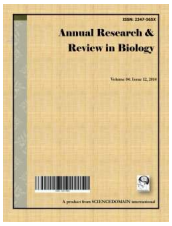

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Primary, Secondary Metabolites, Biochemical and Antioxidant Activity of *Orthosiphon staminues Benth* (Misai Kucing) Under Cadmium Exposure

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHI and NAMZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AI and HO managed the analyses of the study. Author MNHMN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was conducted to evaluate the effects of cadmium (0, 3,6, 9 mg/kg) on the primary, secondary metabolites, biochemical and antioxidant activity in *Orthosiphon stamineus*.
Study Design: *Orthosiphon stamineus* seedlings were exposed to four levels of Cd (0, 3, 6 and 9 mg/kg) in the form of $CdCl_2$ and were applied to the media during preparation. The experiment was organized in a randomized complete block (RCBD) design with three replications. Each experimental unit consisted of five seedlings, and there was a total of 135 seedlings used in the experiment.
Place and Duration of Study: Department of Biology, Faculty of Science Universiti Putra Malaysia

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between August 2013 to September 2013.
Methodology: Total soluble sugar and starch were determined spectrophometically using anthrone reagent, Total phenolics and flavonoid were determined using Folin-Ciocalteu reagent, hydrogen peroxide (H_2O_2) and lipid peroxidation were measured using potassium iodide (KI) and thiobarbiturate acid (TBA). The antioxidant activity was determined by DPPH reagent. Phenyll alanine lyase activity (PAL) was determined by using transcinnmic acid and cadmium determination by using inductively coupled plasma emission (ICPMS).
Results: Increased in Cd from 0 > 9 mg/kg, the production of total phenolics and flavonoid was enhanced in the shoot compared to the root parts. It was found that enhancement of total phenolics and total flavonoids might be contributed by enhancement of PAL and total non-structural carbohydrate (TNC) activity under cadmium exposure. Although total phenolic and total flavonoids increased, the antioxidant activity (DPPH) was reduced with high levels of Cd. It was also found that cadmium accumulated more in roots compared to the shoot. There was accumulation of H_2O_2 and lipid peroxidation as levels of Cd increased from 0 > 9 mg/kg indicating DNA damage under excess levels of cadmium. Total chlorophyll content also was reduced with increasing levels of cadmium indicating that cadmium disrupt nitrogen uptake of *O. stamineus*. The study showed that *O. stamineus* exposed to cadmium accumulate cadmium above the level recommended by WHO indicating there is a safety hazard when this plant is consumed for medicinal purposes.
Conclusion: The current study revealed that cadmium contamination would reduce the medicinal properties of *O. stamineus* although the production of secondary metabolites increased. This is explained by enhanced production of total phenolics, total flavonoids, but lower DPPH values.

Keywords: Medicinal plants; cadmium; plant primary and secondary metabolites; biochemical changes; antioxidant activity.

1. INTRODUCTION

Heavy metal pollution is a global problem that has impacts on ecological, evolutionary, nutritional and environmental reasons. The pollution is introduced to the environment by many anthropogenic activities, such as mining, fertilization, the use of metal-based pesticides and a wide range of industrial activities [1]. Exposure to heavy metals leads to physiological disorders in plants such as an accumulation in reactive oxygen species (ROS). Plants have adapted a number of strategies to counteract the toxic effects in the event of heavy metal stress by activating certain intermediary metabolic activities and making physiological adjustments. These mechanisms protect the plant against free radicals which might disrupt cellular metabolism as well as prevent damage to biomolecules such as lipids, proteins and nucleic acids [2]. Some of the strategies include accumulation of antioxidant enzymes, proline, glutathione and phenolic compounds [3].

Cadmium (Cd) is one of the most hazardous metals in plant because of its accumulation in the environment. It is widespread in soils, water and atmosphere. Due to its high mobility in the plant-soil system, Cd can enter the food chain by being taken up by crop plants used for feeding animals and humans [4]. In plants, Cd has no known function as a nutrient and seems to be more or less toxic. Elevated content of Cd in the environment can lead to various toxicity symptoms in plants that might be showed, including growth inhibition, disturbances in photosynthesis, water and mineral uptake, or stimulation of oxidative stress [5]. Cadmium as a redox inactive metal does not directly participate in cellular redox reactions, since it does not take part in Fenton and Haber-Weiss reactions [6]. However, it can indirectly activate NADPH oxidases in membranes, giving rise to an enhanced formation of superoxide radical and hydrogen peroxide and eventually, to an oxidative burst [4]. Cadmium may also induce expression of lipoxygenases in plant tissues and thus indirectly cause oxidation of polyunsaturated fatty acids [5].

