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HEAVY METAL ACCUMULATION AND ANTIOXIDANT PROPERTIES OF *NEPHROLEPIS BISERRATA* GROWING IN HEAVY METAL-CONTAMINATED SOIL

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ABSTRACT

Antioxidant defense mechanisms are crucial for plants to survive under stress conditions. We investigated the capacity of a wild fern species, *Nephrolepis biserrata,* growing in the vicinity of industrial land to accumulate heavy metals, and assessed its antioxidative response under metal stress. The soils in this particular area were highly contaminated with zinc followed by lead and copper. As control, *N. biserrata* located 10 km away from the industrial area were collected and assessed. *N. biserrata* from the contaminated sites accumulated metals in their tissues in similar descending order of zinc>lead>copper. The values of bioaccumulation factor between 0 to 0.1 indicate *N. biserrata* as a moderate accumulator for the tested metals. For the enzymatic antioxidant assays, the activities of catalase and ascorbate peroxidase were significantly higher in *N. biserrata* from contaminated soil compared to control, while the activity of superoxide dismutase was not differ significantly in plants from both sites. We also detected higher contents of total phenolics and total flavonoids in *N. biserrata* collected from contaminated site compared to control. Our HPLC analysis revealed higher levels of myricetin and kaempferol in plant samples from the contaminated area. Our study verified the capacity of *N. biserrata* to scavenge oxygen radicals when exposed to heavy metal stress. Such ability to tolerate stressful condition suggests that the plant is a potential metal phytoremediator.

Keywords: *Nephrolepis biserrata*, Heavy metals, Antioxidants, Phytoremediators

1. Introduction

Heavy metals are undegradable, hence they are a constant environmental stress factor to plants. Although some metal elements such as zinc (Zn) and copper (Cu) are essential for plants as micronutrients, both are highly toxic to cells when present at excessive levels (Singh *et al.*, 2011). Unlike Zn and Cu, metal such as lead (Pb) is non-essential for plants and is normally toxic to living organisms (Sengar *et al.*, 2008). In this study, we

focused on Zn, Cu and Pb as these elements appeared to be the most prevalent pollutant components in the runoff and have highly accumulated in the tissues of mussels sampled at the sea area near industrial lands in Pasir Gudang, Malaysia (Yap *et al.*, 2004).

Metal toxicity in plants causes cellular redox imbalance. This increases the cellular level of reactive oxygen species (ROS), resulting in oxidative stress that disturbs metabolic pathways (Hegedüs *et al.*, 2001, Sharma and Dietz, 2009). The effects can be observed through plant physiological changes and alteration of plant biochemical properties. In response, plants normally activate their antioxidant defense mechanisms to attenuate oxidative injury (Gupta and Sharma, 2006; Sharma and Dietz, 2009).

Enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are among the first-line defense system that protects plants against early events in oxidative damage. These enzymes control the formation of hydroxyl radicals by the combination of dismutation reactions (Tang *et al.*, 2006). SOD catalyzes the dismutation of superoxide molecules to oxygen and hydrogen peroxide. APX reduces hydrogen peroxide to water using ascorbate as an electron donor. CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen. In the second line of plant defense, non-enzymatic antioxidants such as flavonoids and other phenolics will scavenge ROS and terminate the chain reactions. During heavy metal stress, such second line antioxidants may also act as metal chelators (Gupta and Sharma, 2006).

The next level of plant defense mechanism is the repair and *de novo* antioxidant production from the proteolytic enzymes (Lobo *et al.*, 2010). These enzymes will prevent the accumulation of oxidized proteins by recognizing and degrading the proteins that have been oxidatively modified. The roles of antioxidants in plants during abiotic stress were discussed in several recent reviews (Ahmad *et al.*, 2010; Foyer and Shigeoka, 2011; Gill and Tuteja, 2010).

In this study, heavy metal accumulation and the antioxidative response of *Nephrolepis biserrata* or Giant Sword Fern growing in contaminated industrial land were investigated. This species has edible rhizomes and young shoots; and has been traditionally used to treat skin problems such as blisters and wounds (Liu *et al.*, 2012; Rani *et al.*, 2010). Due to their abundances, *N. biserrata* could be alternatively utilized as an agent to clean up heavy metal-contaminated soil. However, its phytoremediation potential has yet to be confirmed. In this study, we had four objectives: first, to determine the level of heavy metals accumulated in *N. biserrata* and the respective soils; second, to determine the activities of enzymatic antioxidants in *N. biserrata* collected from the contaminated soil; third, to determine the total phenolic and flavonoid contents of *N. biserrata*, and fourth, to quantify the contents of selected flavonoids (myricetin, rutin and kaempferol) in *N. biserrata* exposed to heavy metal stress.

