment 1	3	0.44	0.147	0.002
Treatment 2	3	0.448	0.149	0.004
Treatment 3	3	1.342	0.447	0.074
Treatment 4	3	0.306	0.102	0.004
Treatment 5	3	0.216	0.072	0.001

There were significant differences between the metal types and the plant parts. The average TFC values for Cd exposure for the root, stem, and leaf samples were, respectively, 0.647, 0.581, and 0.663, with variance values of, 0.269, 0.285, and 0.296. The average TFC values for Cu exposure for the root, stem, and leaf samples were 0.119, 0.593, and 0.47, respectively, with variance values of 0.005, 0.361 and 0.251, respectively. The average TFC values with exposure to As for the root, stem, and leaf samples were 0.397, 0.332, and 0.216, with variance values of 0.244, 0.223, and 0.012, respectively. The average TFC values for combined metal exposure for the root, stem, and leaf samples were, respectively, 0.104, 0.181, and 0.266, with a variance value of 0.097 as shown in Table 10. The metal type had a substantial impact on TFC, according to the Two-Way ANOVA results. The interaction impact between metal type and plant part has an F-value of 0.534 and a P-value of 0.779. Overall, the results indicate that the TFC is significantly influenced by the type of metal but the plant part as well as their interaction did not significantly influence TFC.

Table-10. Effect of metal type and plant part on Total Flavonoids content at 510 nm in *H. umbellata* L.

Cd				
	Root	**Stem**	**Leaf**	**Total**
Count	5	5	5	15
Sum	3.2362	2.904	3.316	9.456
Average	0.647	0.581	0.663	0.630
Variance	0.269	0.285	0.296	0.244
Cu				
Count	5	5	5	15
Sum	0.594	2.596	2.35	5.54
Average	0.1188	0.519	0.47	0.369
Variance	0.005	0.361	0.251	0.211
As				
Count	5	5	5	15
Sum	1.985	1.658	1.08	4.723
Average	0.397	0.332	0.216	0.315
Variance	0.244	0.223	0.012	0.143
Comb				
Count	5	5	5	15
Sum	0.521	0.903	1.328	2.752
Average	0.104	0.1806	0.266	0.183
Variance	0.008	0.007	0.079	0.032
Total				
Count	20	20	20	
Sum	6.336	8.061	8.074	
Average	0.317	0.403	0.404	
Variance	0.163	0.211	0.167	

CART models

Two prediction models were developed in this study, using CART approach. One of them was for predicting TPC and the other for predicting TFC which are shown in Figures 2 and 3, respectively. CART models, due to their branching structure, can efficiently clarify the interrelationships of variables and their impact on the output variable (TPC or TFC, in this case). CART models showed the condition for each node and the average TPC or TFC values for that condition. For example, the top node showed the overall average of the data for both cases. Whereas for the CART for predicting TPC (Figure 2), the node on the extreme left-hand side in the third level showed the average TPC content of 1.12 when As was used as the metal regardless of any other factor. For the convenience of the reader, Tables 11 and 12 showed the statements which were extracted from CART models and the average TPC and TFC content at that condition.

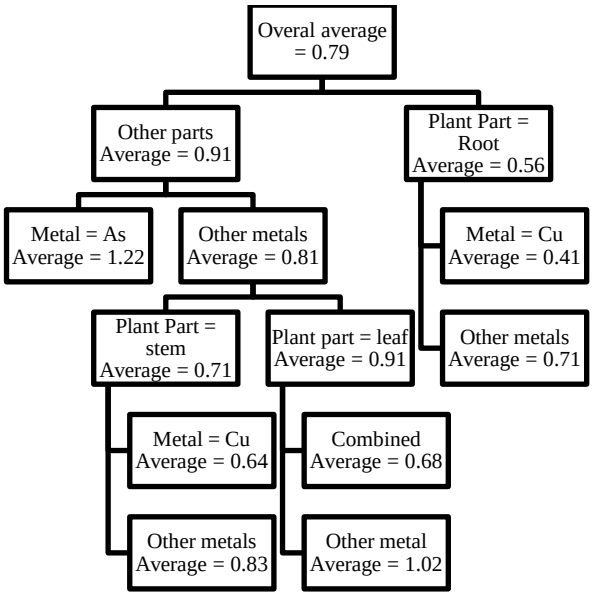

Figure-2: CART model for predicting TPC

The CART model for predicting TPC showed that the highest TPC was observed when leaf is exposed to Cd or Cu which was 1.02. Whereas the lowest TPC observed for root was exposed to Cu which was 0.41, as shown in Table 11. The CART model for predicting TFC showed that the highest observed TFC value was for Cd irrespective of plant part, this value is 0.63. On the other hand, lowest TFC value was observed when plant root or stem was exposed to Cu. These values are shown in Table 12. Based on above findings, it can be concluded that Cu normally produces a lower reaction in terms of TPC and TFC in the plant while Cd produced a higher reaction in the plant for its stabilization.

