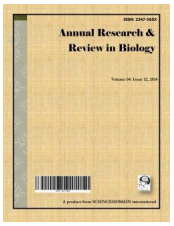

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Application Effects of Cadmium and Humic Acid on the Growth, Chlorophyll Fluorescence, Leaf Gas Exchange and Secondary Metabolites in Misai Kucing (*Orthosiphon stamineus*) Benth

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Authors' contributions

This work was carried out in collaboration between all authors. Author MHI 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 NAMZ managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This experiment was conducted to investigate and distinguish the relationships in the production of total phenolics, total flavonoids, chlorophyll content, total biomass, leaf area, leaf nitrate, proline, net photosynthesis and chlorophyll fluorescence parameters under three levels of cadmium application (0, 3,6 mg/kg) and three concentration of humic acid (HA) (0, 50, 100 mg/L) for 12 weeks in *Orthosiphon stamineus* Benth.
Study Design: Stem cuttings of *O. staminues* were propagated for two weeks in small pots and

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then transferred to pots filled with a soilless mixture of burnt rice husk and coco peat (ratio 3:1). *Orthosiphon stamineus* seedlings were exposed to three levels of Cd (0, 3 and 6 mg/kg in the form of $CdCl_2$) during media preparation and three levels of humic acid (HA) (0, 50 and 100 mg/ L). Soluble humic acid as potassium-humate (90% humic acid, 11–13% K2O) was used. This factorial experiment was organized in a randomized complete block (RCBD) design with three replications.
Place and Duration of Study: Department of Biology, Faculty of Science Universiti Putra Malaysia between August 2013 to September 2013.
Methodology: The experiment was performed for 12 weeks using 135 plants. The measurement of photosynthesis was obtained from a closed infra-red gas analyzer LICOR 6400XT Portable Photosynthesis System (IRGA, Licor Inc., USA). Total phenolics and flavonoid was determined using Follin–Ciocalteau reagent, Nitrate using Cardy nitrate meter, Chlorophyll fluorescence was measured using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech Instruments Ltd., Norwich, UK) and Proline was determined by using Glacial acetic acid and ninhydrin reagent.
Results: It was found that all parameters except chlorophyll fluorescence parameters were influenced by interaction effects between cadmium and humid acid ($P \leq 0.05$). As the concentration of cadmium increased (0 > 6 mg/kg) the production of plant total phenolics, flavonoids and proline increased but the production of total biomass, leaf area, net photosynthesis, total chlorophyll content and nitrate uptake was reduced. The application of humid acid can reduce negative effects of cadmium. As humid acid level increases from 0 to 100 mg/L the negative effects of cadmium on total biomass, leaf area, net photosynthesis and total chlorophyll were decreased.
Conclusion: This work reveals that the use of cadmium can enhance the production of secondary metabolites in *O. stamineus* (total phenolics and flavonoids). The study showed the negative effects of cadmium on plant growth, Gas exchange and chlorophyll fluorescence can be reduced by enhancing application of humic acid. The application of humic acid also was found to reduce the cadmium uptake of this herb. Present study showed that high levels of cadmium can lowered the nitrate concentration in this plant. This showed that application of cadmium and humic acid can serve to be a useful tool to enhance secondary metabolites properties of this plant.

Keywords: Medicinal plants; cadmium; humic acid; growth; biochemical; leaf gas exchange.

1. INTRODUCTION

Orthosiphon stamineus Benth. (Lamiaceae), commonly known as misai kucing, is a popular medicinal plant in Southeast Asia and is widely used for the treatment of eruptive fever, epilepsy, gallstones, hepatitis, rheumatism, hypertension, syphilis and renal calculus [1]. In Malaysia and Indonesia, *Orthosiphon stamineus* leaves are prepared in the form of infusion, popularly known as Java tea, and consumed as a beverage to improve overall health and for treatment of kidney, bladder inflammation, gout and diabetes [2]. Several classes of compounds have been identified from this plant, including flavonoids, terpenoids, saponins, hexoses, organic acids, caffeic acids derivatives, chromene and myo-inositol [3-5]. Among these compounds, the polyphenol derivatives were found to possess potential therapeutic properties as they were shown to exert diuretic and uricosuric actions in rats [3].

Flavonoids and other phenolic acids are deemed to be responsible for the wide spectrum of pharmacological activities attributed to the herb [6]. Flavonoids are polyphenolic compounds that contain a C15 flavone skeleton (diphenylpropane). They consist of flavones, flavonols, flavanols, flavanone and flavanonols, and represent the majority of plant secondary metabolites. Flavonoids are thought to play a role in protection of plants from microbial and insect attack. Moreover, flavonoids have remarkable health promoting effects, such as anti-inflammatory, anti-microbial, antioxidant, anti-cancer activity as well as the prevention of osteoporosis [7-10]. Besides flavonoids, phenolic acids including gallic acid, benzoic acids and cinnamic acids, constitute another major group of plant secondary metabolites. Nowadays, phenolic acids receive considerable attention because of their protective role against cancer and heart disease. This role may be attributed to their antioxidant activity, which was reported to be higher than vitamins C and E, against reactive oxygen species [11].

