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Watermilfoil *Myriophyllum spicatum* extract attenuates cadmium toxicity in the kidney of *Bufo regularis*

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ABSTRACT

Pollution becomes one of the uppermost environmental concerns worldwide, especially chemical pollution, which affects the environment, public health of man and threaten other species life. Chemical pollution is frequently a consequence of daily human activities beside manufacturing and disposing of chemicals. *Bufo regularis* are an example of amphibians in its way to extinction because of chemical pollution.

The existing work designed to find a naturally produced substance for protection of *Bufo regularis* against the chemical pollution. The antioxidant activity of the methanolic extract of *Myriophyllum spicatum* (MEM) was assessed *in vitro* using 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay. The value of inhibitory concentration 50 (IC_{50}) for DPPH radical scavenging was 28.77 ±1.58 µg/mL and the total antioxidant activity of MEM was 191.17±7.1 mg AA equivalent/g extract. For *in vivo* studies, 40 adult male toads were divided into 4 groups. Levels of malondialdehyde (MDA), proinflammatory cytokines and iNOS showed significantly higher expression in the kidney homogenates of Cd group.

These inflammatory alterations accompanied with marked tissue damage in the kidney of Cd-group and moderate improvement of kidney architecture up on administration of the MEM extract as demonstrated by light and electron photomicrographs. In conclusion, current data confirmed that the *Myriophyllum spicatum* extract has a potent antioxidant activity against renal toxicity induced by Cd in *B. regularis*.

INTRODUCTION

Amphibians are among the most modern habitants vulnerables for the risk of extinction globally as previously reported (Sparling et al. 2000). Their life qualities depend on cleanness and refinement of their living environment considering that the terrestrial and aquatic environments (Kucken et al. 1994). Physical alteration of the environment alongside with the chemical substances, particularly with heavy metals are the foremost roots which can describe the deterioration in their different species globally (Collins and Storfer 2003, Collins 2010). Electronic waste (EW) contains different toxic elements (substances), amid them cadmium (Cd), a heavy metal heavily consumed in rechargeable batteries for computers and mobile phones and many other devices. Cd aggregates everywhere in the water, air, and soil.

It can remain with its harmful effects for the local habitants for about 10–30 years (Gervais 2011). Cadmium is obtainable for adult amphibians through numerous

routes whether their skin, digestive and respiratory systems. Different hematological parameters changes in *Bufo regularis* and *Bufo raddei* have been reported (Hilmy et al. 1986, Zhang et al. 2007).

Cadmium is mainly built-up in the liver and kidney, which results in different histopathological aberrations, nevertheless, it is important to note that the observable effects are not mutual toward the entire amphibians, so the effects may due to interspecific or intraspecific effects (Birge et al. 2000, Capaldo et al. 2016). Cadmium affects all living organisms anywhere it can found, its toxicity induces severe nephrotoxicity (Savolainen 1995) and mainly deposited in the kidneys of fishes, amphibians and mammals (Farkhunda *et al.* 2016, Medina et al. 2016, Gil-Manrique et al. 2017). In addition, several biochemical and histological changes such as glycogen, lipids, lactate and proteins were observed in toads when treated with Cd (Loumbourdis and Vogiatzis 2002, Wu et al. 2017). Moreover, Cd toxicity in mammals includes inflammatory degenerative responses in the liver, kidney, lungs along with severe pathological conditions and immune system dysfunctions (Farkhunda et al. 2016).

The aquatic plants are an important habitat providing substrate, primary food source and shelter for birds, toads, fish and invertebrates (Martin and Murray 2011, Watling et al. 2011, Rogalski and Skelly 2012). *Myriophyllum spicatum* (Eurasian watermilfoil) is a known European, Asian, and North African invasive submerged perennial macrohydrophyte, which is found naturally in the shallow water bodies where become in a close proximity to amphibians. *Myriophyllum spicatum* is endowed by synthesis of a variety of phytochemical compounds, which often known by the "secondary metabolites" that include several classes such as phenols, flavonoids, alkaloids, coumarins, glycosides and other compounds. Phenolic compounds could reach up to 10% of the dry weight of the *Myriophyllum* plant (Hogstrand and Haux 1991, Sivaci et al. 2008). Flavonoids and phenolic acids are the most compounds that have antioxidant activity in the plants, specifically phenolic acids have been repeatedly implicated as natural antioxidants in fruits, vegetables, and other plants (Pyo et al. 2003). The natural phenol antioxidants (was) produced by *Myriophyllum spicatum* reduce oxidative stress and lessen the resultant cellular damage (Vattem and Shetty 2005). Therefore, the present study is an attempt to gain novel insights on the mechanism that induce cytoprotective effects of *Myriophyllum spicatum* against Cd induced renal toxicity in *Bufo regularis*.