Plants produce various compounds which can reduce oxidative stress. Phenolic compounds are widely distributed and structurally diverse metabolites involved in many stress responses [7]. They have antioxidative properties and scavenge reactive oxygen species (ROS) directly or through enzymatic reactions [8]. Moreover, they function as metal chelators and may have important roles in Cd uptake and translocation [9,10]. Plant secondary metabolites are often referred to as compounds that have no active role in basic metabolic processes in plants.

However, they are important for plant interactions with the environment [11]. Accumulation of secondary metabolites often occur in plants subjected to environmental stresses including heavy metals [12]. In the case of heavy metal stress, plants can accumulate phenolics and flavonoids as a protective mechanism which prevents deleterious molecules from eliciting oxidative damage to the membrane components thereby maintaining membrane integrity [13]. Phenolics and flavonoids are groups of phytochemical compounds that possess various pharmacological properties [14]. Hence, any environmental factors that affect the synthesis and accumulation of these compounds may also influence the pharmacological properties of the plant. Some of the roles of secondary metabolites include plant protection (e.g. as antioxidants, free radical-scavengers, UV light-absorbing, and anti-proliferative agents) and defense against microorganisms such as bacteria, fungi, and viruses [15]. Secondary metabolites have significant practical applications in the fields of medicine, nutrition and cosmetics [16].

Orthosiphon stamineus Benth. (Lamiaceae), usually known as misai kucing, is a general used medicinal plant in Southeast Asia region and is extensively used for the healing of various illness such as fever, epilepsy, gallstones and hepatitis [17]. Apart from medicinal uses, the plant is also a good ornamental plant. In Malaysia and Indonesia, *O. stamineus* leaves are consumed as a drink to recover health and for treatment of ingestion, irritation, and *diabetes melitus*. Some compounds have been identified from this herb, including caffeic acids derivatives, organic acids, flavonoids, terpenoids, saponins, hexoses and, chromene [18]. Among these chemicals, the polyphenol components were found to possess potential healing properties as they were shown to possess antimicrobial and immunomodulatory activities [19]. The curative effects of *O. stimaneus* have been attributed mainly to its polyphenols, the principal constituent in the leaf, which has been described to be efficient in reducing oxidative stress by preventing the formation of lipid peroxidation in biological systems [20]. Polyphenols are very essential in the control and inhibition of tissue damage done by stimulated oxygen species [21]. The functions of phenolic compounds in plant physiology and interactions with biotic and abiotic environments are numerous [22]. Phenolic compounds and other secondary metabolites have numerous pharmacological properties and thus any environmental condition that affects either the quantity or composition of phytochemical compounds may potentially influence the efficacy of the medicinal plant product [23,24]. There is limited information on specific physiological responses of medicinal plants to heavy metals in soils and the resulting changes in the efficacy of the plant. There was also no documentation on the growth, leaf gas exchange and antioxidant activities of this plant under Cd influences. The PAL activity also was never documented. The objective of this study was to assess the effects of different cadmium concentration on growth, gas exchange, chlorophyll, antioxidant activity, cadmium uptake, PAL activity, primary and secondary metabolite production in *O. staminues*.

2. MATERIALS AND METHODS

2.1 Plant Material and Maintenance

This research was conducted in Taman Pertanian Universiti, Glasshouse Complex, Universiti Putra Malaysia. Stem cuttings of *O. staminues* were propagated for two weeks in pots and then moved to pots filled with a mixture of top soil and sand. All the seedlings were watered using overhead mist irrigation, given four times a day or when necessary. *Orthosiphon stamineus* seedlings were exposed to four levels of Cd (0, 3, 6 and 9 mg/kg) in the form of $CdCl_2$ and were applied to the media during preparation. The experiment was organized in a randomized complete block (RCBD) design with three replications. Each experimental unit consisted of five seedlings, and there was a total of 135 seedlings used in the experiment. All the plants were harvested after 12 weeks of treatment.

2.2 Total Phenolics and Total Flavonoids Quantification

The method of extraction and quantification for total phenolics and flavonoids contents followed methods by Jaafar et al. [25] using gallic acid and rutin as a standard.

2.3 Total Soluble Sugar Determination

Total soluble sugar was measured spectrophotometrically using the method of Edward [26]. Sucrose was used as a standard stock solution to prepare a standard curve for the quantification of sucrose in the sample. The

sample was measured at an absorbance of 620 nm using a spectrophotometer.