2. Materials and Methods

2.1. Plant Materials and soil samples

Matured *Nephrolepis biserrata* at approximately 60 cm height were collected from the roadside at Pasir Gudang Industrial Park, Malaysia in February, 2013. Pasir Gudang is in southern region of Peninsular Malaysia where various types of factories are located (Teh *et al.*, 2014). The sampling location was about 20 m from factories that potentially produce wastes that may contain heavy metals. Respective soil samples at the top 20 cm layer were collected and placed into a large ziplock bag. Plant and soil samples were cleaned by removal of dead leaves, debris and insects. Samples were brought to the laboratory for analysis. Plants were stored at -20 ºC while soil samples were kept at 4 °C prior to analysis. The same plant species and respective soil samples were collected 10 km away from the industrial area as control. Plant species were authenticated by Prof. Dr. Wee Yeow Chin, a botanist and founder of Bird Ecology Study Group of the Nature Society, Singapore.

2.2. Determination of heavy metal content

Prior to heavy metal analysis, both plants and soil samples were dried in the oven at 80 °C for 72 hours. Dried samples were pulverized into powder and approximately 0.5 g samples were subjected to acid digestion process. Nitric acids followed by perchloric acid (2:1) were added to the powdered plant samples. Soil samples were treated similarly. Digested sample was further diluted and aliquots were used for estimation of Zn, Cu and Pb concentrations. The measurements of these metal elements were conducted using Atomic Absorption Spectrophotometer Model AAnalyst 400. Sample preparation and heavy metal analysis were conducted based on the standard method by American Public Health Association (APHA - AWWA - WPCF, 1980).

2.3. Heavy metal analysis

The Biological Concentration Factor (BCF) of Zn, Cu and Pb for *N. biserrata* were determined as: BCF = metal concentration in plants/ metal concentration in soil (Yoon *et al.*, 2006).

2.4. Sample preparation for enzymatic antioxidant activity

Approximately 500 mg of plant samples were homogenized in cold extraction buffer (50 mM potassium phosphate pH 7.0, 1% (w/v) polivinyl pyrolidone) using mortar and pestle. The homogenates were subjected to centrifugation at 15,000 x g for 10 minutes at 4ºC and the supernatant were kept as enzyme extract.

2.5. Assay of superoxide dismutase (SOD) activity

Superoxide Dismutase Assay Kit II (Catalog number 574601) was obtained from Calbiochem (SanDiego, CA) and SOD activity was determined according to manufacturer's protocol. This assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance was read at 450 nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

2.6. Assay of catalase (CAT) activity

CAT activity was assayed in a reaction solution containing 50 mM phosphate buffer (pH 7.0), 150 mM H_2O_2 and 200 µl of enzyme extract (Dhindsa *et al.*, 1981). The activity of catalase was estimated by the decrease of absorbance at 240 nm for 1 min as a consequence of H_2O_2 consumed.

2.7. Assay of ascorbate peroxidase (APX) activity

APX activity was determined as previously described (Nakano and Asada, 1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 6 mM H_2O_2 and 200 µl enzyme extract. The activity was measured by the decrease in the absorbance of ascorbate at 290 nm.

2.8. Preparation of aqueous extracts

Powdered plant sample (2.5 g) was added with 150 mL of *n*-hexane and shaken on an orbital shaker for 24 hours. The solvent was decanted and the hexane treatment was repeated one more time. Next, after the hexane was removed by vacuum filtration, residual hexane in the plant powder was evaporated away in a fume hood. Absolute ethanol (100 mL) was added to the hexane-defatted powder and the mixture was shaken for 2 days. The mixture was then filtered via vacuum suction and the ethanol extract was collected. The collected extract was centrifuged at 7830 rpm for 5 minutes. The supernatant was concentrated by using a rotary evaporator. The concentrated extract was dried to constant weight at 40 ºC in a fan-forced oven. The extract was then re-dissolved with ethanol to obtain 20 mg ml^{-1} aliquots and was stored at 20 °C until further use.