The concentration of polyphenols was considered to be affected by environmental conditions such as light intensity, heavy metals, carbon dioxide levels, temperature, fertilization,

and biotic and abiotic factors, which can alter the concentration of these active constituents [12]. Heavy metals such as cadmium well known for regulate not only plant growth and development, but also in the biosynthesis of both primary and secondary metabolites [13]. Cadmium is a toxic metal that normally occurs in low concentration in soils but can enter the environment mainly from industrial processes and phosphate fertilizers, and then is transferred to the food chain [14,15]. Cadmium is absorbed rapidly by the roots and can be loaded into the xylem for its transport into leaves. The amount of Cd accumulated in roots or translocated to leaves differs considerably among species. Most plants are prone to low Cd concentrations, which inhibit root and shoot growth, as a consequence of alterations in the photo-synthesis rate, uptake and distribution of macronutrients and micronutrients [16].

Humic acid (HA) is the fraction of naturally occurring organic materials commonly found in soils, sediments and natural waters, which derive from the decomposition of plant and animal residues [17]. Research have shown that the effects of humic acid extracted from organic waste materials, like animal manure, food waste, paper-waste vermicomposts [18-19], aerated fermentation extracts of compost [20] and natural waters [21,22] on plant growth and yield were stronger than commercial humic acid and inorganic fertilizer [23]. Humic acid is a suspension, based on potassium humates, which can be sprayed directly to the plant foliage in liquid form as a plant growth stimulator. It stimulates plant growth by acting on the mechanisms involved in cell respiration, photosynthesis, protein synthesis, water and nutrient uptake, enzyme activities [24, 25]. Many researchers concluded the enhancing effect of humic acid on growth, nutrient uptake and yield of bean plants [26-28].

It is well known, that plant exposed to cadmium would decrease their growth due to reduction in photosynthesis and enhance the production of plant secondary metabolites [14,15]. Many studies have investigated the effects of cadmium on the growth and plant primary and secondary metabolism but relatively few studies have investigated the response of cadmium and Humic acid on the medicinal value of local Malaysian herb *O. stamineus*. The application of humic acid can ameliorate the effects heavy metal especially cadmium by reducing the uptake of the metal, due to formation of Cd-HA complexes [29,30]. Some studies have shown that bioavailability to plants of heavy metals to plants is strongly reduced in the presence of HA [31,32]. Furthermore, there is inadequate information on the effect of HA and Cd on the growth, secondary metabolites and Cd uptake on local Malaysian herbs. Therefore the objective of this study was to examine the effects of different cadmium and humic acid on the growth, biochemical (total phenolics, flavonoids, proline, nitrate) and leaf gas exchange in *O. stamineus.*

2. MATERIALS AND METHODS

2.1 Plant Materials and Maintenance

The experiment was conducted in glasshouses at the Faculty of Science, Universiti Putra Malaysia (longitude 101°44'N and latitude 2°58'S, 68 m above sea level) with a mean atmospheric pressure of 1.013 kPa. Stem cuttings of *O. staminues* collected from MARDI serdang (MOS 2 variety) were propagated for two weeks in small pots and then transferred to pots filled with a soilless mixture of burnt rice husk and coco peat (ratio 3:1). *Orthosiphon stamineus* seedlings were exposed to three levels of Cd (0, 3 and 6 mg/kg in the form of $CdCl_2$) during media preparation and three levels of humic acid (HA) (0, 50 and 100 mg/ L). Soluble humic acid as potassium-humate (90% humic acid, 11–13% K_2O) was used. Different levels of HA were applied as irrigation water that were applied every time of watering. Pots were irrigated with tap water every two weeks to wash the media. This factorial experiment was conducted in a randomized complete block (RCBD) design with three replications. Each experimental unit consisted of five seedlings, and there were 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 Flavonoid Quantification

The method of extraction and quantification for total phenolics and flavonoids contents followed after Jaafar et al. [33]. An amount of ground tissue sample (0.1 g) was extracted with 80% ethanol (10 mL) on an orbital shaker for 120 minutes at 50°C. The mixture was subsequently filtered (Whatman™ No.1), and the filtrate was used for the quantification of total phenolics and total flavonoids. Folin – Ciocalteu reagent (diluted 10-fold) was used to determine the total phenolics content of the leaf samples. Two

hundred µl of the sample extract was mixed with Follin–Ciocalteau reagent (1.5 mL) and allowed to stand at 22°C for 5 minutes before adding NaNO3solution (1.5 mL, 60 g L^{-1}). After two hours at 22°C, absorbance was measured at 725 nm. The results were expressed as mg/g gallic acid equivalent (mg GAE/ g dry sample; r^2= 0.934).For total flavonoids determination, sample (1 mL) was mixed with NaNO3 (0.3 mL) in a test tube covered with aluminium foil, and left for 5 minutes. Then 10% $AlCl_3$ (0.3 mL) was added followed by addition of 1 M NaOH (2 mL) and the absorbance was measured at 510 nm using rutin as a standard (mg rutin/ g dry sample; r^2 = 0.956).