2.4 Starch Determination

Starch content was determined spectrophometrically using glucose as a standard using a method described by Thayumanavam and Sadasivam [27]. The sample was read at absorbance of 630 nm to determine the sample starch content.

2.5 Total Non-structural Carbohydrate (TNC)

The total non-structural carbohydrate was calculated as the sum of total soluble sugar and starch content.

2.6 Phenylalanine-Ammonia-lyase (PAL)

Phenylalanine-ammonia-lyase (PAL) activity was measured spectrophotometrically using the method described by Martinez and Lafuente [28]. In this method, Trans-cinnamic acid yield was estimated by measuring increase in the absorbance at 290 nm.

2.7 Chlorophyll Content

For the measurement of chlorophyll concentration, the weighed fresh leaves (200 mg) were grinded using a mortar and pestle and immersed in 10 mL of 100% acetone. Samples then homogenized with homogenizer at 1000 rpm for one minute. The homogenate was filtered through two layers of cheesecloth, and was centrifuged at 2500 rpm for 10 minutes. The two wavelengths of 662 nm and 645 nm were used as the peak absorbance of chlorophyll-a and chlorophyll-b. The total amount of chlorophyll-a and chlorophyll-b were then calculated according to the formulas of Lichtentaler and Wellburn [29].

2.8 Leaf Cd Analyses

To measure cd, one gram of dried plant material samples was digested in nitric acid (HNO_3; 65%). Cadmium concentration of the extracted solution was measured using an inductively coupled plasma emission (ICP-MS, 7500a, Agilent, USA).

2.9 Measurement of H_2O_2 and Malondialdehyde (MDA) Content

Hydrogen peroxide content of the plant parts was measured spectrophotometrically after reaction with potassium iodide (KI) [30] and Lipid peroxidation of plant parts was estimated by the level of malondialdehyde (MDA) production using thiobarbituric acid (TBA) method as described by Heath and Packer [31].

2.10 DPPH Radical Scavenging Assay

The DPPH free radical scavenging activity was determined according to the method described by Joyeux et al. [32]. Initially, a solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm. An aliquot (40 µL) of an extract was added to 3 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured after 30 min. The antiradical activity (AA) was determined using the following formula:

AA% = 100 −[(Abs:sample −Abs:empty sample)] × 100)/Abs:control

The optic density of the samples, the control and the empty samples were measured in comparison with methanol. One synthetic antioxidant, BHT (butylhydroxytoluene) and α-tocopherol, were used as positive controls. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmol Trolox equivalent per gram of dried plant material.

2.11 Statistical Analysis

Data were analyzed using analysis of variance by SAS version 17. Mean separation test between treatments was performed using Duncan multiple range test and standard error of differences between means was calculated with the assumption that data were normally distributed and equally replicated.

3. RESULTS AND DISCUSSION

3.1 Total Phenolics and Total Flavonoids Profiling

Heavy metal stresses can regulate plant growth and development, limit plant production, and alter the physiological and biochemical properties of plants. The accumulation of secondary metabolites is identified as the adaption and protection of plants against environmental stresses. For the medicinal plants, the secondary metabolites are usually considered as the active components [32]. In the current study, different