We assessed several parameters as paradigms to evaluate the health quality of such amphibians. Histochemical and ultra-structural studies were carried out to show up the effects on tissue integrity. Furthermore, biochemical and molecular studies were done to whether confirm or explain the relation between the overall health quality and tissue integrity.

MATERIALS AND METHODS

Plant collection and extraction

Myriophyllum spicatum plant was collected from the River Nile at the area of Aswan city. The plant was identified according to (Täckholm 1974). Samples washed and dried at ambient temperature. Sixty gm of pulverized samples was flooded overnight in 100% methanol. Next day, methanol dried out at 40°C under vacuum pressure. Crude extract was dissolved in distilled water that used for further experimental procedures. Voucher specimen of the plant was deposited at Herbarium of Botany Department, Faculty of Science, Aswan University under number 11816.

Plant extract analysis

Total phenolic and flavonoids concentrations were determined using the Folin–Ciocalteu reagent and $AlCl_3$ using colorimetric method, respectively (Singleton et al. 1999, Zhishen et al. 1999). While total saponins was assessed using 1 mL of plant extract (10 mg/mL) mixed with 2 mL of vanillin reagent. Subsequently, samples heated at 60°C for 1 hour and chilled out for 10 min and the absorbance was measured at 473 nm. The saponins content in samples was calculated as previously described (Ebrahimzadeh and Niknam 1998).

Condensed tannins were assessed using vanillin according to Sun & colleagues, the amount is expressed as mg tannin equivalent/g extract (Sun et al. 1998). Moreover, Free radical scavenging activity was evaluated spectrophotometrically by *in vitro* DPPH (1,1- diphenyl, 2-picryl hydrazyl) assay at 516 nm and the IC_{50} was calculated (Anil and Suresh 2011).

Assessment of the oral LC50

To identify the Lethal Concentration 50 of MEM, a constant volume of extract (250 µl/g body weight) was used to determine the dose that kills 50% of the tested groups. The animals were clinically observed for up to 96 hours (Gulec et al. 2013).

Animals and Experimental design

Forty adult male toads (*Bufo regularis*) weighing 60-65 gm were collected from Aswan Governorate, Egypt. Animals were placed in plastic cages (120x65x60 cm) with a perforated cover at 25°C. Experimental groups arbitrarily separated into four groups, 10 animals each. All groups were treated orally for 21 day:

Group I: 250 µL of dechlorinated tap water as a negative control (**NC**)

Group II: 250 µL of 0.1g w/v MEM as1/20 of LC_{50} (**MC)**

Group III: 250 µL of Cd at 0.62 mg/kg BW represents 1/10 of LC_{50} (**CdC**)

Group IV: 250 µL of MEM and Cd (Cd MEM)

Twenty-four hours after the last dosage, animals were handled in accordance with the code of ethics for Aswan University. Cadmium dose was (6.2 mg/kg), it is based on previous study reported that dose cause changes in blood profile accompanied with alterations in the reproductive system of *Bufo regularis* (Hilmy et al. 1986).

Histology and Ultra-structural studies

Kidney samples were fixed in 10% formalin, embedded in paraffin, tissues were sectioned into 5µm thick and stained with HE (Bleyl 1976).

For the electron microscopic study, the kidney tissues were processed and cutted in ~1 mm^3 thick and fixed in 2.5% glutaraldehyde for 2 hr. Tissues samples were rinsed with PBS and then fixed in 1.5% osmium tetroxide, dehydrated and implanted in *epon 812* and the sections were stained with uranyl acetate continued by lead citrate. Images acquisitions were examined by Jeol 100Cx II TEM (Jeol, MA, USA) at the Electron microscopy Unit, Assiut University, Egypt.