2.3 Chlorophyll Content

Total chlorophyll content was measured by the method from Idso et al. [34] using fresh weight basis. Prior to each destructive harvest, each seedling was analyzed for the leaf chlorophyll relative reading (SPAD meter 502, Minolta Inc, USA). The leaves of O. stamineus with different greenness (yellow, light green and dark green) were selected for analysis and total leaf chlorophyll content was analyzed. For each type of leaf greenness, the relative SPAD value was recorded (five points/leaf) and the same leaves sampled for chlorophyll content determination. Leaf disk 3 mm in diameter was obtained from leaf sample using a hole puncher. For each seedling the measurement was conducted on the youngest fully expanded leaves on each plant, generally the second or third leaf from the tip of the stem was used. The leaf disks were immediately immersed in acetone (20 mL) in an aluminum foil-covered glass bottle for approximately 24 hours at 0°C until all the green colour had bleached out. Finally, the solution (3.5 mL) was transferred to measure at absorbances of 664 and 647 nm using a spectrometer (UV-3101P, Labomed Inc, USA). After that the least squares regression was utilized to develop predictive relation between SPAD meter readings and pigment concentrations (mg / g fresh weight) obtained from the chlorophyll destructive analysis.

2.4 Photosynthesis Rates

The measurement was obtained from a closed infra-red gas analyzer LICOR 6400 Portable Photosynthesis System (IRGA, Licor Inc., USA). Prior to use, the instrument was warmed for 30 minutes and calibrated with the ZERO IRGA mode. Two steps are required in the calibration process: first, the initial zeroing process for the built-in flow meter; and second, zeroing process for the infra-red gas analyzer. The measurements used optimal conditions set by Ibrahim and Jaafar et al. [35] of 400 µmol/mol CO_2 30°C cuvette temperature, 60% relative humidity with air flow rate set at 500 cm^3 min^{-1}, and modified cuvette condition of 800 µmol/m/s photosynthetically photon flux density (PPFD). Measurements of gas exchange were carried out between 09:00 to 11:00 a.m. using fully expanded young leaves to record net photosynthesis rate (A). The operation was automatic and the data were stored in the LI-6400 console and analyzed by "Photosyn Assistant "software (Version 3, Lincoln Inc., USA). Several precautions were taken to avoid errors during measurements. Leaf surfaces were cleaned and dried using tissue paper before enclosed in the leaf cuvette.

2.5 Plant Total Biomass and Leaf Area

Total plant biomass was taken by calculating the dry weight of root, stem and leaf per seedling. Plant parts were separated and placed in paper bags and oven dried at 80 °C until constant weight was reached before dry weights were recorded using electronic weighing scale (Mettler-Toledo Model B303-S, Switzerland). Leaf area per plant was measured using a leaf area meter (LI-3100, Lincoln Inc, USA). The leaves were arranged within the field of view, and overlapping of adjacent leaves was avoided.

2.6 Nitrate Determination

Fresh leaf samples were collected and kept in a refrigerator prior to analysis. The fresh leaves were cut to small pieces and squeezed in a stainless steel press to obtain the sap. Sap was then used to measure nitrate concentration using a Horiba® Cardy Twin Nitrate Meter (Model B-343; Horiba Scientific Inc., Trenton, NJ, USA).

2.7 Determination of Proline Concentration

Samples (from the fourth leaf which was still growing) were collected (between 7 and 8 am) from the five plants of each treatment. An extraction procedure and colorimetric determination with acidic ninhydrin reagent, (2.5 g ninhydrin /100 mLof a solution containing glacial acetic acid, distilled water and ortho-phosphoric acid 85%, at a ratio of 6:3:1) were carried out. After the addition of a small amount

of quartz sand and 10 mL of a 3%(w/v) aqueous sulfosalicylic acid solution, the five leaves collected from each replicate were bulked and 1 g of fresh samples were ground in a mortar. The homogenate was filtered through two layers of glass-fiber filter, and the clear filtrate was then used in the assay. Glacial acetic acid and ninhydrin reagent (1 mL each) were added to 1 mL of the filtrate. The closed test tubes with the reaction mixture, were kept in a boiling water bath for 1 hour. The reaction was terminated in a water bath at 21°C for 5 minutes. Readings were taken immediately at a wavelength of 546 nm. The proline concentration was determined from a standard curve and was calculated on a fresh weight basis (µmol /proline /g fresh weight; r^2= 0.914).