2.9. Determination of total phenolic and flavonoid contents

Total phenolic contents of the extracts were determined by using a Folin-Ciocalteu colorimetric assay (Waterhouse, 2001), expressed as mg gallic acid equivalents (GAE)/g extract. A standard curve was prepared with 0 - 100 µg ml^{-1} gallic acid. Total flavonoid content was determined by using an aluminium chloride colorimetric assay (Zou *et al.*, 2004), expressed as mg rutin equivalents (RE)/g extract. A standard curve was prepared with 0 - 500 µg ml^{-1} rutin.

2.10. RP-HPLC method

Reverse-phase High Performance Liquid Chromatography (RP-HPLC) analysis was carried out by using Shimadzu LC-20AC Prominence Liquid Chromatography pumps, outfitted with Shimadzu Prominence UV/Vis Detector, Shimadzu CTO-10AS VP Column Oven, and Shimadzu DGU-$20A_3$ Prominence Deagasser. Chromatographic separations were carried out by using Phenomenex Gemini 5U C-18 110A column (150 x 4.6 mm, 5 micron). The binary system described by Onanong *et al.*, (2011) was applied with minor adjustments. The mobile phase consisted of two solvents, namely deionised water acidified to pH 2.8 with acetic acid (solvent A) and HPLC-grade acetonitrile (solvent B). The gradient elution was programmed as follows: linear gradient from 5% to 9% solvent B from 0 to 5 minutes; 9% solvent B from 5 to 15 minutes; linear gradient from 9% to 11% solvent B from 15 to 22 minutes; linear gradient from 11% to 18% solvent B from 22 to 38 minutes; linear gradient from 18% to 23% solvent B from 38 to 43 minutes; linear gradient from 23% to 90% solvent B from 43 to 44 minutes; linear gradient from 90% to 80% solvent B from 44 to 45 minutes; isocratic at 80% solvent B from 45 to 55 minutes; lastly, linear gradient from 80% to 5% solvent B from 55 to 60 minutes and 5% solvent B used between individual runs with a re-equilibration period of 5 minutes. Optimized operating conditions were as follows: injection volume of 20 µl, column temperature of 38 °C, flow rate of 0.8 ml/minute and UV detector set at 370 nm for flavonoid detection. An external standard method was used to identify flavonoids by comparing the relative retention times of peaks in the UV spectra of samples and those of standard compounds. The standard compounds used were myricetin, rutin, and kaempferol.

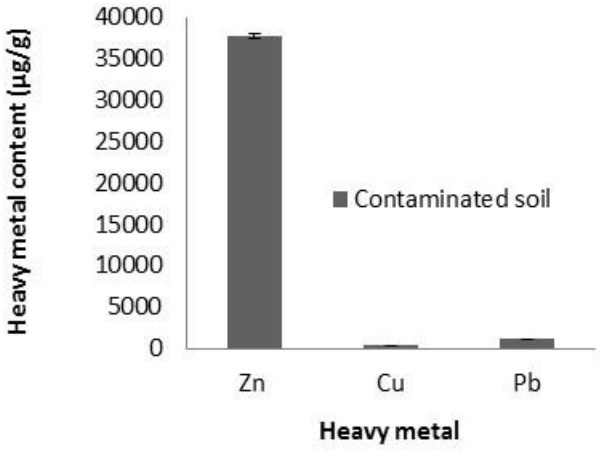
2.11. Data Analysis

Experiments were carried out in triplicates. Data were analyzed using Microsoft Excel 2007 and reported as mean $\pm$ standard error. Student's t test was used at the 0.05 level of probability for comparison of two set of means.

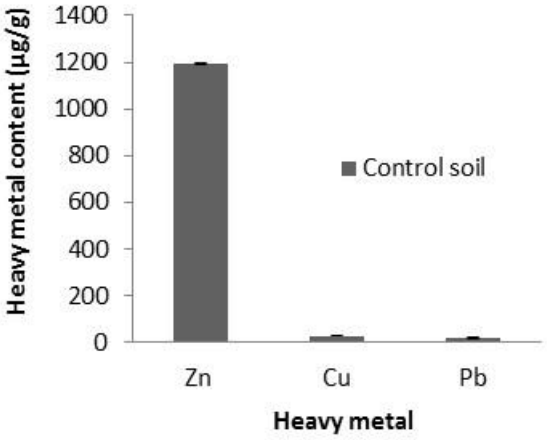
3. Results

3.1. Zn, Cu and Pb contents in soil samples

Soil collected in the vicinity of the industrial land was markedly contaminated with Zn, followed by Pb and Cu, whereas soil from the control site contained Zn followed by Cu and Pb in much lower amounts (Figure 1). In contaminated soil, Pb content was 55-fold, Zn was 32-fold and Cu was 14-fold higher compared to the respective metal content in the control soil.