Table-11. Statements Extracted from CART model for predicting TPC

Statement	Average TPC at 760nm
If plant part is leaf or stem and metal is As	0.91
If plant part is stem and metal is Cu	0.64
If plant part is stem and metal is Cd or Combined	0.83
If plant part is root and metal is Cu	0.41
If plant part is root and metal is Cd, As, or Combined	0.71
If plant part is leaf and metal is Combined	0.68
If plant part is leaf and metal is Cd or Cu	1.02

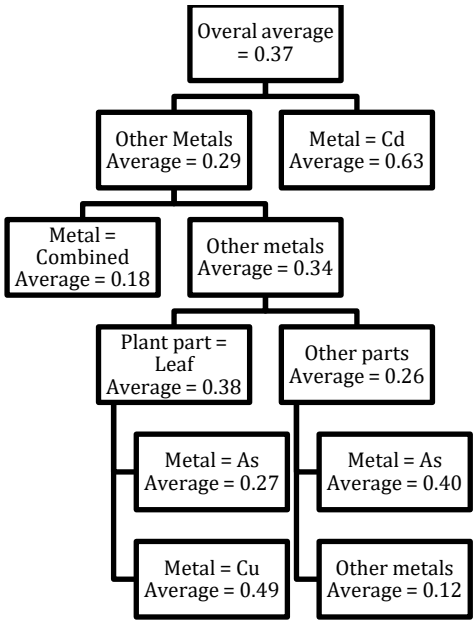

Figure 3: CART model for predicting TFC

Table-12. Statements extracted from CART model for predicting TFC

Statement	Average TFC
If metal is Cd	0.63
If metal is combined	0.18
If plant part is leaf and metal is As	0.27
If plant part is leaf or root and metal is Cu	0.49
If plant part is stem or root and metal is As	0.40
If plant part is stem or root and metal is Cu	0.12

Figures 4 and 5 showed the comparison of predicted and observed values for the CART models. It can be observed that models were able to capture the trend of variation for both cases. Hence, use of CART models is highly recommended for future studies in this area. Furthermore, it can be observed that the trends revealed through multiple ANOVA tests are more convenient and efficiently incorporated in the CART models for each case. This would be another reason for employing these models in similar studies comprising of experimental data.

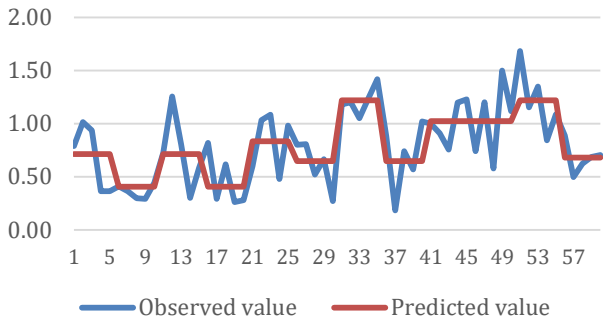

Figure-4. Comparison of predicted and observed TPC Values

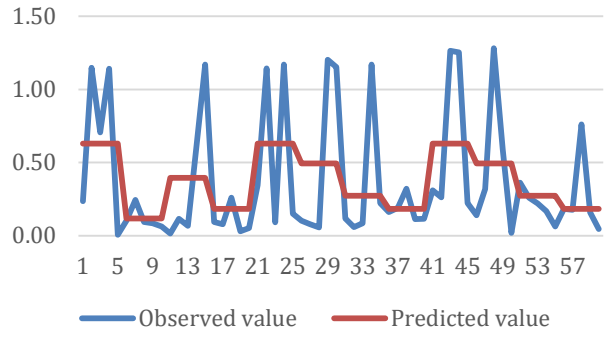

Figure-5. Comparison of predicted and observed TFC values

Discussion

HMs have toxic effects on plants, and these effects vary depending on the type of metal, the concentration of metal, plant part and the plant's tolerance towards that metal. Production of certain phytochemicals is one of the defense mechanisms against the stress caused by metal. ROS are generated as a result of HM exposure, and results in the production of phenolic and flavonoids in plants**.** Phenolic molecules distinguish themselves in plants by serving a variety of purposes, showcasing their antioxidant potential under varied stress circumstances (Dehghanian et al., 2022). Abiotic stress can cause a drop or rise in phenolic compounds in plants (Król et al., 2014). Production of secondary metabolites in plants differ with the type of metal used.