2.8 Leaf Cadmium Analysis

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 Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence were taken from fully expanded second leaves. Leaves were darkened for 15 min by attaching light-exclusion clips to the central region of the leaf surface. Chlorophyll fluorescence was measured using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech Instruments Ltd., Norwich, UK). Measurements were taken at >3,000 µmol/m2/s and recorded for up to 5 seconds. The fluorescence responses were induced by emitting diodes. Measurement of f_o (initial fluorescence), f_m (maximum fluorescence) and f_v (variable fluorescence) were obtained from this procedure. fv is derived as the difference between f_m and f_o. Fluorescence values recorded, include the initial/minimal fluorescence (f_o), the ratio of variable to maximum fluorescence (f_v/f_m) which represents the maximum quantum yield of photosystem II (PS II), and the ratio of variable to minimum fluorescence (f_v/f_o) which estimates the maximum primary yield of photochemistry of PS II. The fm is the maximal fluorescence value, and fv is the variable fluorescence calculated as $f_m - f_o$ [35].

2.10 Statistical Analysis

Data was analysed using the analysis of variance procedure in SAS version 17. Means separation test between treatments was performed using Duncan multiple range test and the 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 Plant Biomass

The interaction effect between cadmium and HA had a significant ($p \leq 0.05$) impact on the production of total biomass in *O. stamineus* (Table 1). As the plant received more HA (0 to 100 mg/L) the production of total biomass was enhanced. The decrease in the production of total plant biomass was considered to be statistically higher in 6 mg/kg than 3 mg/kg cadmium. Lower total biomass with enhanced application of cadmium showed that application of more than 3 mg/kg cadmium has induced toxicity to the plant [33,36]. Usually, the reduction in biomass would be found in plant under high levels of cadmium [37,38]. The enhancement of total biomass was optimized when *O. staminues* was exposed to high HA (100 mg/L) compared to 50 mg/L and the control. The present result indicated the production of total biomass can be increased by application of HA under cadmium application, thus showed that HA can ameliorate cadmium adverse effect on *O. stamineus*. The enhancement of plant biomass with HA application under cadmium application has been observed by Maryam et al. [39] on lettuce. They came to a similar conclusion as in the present study, where they observed the imposition of HA had enhanced production of lettuce fresh biomass under cadmium application (2 -4 mg/L) by 13-27%. Previous studies have indicated that, application of HA can ameliorate plant stress on tobacco, corn and oat [40-42]. This suggests a potential role of humic acid in reducing the negative impact of cadmium application on *O. stamineus.*

3.2 Total Leaf Area

The interaction of HA and Cd on the plant leaf area of *O. stamineus* was significant ($P \leq 0.05$; Fig. 1) and the effect of HA on leaf area was dependent on the Cd concentration. Without any HA added, plant leaf area decrease as added Cd concentration increased. The application of 50 and 100 mg/L HA increased plant leaf area and negated the effect of added Cd. The increase in plant leaf area with increasing level of humic acid in the present study might be due to the formation of Cd-HA complex might down regulate

the uptake of cadmium in the present study thus increase plant growth that is justified by the increase in plant leaf area. In line with this result, Asik et al. [43] showed that treating plants with HA progressively under salt stress has increased wheat plant growth. Previous studies found HA increased shoot dry matter yield and length of durum wheat roots under environmental stress [44]. It was found also that application of HA had a positive effect on growth and yield of *Pisum Sativum* [45]. The present result indicated that application of humic acid can mitigate the negative effects of cadmium on plant growth especially leaf area in *O. stamineus*. Humic acid (HA) is a growth stimulator compound that also decreases plant uptake of Cd probably due to formation of Cd-HA complexes that reduce the uptake of Cd ion [29,30]. Previous studies showed HA can stimulate shoot and root growth and improve plants' resistance to environmental stresses especially heavy metal stress [46]. The result indicated that reduction in leaf area by cadmium can be reduced by addition of HA to *O. stamineus*