(A)

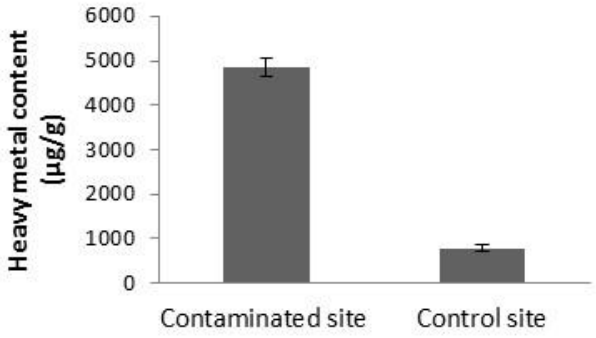


(B)

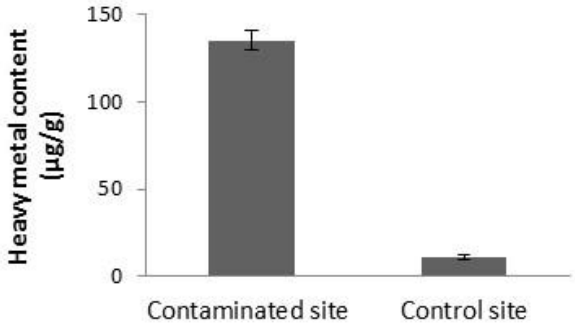
Figure 1. Contents of Zn, Cu and Pb in contaminated soil (A) and control soil (B). Data are means ± standard errors (n =3).

3.2. Heavy metal accumulation in N. biserrata

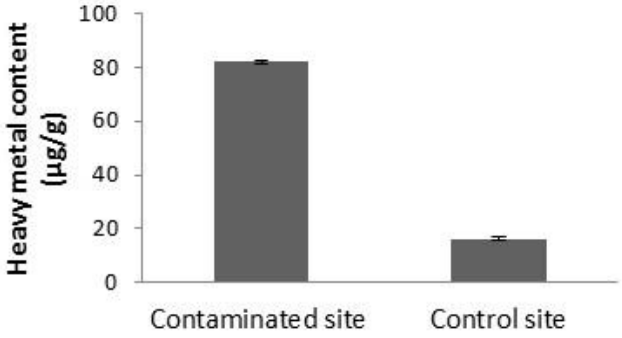
N. biserrata taken from the contaminated site accumulated the three heavy metals in the descending order of Zn>Pb>Cu, whereas metal accumulation in *N. biserrata* from the control site was in the descending order of Zn>Cu>Pb (Figure 2). The sequence of metal contents in plant tissues were corresponded to the level of metals in soil collected from the respective area. Among the tested metals, Pb was highly absorbed by the plant. The level of Pb was 92% higher in plants from the contaminated site compared to control site. Zn was 83.7% and Cu was 80.3% higher in plants from contaminated site compared to plants from the control site.



(A)



(B)



(C)

Figure 2. Contents of Zn (A), Pb (B) and Cu (C) in *Nephrolepis biserrata* collected from contaminated and control soils. Data are means ± standard errors (n =3)

3.2. Bio-concentrations of heavy metals in N. biserratat

The capacity of *N. biserrata* to accumulate heavy metals was determined using plant-soil Biological Concentration Factor (BCF). Calculated values that were in the range of 0.1 to 1.0, the BCF for plants from the contaminated site was lower than that of plants from the control site (Table 1).

Table 1. Biological concentration factor (BCF) of Zn, Cu and Pb in *Nephrolepis biserrata.*

Plant-Soil Origin	Zn	Cu	Pb
Contaminated	0.1	0.2	0.1
Control	0.7	0.6	0.5

3.3. Enzymatic antioxidants

Overall, higher levels of antioxidant activities were detected in plants from the contaminated site, compared to the control site. APX and CAT activities in plants from the contaminated site were two-fold and 3.2-fold higher, respectively, when compared with the control site (Table 2). However, no statistically significant differences were found for SOD activity in *N. biserrata* samples taken from either sampling site (Table 2).

Table 2. SOD, CAT and APX specific activities of *Nephrolepis biserrata* samples.