cd concentrations had a significant (P ≤0.05) impact on the production of total phenolics and flavonoids (Table 1). As Cd concentrations increased from 0 to 9 mg/kg, the *O. stimaneus* plants produced more total phenolics and flavonoids. The accumulation of secondary metabolites was also found to be equal in all plant parts. As the Cd concentrations increased from 3 to 6 and then 9 mg/kg the leaves total phenolics elevated subsequently by 34, 64 and 154%, respectively, compared to control at 0 mg/kg. Same reducing trend was also observed for total flavonoids that not exposed to cadmium showing the lowest value of total flavonoids production (0.61 mg rutin/g dry weight), compared to 9 mg/kg, which recorded 1.88 mg rutin/g dry weight. The rise in total plant flavonoids and total phenolics under Cd application was also reported in previous studies in *Hypoxis hemerocallidea* and *Drimia elata* [33,34]. The upregulation in total phenolics and flavonoids under excess Cd might be due to an increase in the production of carbohydrates (TNC), as revealed by the high correlation coefficient (r^2= 0.921; total phenolics and r^2= 0.962; total flavonoids; P ≤0.05) in Table 2, the increase in starch content might have more effect on the increase of plant metabolites, compared to sucrose, which showed higher correlation coefficients with both the total flavonoids (r^2= 0.860 vs r^2 = 0.770; P ≤0.05) and phenolics (r^2= 0.890 vs.0.791; P ≤0.05). The data indicate, that increase in starch content in *O. stamineus* might have more profound effect than sucrose in regulating the increase in production of total flavonoids, and phenolics in the present study. Guo et al. [35], proposed that the increase in bio-metabolites production in their study on broccoli might be due to increase in production of starch compared to sucrose. Meanwhile, Kuo *et al*. [36] suggested that the increase in carbon based secondary metabolites production (total phenolics and flavonoids) under heavy metal especially cadmium application was due to increased availability of phenyl alanine under application of 5 – 25 mg/L Cd in rice seedlings. In the current study, strong significant positive correlation was observed between PAL and production of total phenolics (r^2 =0.872; P≤0.05) and flavonoid (r^2 =0.812; P≤0.05). This indicates that the increased production of total phenolics and flavonoids of *O. stamineus* under Cd application might be due to increase in PAL activity that stimulate the production of total phenolics and flavonoid. It can be concluded that the enhancement of carbon based secondary metabolites of *O. stamineus* under Cd application might be due to upregulation of total non structurable carbohydrate and PAL activity that were observed in the present study [37]. This suggests that heavy metal especially Cd can regulate production of secondary especially in *O. stamineus*.

3.2 Carbohydrate Content

Carbohydrates content was influenced by the cadmium rates applied to *O. stamineus* (P ≤0.01;Table 3). The carbohydrates in different parts of the plant showed a inclining order of leaf > stem > root. Generally, as Cd rates increased,

Table 1. Accumulation and partitioning of total flavonoids (TF) and total phenolics (TP) in different plant parts of *O. stamineus* under different Cd concentrations

Cadmium rates (mg/L)	Plant parts	Total phenolics, TP (mg gallic acid/g dry weight)	Total flavonoids, TF (mg rutin/g dry weight)
0	Leaves	1.31±0.14^{d}	0.61±0.04^{f}
	Stems	1.23±0.24^{d}	0.65±0.02^{f}
	Roots	1.18±0.11^{e}	0.62±0.03^{f}
3	Leaves	1.67±0.04^{c}	0.87±0.01^{e}
	Stems	1.81±0.14^{c}	1.11±0.04^{d}
	Roots	1.71±0.16^{c}	1.23±0.05^{d}
6	Leaves	1.76±0.08^{b}	1.32±0.06^{d}
	Stems	2.15±0.31^{b}	1.71±0.02^{c}
	Roots	2.21±0.32^{b}	1.81±0.01^{b}
9	Leaves	3.34±0.15^{a}	1.88±0.04^{b}
	Stems	3.21±0.21^{a}	2.01±0.02^{b}
	Roots	3.41±0.32^{a}	2.31±0.05^{a}

All analyses are mean ± standard error of mean (SEM). N = 40. Means not sharing a common letter were significantly different at P ≤0.05

the carbohydrate content increased (Table 3). It was discovered that, the content of sucrose and starch recorded the lowest values in control (0 mg/kg) cadmium compared to other treatments. In the leaves, the control 0 mg/L (37.81 mg sucrose/g dry weight), 3 mg/kg (42.11mg sucrose/g dry weight) and 6 mg/kg (47.21 mg sucrose/g dry weight) treatments produced less sucrose than the 9 mg/kg treatment which produced 57.11 mg sucrose/g dry weight. The starch content in the leaves at 0 mg/kg Cd was statistically lower than other treatments. In the experiment, the leaves starch contents at 3,6,9 mg/kg treatments were 44.11, 52.21 and 67.80 mg glucose/g dry weight, respectively, compared to only 37.82 mg glucose/g dry weight at 0 mg/kg treatments. In all plant parts, the increase in starch content was higher than the increase in sugar content. The current results imply that high Cd concentration were able to enhance the production of carbohydrate, which concurrently enhanced the TNC [38,39]. Devi et al [40] have attributed the increase in production of carbohydrates under Cd in pea was due to increasing activity of sucrose synthase and sucrose phosphate synthase (SPS) activities in roots and shoots. This indicate that this enzyme activity might be enhanced under cadmium application in *O. stamineus* [41]. Carbohydrates are the essential compounds necessary to produce plant secondary metabolites in the shikimic acid pathway [42]. Ibrahim and Jaafar [43] found that upregulation of secondary metabolites in *L .pumila* was due to increment in TNC production. The increase in phenolics and flavonoids production in the present study might be related to the greater the source-to-sink ratio in *O. staminues* due to higher production of carbohydrate content that correspond to the greater would production of plant secondary metabolites [44].