3.3 Total Chlorophyll Content and Net Photosynthesis

Leaf chlorophyll content and net photosynthesis was influenced by interaction between humic acid and cadmium content ($p \leq 0.05$; Table 2 and 3). In general, total chlorophyll content decreased as cadmium content increased from 3 to 6 mg/kg. The application of humic acid was found to enhance the production of total chlorophyll as the HA concentration increased from 50 to 100 mg/kg. The highest production of total chlorophyll was found in 0 mg/kg cadmium + 50 mg/L HA and the lowest total chlorophyll content was observed in 6 mg/kg cadmium + 100 mg/L HA. The reduction of total chlorophyll content with increasing cadmium content has been observed by Sheoran et al. [45]. They observed chlorophyll chlorosis was 26% higher in pigeon pea compared to the control (without cadmium exposure). The positive effects of HA on leaf total chlorophyll content are functions of the cadmium levels. The highest leaf total chlorophyll was obtained with application of 50 and 100 mg/L exposed to 3 mg/kg Cd. This result was in agreement with Nardi et al. [46] where they observed the application of humic acid has increased the total chlorophyll and net photosynthesis in the plant samples. From the present study, it was observed that cadmium significantly reduced the net photosynthesis of *O. stamineus.* As cadmium content increased from 0 to 6 mg/kg, net photosynthesis was statistically reduced in *O. staminues*. In the present study, the application of HA was found to ameliorate the cadmium effects in net photosynthesis. As HA application increased from 50 to 100 mg/L net photosynthesis was steadily increased under 3 and 6 mg/L cadmium application. Previous studies by Lakshaman and Surinder [47] indicated that reduction in photosynthesis of plant under cadmium exposure was due to degradation of chlorophyll and disturbance of plant photochemical and Calvin cycle enzyme. According to Siedlecka et al. [48], cadmium disrupt photosynthesis by two key enzymes of CO_2 fixation: ribulose-1,5-bisphosphate carboxylase (RuBPCase) and phosphoenolpyruvate carboxylase (PEPCase). Cadmium ions lower the activity of RuBPCase and damage its structure by substituting for Mg ions, which are essential cofactors of carboxylation reactions and also Cd can shift RuBPCase activity towards oxygenation reactions. Stiborova [49] and Malik et al. [50] demonstrated that Cd caused an irreversible dissociation of the large and small subunits of RuBPCase, thus leading to total inhibition of the enzyme thus reduce the photosynthesis of plants. This showed, that reduction in total chlorophyll content and photosynthesis can be reduced by application of HA in plant under cadmium toxicity.

Table 1. The effects of humic acid and cadmium addition on plant total dry weight (g) of *O. staminues* after 20 days of application. N= 15. Means followed by the same letters are not significantly different by DNMRT test ($p \leq 0.05$)

Humic acid concentrations (mg/L)	Cadmium concentration (mg/kg)		
	0	3	6
0	7.35 ± 0.23^{b}	6.67 ± 0.31^{c}	5.45 ± 0.14^{d}
50	8.09 ± 0.12^{a}	6.89 ± 0.03^{c}	4.35 ± 0.17^{e}
100	8.24 ± 0.08^{a}	6.76 ± 0.27^{b}	4.12 ± 0.22^{e}

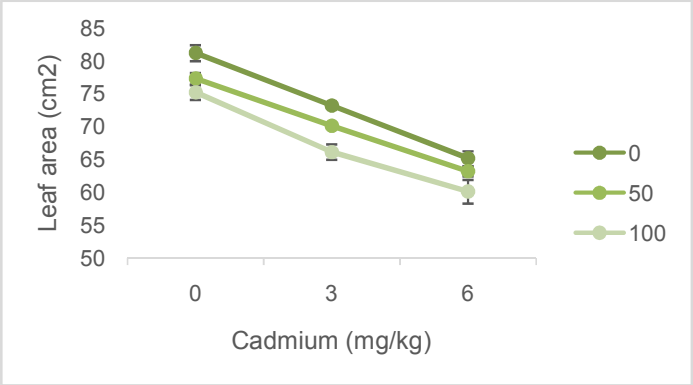

Fig. 1. Impact of cadmium and humic acid on total leaf area of *O. stamineus*. N= 15. Bars respresent standard error of differences between means (SEM)

Table 2. The effects of humic acid and cadmium addition on the leaf chlorophyll concentration of *O. staminues* after 20 days of application. N= 15. Means followed by the same letters are not significantly different by DNMRT test (p ≤0.05)

Humic acid concentrations (mg/L)	Cadmium concentration (mg/kg)		
	0	3	6
0	37.23± 2.11^{a}	32.32±0.23^{c}	28.26±2.17^{e}
50	38.14±1.67^{a}	34.21±1.32^{c}	30.12±3.18^{d}
100	37.24±1.88^{a}	36.12±3.21^{b}	29.32±0.25^{f}

Table 3. The effects of humic acid and cadmium addition on the net photosynthesis of *O. staminues* (µmol/m2/s) after 20 days of application. N= 15. Means followed by the same letters are not significantly different by DNMRT test (p ≤0.05)

Humic acid concentrations (mg/L)	Cadmium concentration (mg/kg)		
	0	3	6
0	10.04± 0.23^{b}	8.24±1.21^{d}	6.35±1.42^{f}
50	10.24± 1.21^{b}	8.79±0.32^{d}	6.47±1.67^{f}
100	11.23±0.67^{a}	9.23±1.31^{c}	7.21±0.54^{e}