Lipid peroxidation assay (MDA) and QRT-PCR analysis

Lipid peroxidation was measured in the kidneys of all groups and the collected data were expressed as nanomoles of MDA/mg tissue as instructed by the manufacturer.

Expression of pro-inflammatory genes, TNF-α, IL-1β and IL-10, GCSF-R, iNOS in renal tissues examined using reverse transcriptase-PCR (RT-PCR). Total RNA was isolated using *Trizol*®. Five micrograms of isolated RNA were converted into cDNA using commercial kit as instructed. PCR primers (Invitrogen, USA) used for all analyzed genes were displayed in Table 1. Expression of genes was normalized to 16S rRNA. Genes were.

Table 1: Primers used to quantitate real time PCR.

Primers	Primer direction and/or sequence
16S rRNA	Forward 5'-AGGTCAAGGTGCAGCAAATG- 3'
	Reverse 5' - TGCTAAATCCGCCTTCCAAC - 3'
IL-10	Forward 5' - TGCTGGATCTTAAGCACACCCTGA - 3'
	Reverse 5' - TGTACAGGCCTTGTTCACGCATCT - 3'
IL-1β	Forward 5' - CATTCCCATGGAGGGCTACA - 3'
	Reverse 5' - TGACTGCCACTGAGCAGCAT - 3'
TNF-α	Forward 5' - TGTCAGGCAGGAAAGAAGCA - 3'
	Reverse 5' - CAGCAGAGCAAAGAGGATGGT- 3'
iNOS	Forward 5' - AACCGTAAGCCAAAGAAGGA - 3'
	Reverse 5' - TGGTTCTGGCAGCCACAGT - 3'

Statistical analysis

All data were measured in triplicate and presented as mean ± standard error. Statistical differences were analyzed using two-way ANOVA followed by the *Tukey's post hoc test*. Statistical significance was accepted at $P < 0.05$.

RESULTS

Myriophyllum spicatum is highly rich in antioxidant secondary metabolites

The whole plant extract revealed a rich content of flavonoids of 391.66±2.46 mg QE/g extract and saponins of 89.50±2.29 mg saponin/g extract. The smallest value of 17.40±1.11 mg TE/g extract was evaluated for tannins as shown in Table 2.

Table 2: Major secondary metabolites quantified in the methanolic crude extract of whole plant of *Myriophyllum spicatum*

Major secondary metabolite	concentration
Total Phenols (mg GAE/g extract)	31.4 ± 2.01
Total Flavonoids (mg QE/g extract)	391.7 ± 2.5
Saponins (mg saponin/g extract)	89.5 ± 2.3
Tannins (mg TE/g extract)	17.4 ± 1.11

***** GAE= Gallic acid equivalent; QE= Quercetin equivalent; TE= tannin equivalent

Moreover, *in vitro* free radical scavenging activity assay exhibited an increase in the extract concentration until a certain point, after which, a plateau was maintained (Fig. 1). By omitting the plateau points and drawing the regression line, the values of IC_{50} were calculated from the regression equation. The smallest IC_{50} value indicated the highest potency of antioxidant properties of the sample was 28.77±1.58 µg/mL in correlation to that of ascorbic acid of a value approximately 5.78 ±0.65 µg/mL. In conclusion, plant extract showed a high content of antioxidant secondary metabolites.

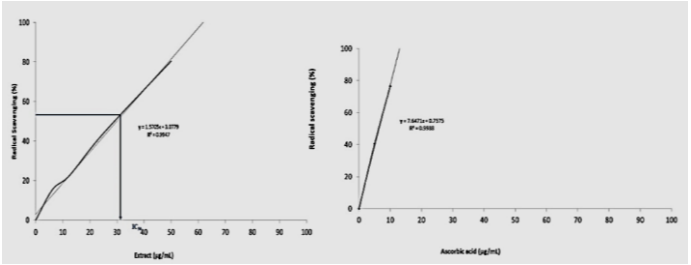

Fig. 1. Radical scavenging activity. The radical scavenging of methanolic crude extract of *M. spicatum* (a) compared to that of Ascorbic acid (b).