3.3 Phenyl-Alanine-Lyase (PAL) Activity

The PAL activity in *O. stamineus* was influenced by Cd rates (P≥ 0.05). Phenyl-Alanine-Lyase Activity was found to be the highest (3.44 nM transcinnamic/mg protein/h) under 9 mg/kg, and the lowest activity was demonstrated at 0 mg/kg which registered a value of 1.21 nMtranscinnamic/mg protein/h (Fig. 1). The present findings were in agreement with Sylwia et al [45] that found Cd increased the PAL activity in *Glycine max* and *Lupinus luteus* seedlings treated with different concentrations of cadmium (Cd^{2+}, 0–25 mg/l). They found the increase in PAL activity in the plants was related to the increased in PAL mRNA levels, thus suggesting increased gene expression of PAL genes under cadmium treatments. The increase in the production of plant metabolites in the present work could be due to the increase in PAL activities under high Cd concentration. This was showed with a significant (P≤ 0.05) positive relationship with total phenolics (r^2= 0.87) and flavonoids (r^2= 0.81), proposing an up-regulation of total phenolics and flavonoids with increased PAL activity. This might be due to the fact that PAL is an enzyme, which a precursor for total phenolics and flavonoids production [46]. This showed, increase in PAL activity correspond to the up-regulation of plant secondary metabolites production in *O. stamineus* under Cd exposure in the present study [47].

3.4 Production of Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA)

The production of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) was influenced by Cd rates imposed onto *O. stamineus* seedlings (P ≤0.01). It was showed, that as the concentration of cadmium increased from 3 to 9 mg/kg the production of H_2O_2 and MDA was up-regulated (Table 4). At all Cd concentrations, the highest contents of H_2O_2 and MDA were observed in leaves, stems, and the smallest in the roots. From the correlation Table 2, the H_2O_2 and MDA production showed a significant positive correlation with total flavonoids and total phenolics, implying that increase in these compounds might be involved in the increase of secondary metabolites production under high Cd concentration. The production of H_2O_2 in plants is an early event of plant response to biotic and abiotic stress, and acts as a signal to the increase production of secondary metabolism in plant [48]. The production of H_2O_2 can change the content of redox agents and the redox state of cells [49]. The increase in H_2O_2 content with corresponding increase in total flavonoids and phenolics was also observed by Jabs et al. [50] in parsley exposed to heavy metal stress. Lipid peroxidation or the formation of malondialdehyde (MDA) was considered as a suitable biomarker for oxidative stress this was due to degradation of fatty acids hydroperoxide [48]. Total MDA activity documented in (9 mg/kg) was significantly higher in the control (0 mg/kg) indicating higher stress levels exhibited by plants increase of H_2O_2 and MDA these suggested that

Table 2. Correlation among the measured parameters in the study

Parameters	1	2	3	4	5	6	7	8	9	10	11
1. Total phenolics	1.000										
2. Total flavonoids	0.432	1.000									
3. Sugar	0.771*	0.792*	1.000								
4. Starch	0.861*	0.892*	0.541	1.000							
5. TNC	0.921*	0.962*	0.445	0.112	1.000						
6. PAL	0.872*	0.812*	0.320	0.231	0.432	1.000					
7. H_2O_2	0.112	0.321	0.223	0.432	0.344	0.302	1.000				
8. MDA	0.222	0.231	0.134	0.102	0.676	0.112	0.872*	1.000			
9. Cadmium	0.004	0.421	0.009	0.302	0.213	0.354	0.812*	0.871*	1.000		
10. TCC	-0.791*	-0.804*	0.235	0.004	0.321	0.098	0.087	0.067	0.211	1.000	
11. DPPH	0.211	0.311	0.021	0.187	0.109	0.087	0.023	0.124	-0.812*	0.098	1.000

*Note TNC = total non structural carbohydrate; PAL = phenyl alanine lyase activity; H2O2 = hydrogen peroxide; MDA = lipid peroxidation; TCC = total chlorophyll content; DPPH = Diphenyl-2-picryl-hydrazyl (DPPH) Assay *, significant at p ≤0.05*

Table 3. Accumulation and partitioning of soluble sugar, starch and total non structurable carbohydrate (TNC) in different plant parts of *O. stamineus* under different Cd concentrations