3.4 Chlorophyll Fluorescence

It was observed that minimal fluorescence (fo) value was highest in 6 mg/L cadmium (556.45), followed by 3 mg/kg (512.34) and 0 mg/kg (444.12; Table 4). This showed that cadmium can increase minimal fluorescence (f_o) values in *O. stamineus*. The f_o is the primary chlorophyll fluorescence yield, that measures the stability of the light harvesting complex [51]. The increase in f_o in the present study showed the disruption of the photosynthetic apparatus. Previous report s have shown that the increase in f_o might be attributed to abiotic stress [52]. The f_v/f_o is an indication of maximum quantum yield of photochemical and non-photochemical processes in photosystem II and correlates with leaf photosynthetic capacity. The reduction in f_v/f_o ratio as the cadmium level increase might be caused by disruption of photosynthesis in the donor part of photosystem 1 and II. Normally, the f_v/f_o value is in the range of 4–6. However, the range can be very different in different plants adapting to different environments [35]. The maximum quantum yield of photosystem II (f_v/f_m) also showed the same trends with f_v/f_o ratio. This indicates that f_v/f_o values correlate with f_v/f_m ratio. The f_v/f_m represent the maximum quantum yield of photosystem II, which is correlated with the quantum yield of photosynthesis. It is usually employed as an indicator of the photo inhibitor or other injury caused to the photosystem II complex [53]. In the present study, the f_v/f_m values were less than 0.81 in 3 mg/kg and 6 mg/kg cadmium. This suggests that the total amount of light energy transformed in the photosystem II reaction centre was decreased and this plant was stressed under these

Table 4. Impact of cadmium on cholorophyll fluorescence parameters of *O. stamineus*. N= 40. Means followed by the same letters are not significantly different by DNMRT test (p ≤0.05)

Cadmium Treatment (mg/kg)	Minimal fluorescence (fo)	Maximum quantum yield of photochemical and non photochemical (fv/fo)	Maximum quantum yield of photosystem II (fv/fm)
0	444.12± 22.12^{c}	3.71±0.12^{a}	0.81±0.01^{a}
3	512.34±12.32^{b}	3.01±0.08^{b}	0.75±0.04^{b}
6	556.45±11.21^{a}	2.71±0.26^{c}	0.69±0.07^{c}

conditions. This showed that reduction in photochemical activity of photosystem II can contribute to the limitation to photosynthesis under cadmium conditions [51]. It can be concluded that from the drop in f_v/f_o and f_v/f_m and the increase in f_o value, the start of cadmium stress can possibly damage the photosynthetic apparatus and lead to disturbances in the photosynthetic process [54]. This have been supported by Sigfridsson et al [55] where they showed cadmium can disrupt photosystem II in plants by replacing the Ca^{2+} in Ca/Mn clusters constituting the oxygen-evolving centres or by modify the quinone (Q_b) binding site thus reduced the f_v/f_o and f_v/f_m value. The current result showed that chlorophyll fluorescence can be used to indicate plant tolerance to cadmium toxicity.

3.5 Total Phenolics and Flavonoids

Plant antioxidants are thought to play a role in protection against a variety of diseases and to delay ageing processes. The health promoting effect of antioxidants from plants could be attributed by their protective effects by counteracting reactive oxygen species (ROS) [56]. There are several compounds which contribute to the antioxidative properties these include phenolics and flavonoids compounds. Production of plant total phenolics and flavonoids is an indication of production of plant secondary metabolites. In the present study, total phenolics and flavonoids content was found to be influenced by the interaction effects between cadmium and humic acid (p ≤0.01; Tables 5;6). It was observed that, 6 mg/kg cd+ 100 mg/L HA, 6 mg/kg cd+ 50 mg/L HA, 6 mg/kg cd+ 0 mg/L HA, 3 mg/kg cd+ 100 mg/L HA, 3 mg/kg cd+ 50 mg/L HA, 3 mg/kg cd+ 0 mg/L HA, 0 mg/kg cd+ 100 mg/L HA, 0 mg/kg cd+ 50 mg/L HA produces more total phenolics and flavonoids than the plant under 0 mg/L cd+ 0 mg/L HA, which produces only 2.04 mg/g Gallic Acid and 1.23 mg/g rutin. Previous studies have showed that the imposition of cadmium have increased the production of plant total phenolics and flavonoids in Brassica rapa, sour fig and yellow morel [57-58]. There was no report before on the synergistic effect of cadmium and humic acid on the production of total phenolics and flavonoids. The current study indicated that the combination of cadmium and humic acid can increase the medicinal properties of *O. stamineus* by increasing the production of total phenolics and flavonoids content.