Parameter	Contaminated site	Control site
SOD (U/mg protein)	1.56 ± 0.19	1.16 ± 0.12
APX (nmole ascorbate oxidized/min/mg protein)	270 ± 20	140 ± 20*
CAT (µmole H_2O_2 decomposed/min/mg protein)	13.69 ± 2.14	4.24 ± 1.24*

*denotes significant difference between the mean values of enzyme extracts prepared from samples taken from contaminated and control sites, as determined by using Student's t test at $P < 0.05$. For SOD, 1 U is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

3.4. Total phenolic and total flavonoid contents

Analysis of ethanol extracts of *N. biserrata* sampled from the contaminated site revealed 33% and 13% higher levels of total phenolic and total flavonoid contents than samples taken from control site (Table 3).

Table 3. Total phenolic and flavonoid contents of *Nephrolepis biserrata* samples.

Parameter	Contaminated site	Control site	
Total phenolics (mg GAE/g)	72.86 ± 0.95	54.98 ± 0.76	*
Total flavonoids (mg RE/g)	8.09 ± 0.18	7.17 ± 0.44	*

* denotes significant difference between the mean values of ethanol extracts prepared from samples taken from contaminated and control sites, as determined by using Student's t test at $P < 0.05$.

3.5. HPLC analysis

The contents of myricetin, rutin, and kaempferol in the ethanol extracts of *N. biserrata* were analyzed using RP-HPLC.

Table 4. Flavonoid contents of *Nephrolepis biserrata* samples.

	Concentration (µg g^{-1})		
Flavonoid	**Contaminated site**	**Control site**	
Myricetin	1052.20 ± 116.22	673.57 ± 44.08	*
Kaempferol	250.03 ± 40.51	80.33 ± 4.88	*
Rutin	300.63 ± 49.94	303.10 ± 26.89	

*denotes significant difference between the mean values of ethanol extracts prepared from samples taken from contaminated and control sites, as determined by using Student's t test at $P < 0.05$.

Among the three flavonoids, myricetin was the most abundant on the basis of per gram extract dry mass, followed by rutin and lastly kaempferol. Myricetin and kaempferol contents in plant sample taken from the contaminated site were 1.56-fold and 3.11-fold higher, respectively, compared with plants sample collected form the control site. Plant samples from both sampling sites did not differ significantly in their rutin contents (Table 4).

4. Discussion

Heavy metals derived from industrial activities represent a continuous threat to living organisms including plants. Distinctively, plants are equipped with their own defense mechanism to continue survival under unfavorable conditions. In this study, a species of wild fern, *Nephrolepis biserrata* growing in heavy metal-contaminated industrial land was examined for their capacity to accumulate heavy metals and antioxidative responses towards heavy metal stress. This species was investigated due to its underexplored antioxidative properties, despite the abundant population regardless the environmental status of the area.

We started the investigation by analyzing the content of metals in the soil samples from this industrial area. The results showed high level of contaminations in the soil particularly from Zn, followed by Pb and Cu. The soil contents of these tested metals greatly exceeded the safe limit of metals in soil based on the European Standard 2002. According to the EU standard, the safe limit for Zn and Pb is 300 μg g^{-1}, while Cu is 140 μg/g (European Union, 2002). Hence, soil clean-up is required to prevent further environmental deterioration.

The amounts of metals accumulated in *N. biserrata* were determined in order to identify its strategy in metal uptake. As expected, *N. biserrata* accumulated significant amount of metals in their tissues, corresponding to the metal contents in the soils of contaminated and control sites. The amounts of Zn, Pb and Cu in *N. biserrata* samples collected from both sites exceeded the safe level set in the Malaysian Food Regulation 1985. This suggests that *N. biserrata*, despite its edible and medicinal properties, is unsafe for human consumption and/or for medicinal uses if it has been harvested from heavy metal contaminated areas. Users need to be more careful in selecting the source of the plants intended for consumption or medicinal purposes. Hence, *N. biserrata* growing in contaminated sites will be better utilized as an agent to clean-up metals from the soil. In a study conducted on the capacity of several forage weeds in removing cadmium and plumbum near the automobile battery manufacturing in Nigeria, *N. biserrata* has been found to be a good accumulator for Pb either in wet or dry season (Adie *et al.*, 2009).

Generally, the value of biological concentration factor (BCF) between 0.1 to 1 indicates the capacity of a plant as a moderate accumulator, while plant with the BCF value greater than one is known as an accumulator (Yoon *et al.*, 2006). Our results suggest that *N. biserrata* can be categorized as a moderate accumulator for Zn, Cu and Pb. Interestingly, we found huge differences of BCF values for *N. biserrata* collected from both sites although the values are still within the range of an accumulator species. It could be that the lower concentrations of metals in the control soil permits additional amount of metals to be taken up by plants. Thus, higher BCF value was recorded for *N. biserrata* from the control site (Table 1). High concentrations of metals in contaminated site might be toxic and hindered the ability of plants to actively take up these elements into their tissues. This is supported by the fact that the capacity of plants to absorb metals is decreasing at higher metal concentrations due to the saturation of metal binding sites (Dönmez and Aksu, 2002).