Cadmium rates (mg/kg)	Plant parts	Soluble sugar (mg sucrose/g dry weight)	Starch (mg glucose/g dry weight)	TNC (mg/g dry weight)
0	Leaves	37.81±7.34^{d}	37.82±4.41^{d}	75.11±10.34^{i}
	Stems	35.21±5.14^{d}	37.51±3.34^{d}	72.21±12.42^{i}
	Roots	33.11±7.21^{d}	34.82±2.24^{d}	67.041±11.34^{j}
3	Leaves	42.11±6.16^{c}	44.11±7.21^{c}	85.83±8.34^{g}
	Stems	39.21±8.13^{c}	43.31±3.04^{c}	81.87±12.30^{h}
	Roots	38.01±2.54^{c}	42.06±1.74^{c}	82.08±7.31^{h}
6	Leaves	47.21±9.14^{b}	52.21±4.14^{b}	98.21±8.36^{f}
	Stems	43.83±7.77^{b}	50.11±5.03^{b}	92.41±6.32^{e}
	Roots	42.09±6.31^{b}	49.12±6.11^{b}	91.33±9.32^{e}
9	Leaves	57.11±4.64^{a}	67.80±4.34^{a}	123.41±13.35^{a}
	Stems	54.31±6.34^{a}	62.83±2.11^{a}	117.21±14.32^{b}
	Roots	52.32±3.94^{a}	60.81±1.76^{a}	112.80±15.31^{c}

All analyses are mean ± standard error of mean (SEM). N = 40. Means not sharing a common letter were significantly different at P ≤0.05

MDA accumulated due to higher amount of H_2O_2 levels. This was supported by a significant positive correlation between MDA and H_2O_2 (r^2 = 0.891; P≤ 0.01). Furthermore, there was significant positive correlation observed between cd concentrations with hydrogen peroxide (r^2 = 0.812; P ≤ 0.01) and MDA (r^2 = 0.871; P ≤ 0.01) in the present study. The increase in hydrogen peroxide and lipid peroxidation usually signifies induction of plant defense systems and this increase could enhance the production of secondary metabolite that was observed in the present study [51]. The increase production of MDA repair DNA damages from H_2O_2 accumulation under cadmium application. DNA damage is caused by Cd involved destruction of nucleic acids, cell membrane, lipids, and proteins; reduction of protein synthesis; and damage of photosynthetic proteins, which affects growth and development of the whole organism [52,53].

3.5 Cadmium Accumulation

Cadmium content was found to be influenced by cadmium concentrations applied (P ≤ 0.01). The accumulation of cadmium was found to be the highest under roots compared to the leaves and

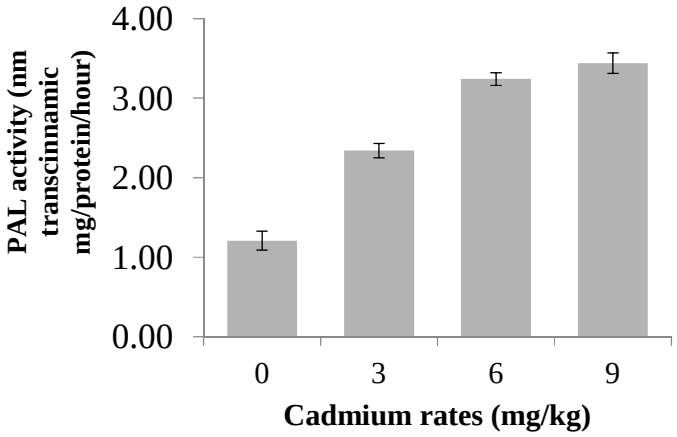

Fig. 1. The effects of different cadmium rates on PAL activity of *O. stamineus*. N = 40. Bars represent standard error of differences between means (SEM)

Table 4. Accumulation and partitioning of H_2O_2 and MDA in different plant parts of *O. stamineus* under different Cd concentrations

Cadmium rates (mg/L)	Plant parts	H_2O_2 (ng/g fresh weight)	Lipid peroxidation (µmol/g fresh weight)
0	Leaves	0.88±0.21^{d}	0.91±0.02^{h}
	Stems	0.76±0.04^{d}	0.84±0.06^{g}
	Roots	0.72±0.05^{d}	0.71±0.03^{f}
3	Leaves	0.96±0.12^{c}	1.25±0.04^{e}
	Stems	0.86±0.15^{c}	1.11±0.08^{d}
	Roots	0.82±0.17^{c}	1.01±0.09^{d}
6	Leaves	1.35±0.19^{c}	1.63±0.05^{c}
	Stems	1.31±0.31^{b}	1.51±0.14^{c}
	Roots	1.27±0.30^{b}	1.50±0.07^{b}
9	Leaves	1.81±0.24^{a}	2.71±0.02^{a}
	Stems	1.72±0.74^{a}	2.61±0.02^{a}
	Roots	1.71±0.14^{a}	2.52±0.04^{a}