3.6 Leaf Nitrate Content

The leaf nitrate content was influenced by the interaction between humic acid and cadmium concentration (P≤0.01; Fig. 2). Generally as cadmium content increased from 0 to 6 mg/kg the nitrate content in the leaves was reduced in all humic acid concentrations (0, 50 and 100 mg/L). The lowest nitrate content was observed in 6 mg/kg cd + 100 mg/L HA (87.32 ppm) and highest in 0 mg/kg cd+ 0 mg/L (control) that recorded 152.23 ppm. The high cadmium level might disrupt the leaf nitrate uptake and lowered the nitrate content in the leaves. The reduction in nitrate content with increase in cadmium content was also observed by Irfan et al. [59] and Arslan et al. [60] in Brasica juncea and Verbascum olympicum respectively. Nitrate has been attributed to the negative effects on human health. Toxicity of nitrate to human can be manifested by headaches, syncope, vertigo and discoloration that manifest in fingers or lips [61]. The results suggest that, high level of cadmium can minimize the nitrate content in plants. The nitrate levels that acceptable to human are 10–100 ppm in drinking water. Intake more than 100 ppm would induce the toxic effects [62]. In the current result, the nitrate value was higher than acceptable recommended levels (>100 ppm). However, most vegetables and fruits contained nitrate levels from 20–200 ppm [63]. Usually, processing of food (e.g., chopping, grinding and heating) would reduce the nitrate content and thus reduce the negative effects [64]. This current result suggests, that the application of cadmium can reduce the nitrate content in *O. stamineus*.

3.7 Leaf Cadmium Content

Fig. 3 shows, that the cadmium content was increased with increasing cadmium application. It was observed that application of humic acid significantly reduced the cadmium content compared from the control without application of humic acid, however there was no statistical significant difference between application of 50 and 100 mg/L HA under 3 and 6 mg/kg cadmium. The increase in cadmium levels in leaf tissue with increased cadmium application indicated that *O. staminues* were a heavy metal tolerant plant. The present result was also observed by Maryam et al. [39] in lettuce where application of humic acid decreased the uptake of cadmium by 37-41% in lettuce. These results are in agreement with those of Bunluesin et al.[65] and Spehar et al [66] that showed Cd uptake and its hazardous symptoms were reduced by addition of HA. Formation of HA-Cd complexes that are less available for plant uptake in comparison with free Cd 2+ is another possible reason for reducing Cd accumulation by the leaves in the presence of HA. It has been reported that HA reduced the availability of heavy metals (HM) in soil and their uptake by plants through the formation of relatively insoluble complexes of HM-HA [67]. Reduced Cd accumulation in lettuce leaves by addition of HA may be linked to a significant increase in plant growth and enhanced leaf biomass (dilution effect) [66]. This showed that, application of HA can reduce the cadmium uptake in plant exposed to high level of cadmium.

3.8 Leaf Proline Content

Fig. 4 gives the leaf proline content in *O. stamineus* under different cadmium and humic acid application. The leaf proline content was significantly higher as cadmium content increased from 3 to 6 mg/kg. At the end of the experiment, leaf proline content in 6 mg/L treatment is 46% higher than the average leaf proline for 0 and 3 mg/L. The high level of proline content in 6 mg/L clearly indicated that *O. stamineus* under high cadmium content have high oxidative stress. The role of proline as an antioxidant was reported in tobacco (*Nicotiana tabacum* L.) cells exposed to Cd stress. Islam et al. [68] reported that tobacco cells exposed to Cd treatment accumulated high levels of proline and by this way they can alleviate the inhibitory effect of Cd on cell growth. The present study was in agreement with Kastori et al. [69] and Zhang et al. [70] that found high accumulation of proline in cadmium treated sunflower and mung bean. This increase may be attributable to a direct induction of proline synthesis [68] or as an enzymatic mechanism to protect plants against water stress induced by Cd [69]. Cadmium inhibits the photoactivation of photosystem II by inhibiting electron transfer. Thus, Cd could lead to the generation of ROS indirectly by production of a disturbance in the chloroplasts. Kastori et al. [69] observed proline accumulation in plant tissues in the presence of Cd. They proposed that this increase was linked with the role of proline in water balance maintenance. Proline mediates the alleviation of water deficit and thereby could

Table 5. The effects of humic acid and cadmium addition on the total phenolics (mg/g Gae) content of *O. staminues* after 20 days of application. N= 15. Means followed by the same letters are not significantly different by DNMRT test (p ≤0.05)

Humic acid concentrations (mg/L)	Cadmium concentration (mg/kg)		
	0	3	6
0	2.04±0.56^{c}	6.24±0.02^{b}	8.34±0.14^{a}
50	2.23±0.72^{c}	6.45±0.72^{b}	8.21±0.24^{a}
100	2.34±0.08^{c}	6.87±0.28^{b}	8.87±0.21^{a}