Adverse environmental conditions initially facilitate plant response mechanisms by the production of enzymatic antioxidants. Our results showed that the activities of enzymatic antioxidants were enhanced in plants sampled from the contaminated site. CAT activity was induced to a greater extent than were SOD and APX in plant samples collected from contaminated site. This may implies that CAT-mediated reaction was favorably adopted by *N. biserrata* to scavenge H_2O_2, compared to the APX-mediated pathway. In plant cells,

SOD-mediated reaction produces ROS in the form of H_2O_2. H_2O_2 will be further decomposed either by CAT- or APX-mediated pathways (Halliwell, 2006). This is consistent with the fact that catalase activity can destroy high concentrations of H_2O_2 (Mhamdi *et al.*, 2010). However, it is interesting to note that our result contradicts with an earlier finding that showed decreased activity of catalase when *N. biserrata* was exposed to biotic stress, indicating a different response pathway of plants involved in biotic and abiotic stress (Golan *et al.*, 2013)**.**

SOD activity in plants from the contaminated site however, did not significantly differ from plants at the control site. This implies that induction of SOD activity to higher levels may not be required for the *N. biserrata* for survival and/or grow in the contaminated site. SOD activity was determined using commercial kit, a reliable method alternative to the conventional technique. This approach is comparable to some previous studies in different plants since the SOD activities recorded were within the range of SOD level determined when plants exposed to stress (Esfandiari *et al.*, 2007; Abedi 2010).

Many studies reported enhanced production of plant phenolics in plants during stress. Flavonoids, together with some other phenolics, contribute to the overall antioxidant activities of plants mainly due to their redox properties and are capable of mitigating metal toxicity. The ability of flavonoids to detoxify free radicals and chelate metals (Symonowicz and Kolanek, 2012) is well established. In this study, increased levels of total phenolics and flavonoids in *N. biserrata* collected from heavy metal-contaminated site implies that flavonoids and other phenolics may play an important role in the growth and survival of *N. biserrata* under heavy metal stress. In other words, besides the enhanced CAT and APX activities, the ability of *N. biserrata* to accumulate heavy metals without severely compromising its survival may be attributed to its ability to upregulate its phenolic production.

To further clarify the potential importance of flavonoids in the response of *N. biserrata* to exposure to heavy metals, we detected, quantified and compared the levels of three flavonoids, namely myricetin, rutin and kaempferol, in plant samples collected from the contaminated and control sites. The three flavonols were previously detected among the major compounds in edible tropical plants (Miean and Suhaila 2001) and ubiquitous in medicinal plants. Enhanced accumulation of myricetin and kaempferol was detected in samples collected from the contaminated site. This observation suggests that myricetin and kaempferol may be crucial to the growth and survival of *N. biserrata* growing in heavy metal-contaminated soil. Supporting this possibility are earlier findings of free radical scavenging and metal chelating activities of myricetin and kaempferol (Jomova and Valko, 2011; Roedig-Penman and Gordon, 1998; Singh *et al.*, 2008). Rutin was abundantly found in *Pteris multifida*, an arsenic hyperaccumulator fern (Lu *et al.*, 1999). Metal, including Pb-chelating activity of rutin has been previously reported (Soczyńska-Kordala *et al.* 2001). However, rutin content was not enhanced in *N. biserrata* growing in heavy metal contaminated soil. This implies that at least in *N. biserrata*, rutin may not play a significant role in enhancing the metal chelating or accumulating ability of the species when growing in heavy metal contaminated soil.

5. Conclusion

Based on BCF value, our study has demonstrated *N. biserrata* to be a moderate accumulator of Zn, Cu and Pb. Enhanced CAT and APX activities as well as increased accumulation of phenolics, may underlie the biochemical basis of the ability of *N. biserrata* to grow in heavy metal-contaminated soil and accumulate Zn, Cu and Pb to different extents in its tissues. Increased production of myricetin and kaempferol may have contributed to the ability of the plant to chelate metal ions and concurrently protected the plant against potential oxidative stress resulting from excess metal accumulation. Altogether our results suggest that *N. biserrata* is a potential species for further exploration and development for phytoremediation purposes.

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