All analyses are mean ± standard error of mean (SEM). N = 40. Means not sharing a common letter were significantly different at P ≤0.05.

stems. Cadmium content in roots of 3, 6 and 9 mg/kg was 0.91, 1.66 and 3.09 mg/kg, respectively compared to only 0.05 mg/kg in 0 mg/kg Cd treatments. In the leaf, Cd content in 9 mg/kg was 78% higher than the average cadmium content in the three cadmium treatment levels values (Table 5). From the Cd accumulation pattern it can be concluded that *O. stamineus* is a hyper-tolerant accumulator plants, in which metals are actively taken up through the root, and largely loaded into xylem for root to shoot transport [54]. The present result suggests that *O. stamineus* develop tolerance strategy, which relies on confinement and detoxification of metals in a controlled way. This permits plants to withstand high metal exposure but also to accumulate metals to sometimes extremely high concentrations [55]. The Cd contents in the plant parts have shown to exceed safety level permitted by WHO in herbal medicines (> 0.5 mg/kg), thus indicating that this plant is not safe to consume [56]. However, the standards are different in different countries that establish the heavy metal limits for the commercial, industrial, residential, and agricultural soil. The maximum Cd concentration in agricultural soil provided by the Canadian Council of Ministers of the Environment is 1.4 mg/kg, while the soil guideline value of cd in allotment land given by the UK Environment Agency is 1.8 mg/kg [57-59].

3.6 Chlorophyll Content

The production of chlorophyll content was influenced by the application of Cd ($P \leq 0.01$). It was found that cadmium significantly affected the chlorophyll content. As cadmium increase in an ascending order 0 > 3 > 6 > 9 mg/kg, chlorophyll a, b and total (a+b) decreased steadily (Table 3). The highest total chlorophyll content was obtained when plants were not exposed to cadmium [0 mg/kg; 33.71 mg/g fresh weight) followed by 3 mg/kg (25.45 mg/g fresh weight), 6 mg/kg (20.89 mg/g fresh weight) and the lowest in 9 mg/kg (14.71 mg/g fresh weight). Previous studies have indicated that reduction on total chlorophyll production under Cd treatment was due to degradation of and interference with chloroplast production of plants and was observed in plant species such as *Triticum aestivum* [60], *Beta vulgaris* [61], and *Spinacea oleracea* [62]. It was found from the correlation (Table 2) that total chlorophyll (TC) had a significant ($P \leq 0.01$) negative linear relationship with the production of secondary metabolites. (TC vs phenolics; $r^2 = -0.790$; TC vs flavonoids = -0.804; $P \leq 0.05$). This negative relationship was explained in protein competition model (PCM) proposed by Jones and Hartley [63] that proposed the secondary metabolites production is controlled by the competition between protein and secondary metabolites production. This negative relationship is a sign of gradual switch-off of investment from protein to biosynthesis of secondary metabolites production [63]. The reduced TC content with high concentration of Cd was also observed in *O. staminues* under low light condition where reduction in TC was due to decreased chlorophyll a and b [64].

Table 5. Accumulation and partitioning of cadmium in different plant parts of *O. stamineus* under different Cd concentrations

Cadmium concentrations (mg/L)	Plant parts	Cadmium content(ppm)
0	Leaves	0.04 ± 0.001^{h}
	Stems	0.03 ± 0.001^{g}
	Roots	0.05 ± 0.001^{f}
3	Leaves	0.82 ± 0.142^{e}
	Stems	0.71 ± 0.121^{e}
	Roots	0.91 ± 0.342^{d}
6	Leaves	1.41 ± 0.213^{c}
	Stems	1.21 ± 0.071^{d}
	Roots	1.66 ± 0.052^{c}
9	Leaves	2.54 ± 0.042^{b}
	Stems	2.21 ± 0.014^{b}
	Roots	3.09 ± 0.025^{a}

All analyses are mean ± standard error of mean (SEM). N = 40. Means not sharing a common letter were significantly different at P ≤0.05.