Table 6. The effects of humic acid and cadmium addition on total flavonoids contents (mg/g rutin dry weight) of *O. staminues* after 20 days of application. N= 15. Means followed by the same letters are not significantly different by DNMRT test (p ≤0.05)

Humic acid concentrations (mg/L)	Cadmium concentration (mg/kg)		
	0	3	6
0	1.23±0.21^{d}	3.34±0.07^{c}	5.43±0.12^{b}
50	1.34±0.16^{d}	3.76±0.03^{c}	6.12±0.24^{a}
100	1.42±0.13^{d}	3.87±0.16^{c}	6.34±0.15^{a}

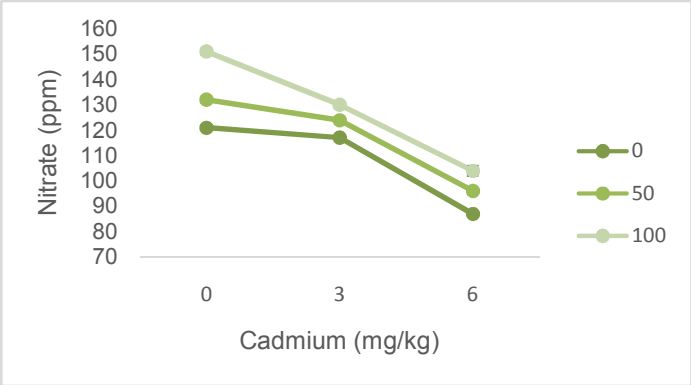

Fig. 2. Impact of cadmium and humic acid on nitrate content of *O. stamineus*. N= 15. Bars respresent standard error of differences between means (SEM)

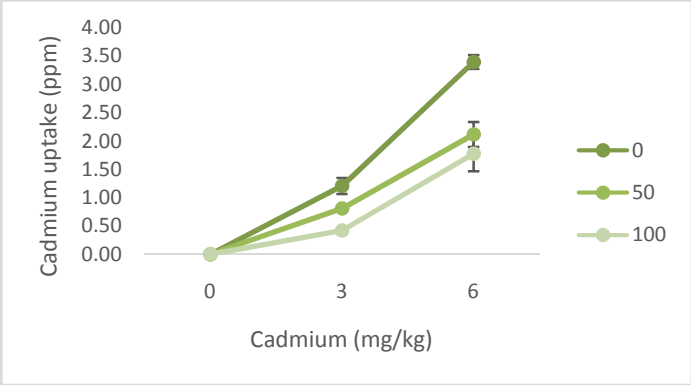

Fig. 3. Impact of cadmium and humic acid on cadmium content of *O. stamineus*. N= 15. Bars respresent standard error of differences between means (SEM)

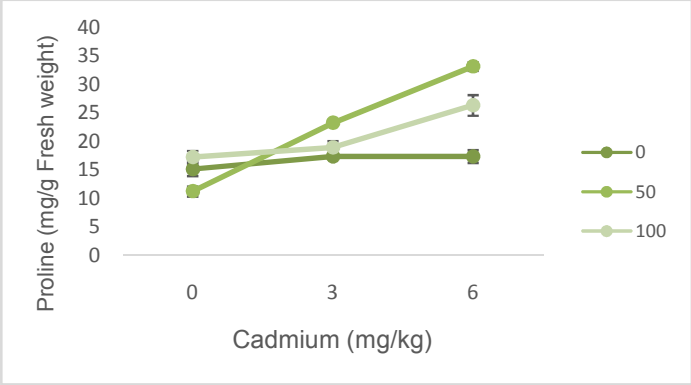

Fig. 4. Impact of cadmium and humic acid on proline content of *O. stamineus*. N= 15. Bars respresent standard error of differences between means (SEM)

substantially enhance tolerance of plants to Cd toxicity. Accumulation of large amounts of osmolytes (proline) is an adaptive response in plants exposed to a stressful environment. Proline accumulation seemed to be a suitable indicator of heavy metal stress [71-73].

4. CONCLUSIONS

This work reveals that the use of cadmium can enhance the production of secondary metabolites in *O. stamineus* (total phenolics and flavonoids). The study showed the negative effects of cadmium on plant growth, Gas exchange and chlorophyll fluorecence can be reduced by enhancing application of humic acid. The application of humic acid also was found to reduce the cadmium uptake of this herb. Present study showed that high levels of cadmium can lowered the nitrate concentration in this plant. This showed that application of humic acid can be a useful tool to enhance secondary metabolites and reduce the negative impact of cadmium on this medicinal plant. The result obtained from this study has laid an important platform that indicates humic acid application can ameliorate the negative effects of cadmium on medicinal plant in *O. stamineus*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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