A key factor determining how plants react and how it affects secondary metabolism is the concentration of heavy metals. In the current study, the concentration of single metal had no significant effect on TPC and TFC but in case of combined metals, stress in TFC and TPC decreases with increasing metal stress. At lower concentrations the production of TPC and TFC were higher but when metal concentration increased, a decline in the production of total phenolics and total flavonoids was noticed. The formation of secondary metabolites is linked to heavy metal stress in plants. However, an excess of metals, especially heavy metals, can be harmful to plants; as a result, plant cells have systems in place to prevent their toxic accumulation (Eghbaliferiz and Iranshahi, 2016). Moreover, as reported by González-Mendoza et al. (2018) that high concentration of HMs causes a drop in the accumulation of phenolic compounds, affected by the plant's inability to synthesize new phenolic compounds and flavonoids. Jan´czak-Pieniaz˙ek et al. (2023) worked on different varieties of winter wheat with various concentrations of Pb and Cu and found that higher concentrations led to a fall in flavonoid levels. But in another study, increase in production of TPC was reported by increasing treatment concentration in leaves and roots of *Kandelia obovate* by application of Cd and Zn (Chen et al., 2019). Production of secondary metabolites is also linked to the plant part after HM accumulation. In the current study, it has also been found that production of secondary metabolites also vary in different parts of *H. umbellata* L. Highest phenolic and flavonoid contents were found in leaves followed by stem and least value was found in roots after application of Cd, Cu, As and combined metals stress. When exposed to Cd or Cu, leaves show the highest TPC, which is 1.02 and the lowest TPC found for a root exposed to copper, which is 0.41. According to the current study, Cd has the highest TFC value, which is 0.63, among all plant parts. On the other hand, when plant roots or stems are exposed to Cu, the lowest TFC value is seen. Cu typically creates a lower reaction in the plant in terms of TPC and TFC, however Cd provided a stronger reaction for the plant's stabilization. Similar results were reported previously by Makuch-Pietraś et al. (2023), in which phenolic and flavonoid contents were highest in leaves than other parts when antioxidant activities in relation to the transport of heavy metals (Cu, Zn, Cd, Pb, Ni and Cr) from the soil to different parts of *Betula pendula* were studied. Similarly, an increase in TPC was observed in leaves of corn when exposed to Cd, Cu & Pb (Kisa et al., 2016). Higher levels of phenolics and flavonoids were noticed in leaves of *Prosopis glandulosa* as compared to control plants under Cd and Cu toxicity (González-Mendoza et al., 2018). Higher content of

total phenolic and flavonoids was also detected in *N. biserrata* collected from contaminated sites as compared to control plants (Manan et al., 2015). Whereas some studies showed decline in total phenolic compounds under heavy metal stress. Kisa et al. (2016) reported a reduction in TPC in leaves of tomato under Cu, Cd and Pb stress and also that decrease can be related to treatment doses. Whereas enhanced TPC and TFCs were reported in *Gynura procumbens* under Cd and Cu stress individually, but under combined stress a reduction was observed in them. Moreover, lower levels of heavy metals encouraged the production of secondary metabolites (Ibrahim et al., 2017).

The correlation between levels of TPC and TFC with the type of metal used is also established in the present study. Overall, in recent studies the average TFC decreases in the order: Cd > Cu > As > Combined metals. Average TPC under different metals decreases in following order As > Cd> Cu > combined metals. The highest average was observed for As in the leaf, while the lowest average was observed for Cu in the root. These findings can be useful in understanding the impact of different metals on different parts of the plant, as well as how they interact with one another. This information can be used to inform future research and potentially guide strategies for managing metal pollution in the environment.

Conclusion

The concentration of single metal had no significant effect on TPC and TFC while in case of combined metals stress TFC and TPC decreased with increasing metal stress. Highest Phenolics and flavonoids were found in leaves followed by stem and least value was found in leaves after the application individual and combined metals stress. The results of the CART model also support these findings. It was concluded that the plant produces significant levels of TPC and TFC for combating HMs stress.

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Contribution of Authors

Saeed SH, Shaheen S & Gul A: Performed experiments and wrote the first draft of manuscript
Gillani GMS & Gazder U: Developed study designs
Arifuzzaman M, Asif AH, Nasrin A & Asaduzzaman M: Edited the draft and revised in the final stage
Mahmood Q: Developed study designs, edited the draft and revised in the final stage

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