3.7 1,1-Diphenyl-2-picryl-hydrazyl,(DPPH) Assay

DPPH is a stable free radical, which can be reduced to yellow colored (α, α-diphenyl-β-picryhydrazine) when mixed with antioxidant. This interrupts the oxidation of the free radical chain by donation of hydrogen from the hydroxyl group that not promote further oxidation of lipids [65]. Generally, DPPH antioxidant activity in *O. stamineus* was found to be the highest on the leaves, stems and lowest on roots in all treatment of cadmium. The DPPH antioxidant activity at 0 mg/kg was the highest (44.51–62.31%), followed by the 3 mg/kg (35.65–54.32%), 6 mg/kg (32.43–47.86%) and the lowest in 9 mg/kg (27.34–44.51%), However, DPPH radical scavenging abilities of the extracts of the plant were less than for those of butylated hydroxyl toluene (BHT) (69.21%) and α-tocopherol (78.22%) (Table 5). This study showed that *O. stamineus* methanolic extract demonstrated inhibiting activity against DPPH free radicals and hence could be used as a radical scavenging agent, acting possibly as the primary antioxidant. This result implies that high cadmium content significantly decreased DPPH radical scavenging activity. From the correlation analysis in Table 2, it was shown that DPPH have a significant negative relationship with cadmium (r^2= -0.821; P≤0.05) that indicate high levels of Cd have the potential to reduce the antioxidant activity of *O. stamineus.* The present result was in agreement with the findings of Sidduque et al. [66], where they found cadmium have significantly reduced DPPH activity (31.86-59.62%) in germinating seeds of *Brasica rapa*. Similar findings were also observed in *Arundo donax* [67], where high exposure to cadmium has significantly increased the DPPH IC_{50} values of the plant. It was found that DPPH was not related to the phenolics and flavonoid content (Table 2), indicating that increase in production of secondary metabolites did not contribute to the antioxidant activity of *O. stamineus* exposed to Cd.

Table 6. The effects of different Cd concentrations on total chlorophyll content in *O. stamineus*

Cadmium (mg/kg)	0 mg/kg	3 mg/kg	6 mg/kg	9 mg/kg
Chlorophyll a (mg/g fresh weight)	6.11±0.34[a]	4.83±0.34[b]	2.61±0.34[c]	1.23±0.34[d]
Chlorophyll b (mg/g fresh weight)	27.01±0.34[a]	20.84±0.34[b]	18.71±0.34[c]	13.95±0.34[d]
Total chlorophyll (mg/g fresh weight)	33.71±0.34[a]	25.45±0.34[b]	20.89±0.34[c]	14.71±0.34[d]

All analyses are mean ± standard error of mean (SEM). N = 40. Means not sharing a common letter were significantly different at P ≤0.05

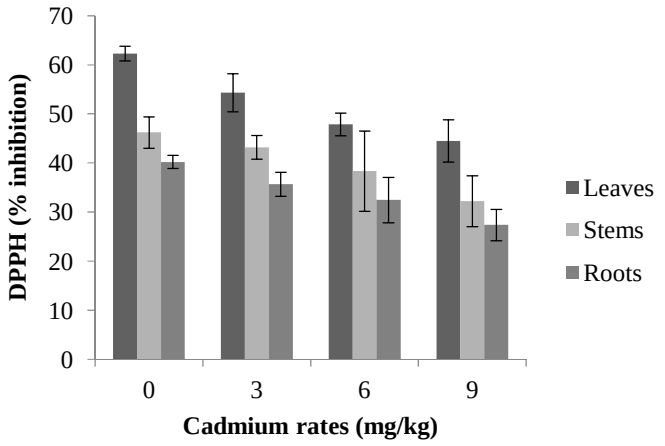

Fig. 2. Effects of Cd on DPPH scavenging activity of *O. stamineus* in different plant parts. BHT and *α*-tocopherol were used as controls and valued at 70.23 and 88.14, respectively N=40. Bars represent standard error of differences between means (SEM)

4. CONCLUSION

The results of this study suggest that Cd stress could have a major impact on the antioxidant capacity of *O. staminues*. Currently, the total phenolics and flavonoids are not responsible for the antioxidant activity in plant tissues. Total phenolics and flavonoid contents of *O. staminues* treated with cadmium were significantly higher than those of untreated plants grown under 0 mg/kg. Our results also indicated that stimulation of total phenolics and flavonoids activity in this plant was due to enhanced production of TNC and PAL activity. The study showed that *O. stamineus* exposed to cadmium accumulate cadmium above the level recommended by WHO indicate there is a safety hazard when this plant consumed for medicinal purposes. Apart from that the production of H_2O_2 and MDA was increased with increasing concentrations of Cd. The present findings indicate that cd contamination would reduce the medicinal properties of this herb.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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