Effect of *Myriophyllum spicatum* extract on histology of the Kidney

Histology of the normal kidney and MEM treated groups were shown as a complex mass of nephric units among which lie blood vessels and capillaries. The nephric unit consists of a renal corpuscle (Bowman's capsule and glomerulus) and the renal tubules (Fig. 2A and B). The kidney sections of the Cd group revealed severe deleterious renal damage represented by shrinkage and glomeruli atrophy with a wide glomerular interspaces. In addition, signs of renal degeneration appeared in the most of renal tissue with leucocytes infiltrations and necrosis (Fig. 2C). Matching with Cd group, the kidney sections of Cd MEM group revealed a remarkable reorganization of the renal parenchyma with apparent recovery of inflammation as evidenced by the noticeable reduction in infiltration of inflammatory cells. A noticeable regeneration of the cell lining of the renal corpuscles and tubules was observed. Nevertheless, few glomeruli underwent some symptoms of injury (Fig. 2D).

Deep insights about structural changes revealed with electron microscopy imaging which revealed the normal ultra-structure of the renal cells in the NC group. Renal tubule showed a number of lining epithelial cells with indistinct cell boundaries, each cell containing a basally located oval nucleus. In addition, the basal part of cells shows infoldings of the basal membrane, dividing the basal part into compartments, containing large perpendicular mitochondria (basal striations) as shown in Figure 3A and B. In contrary, the Cd group displayed noticeable structural degeneration. There were marked alterations of the fine structures of the renal tubule lining cells, which acquired irregular shapes, collapse of nuclei and mitochondrial degeneration together with the presence of degenerative vacuoles. In addition, short infoldings of the basal membrane as well as the lateral cellular interdigitations appeared in abnormal shape with nearly complete loss of organelles (Fig. 3C). Again, kidneys of Cd MEM group showed a significant structural reorganization and improvement of the structural deformation retaining the normal structure of the kidneys to a broad extent (Fig. 3D).

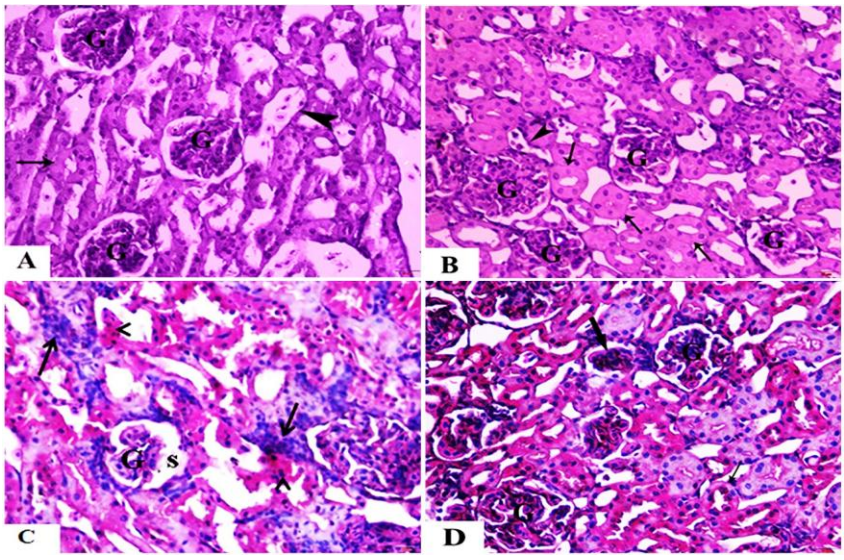

Fig. 2: Light micrographs of the kidney sections of the control and treated groups. Normal histology of the kidney in control group and MEM treated group with normal glomeruli (G) and renal tubules (arrows) depicted in (**A and B**). Clear glomerulus atrophy (G) forming large space (S), cellular necrosis of renal tubules (arrowhead) while arrows refer to inflammatory cell infiltration (**C**). Nearly normal appearance of glomerulus (G) and renal tubules (thin arrows) with few necrotic signs in the glomeruli (thick arrow) and renal tubules (*) shown in **D**. Scale bar = 50µm; sections were processed equally in time and washing using standard HE stain.

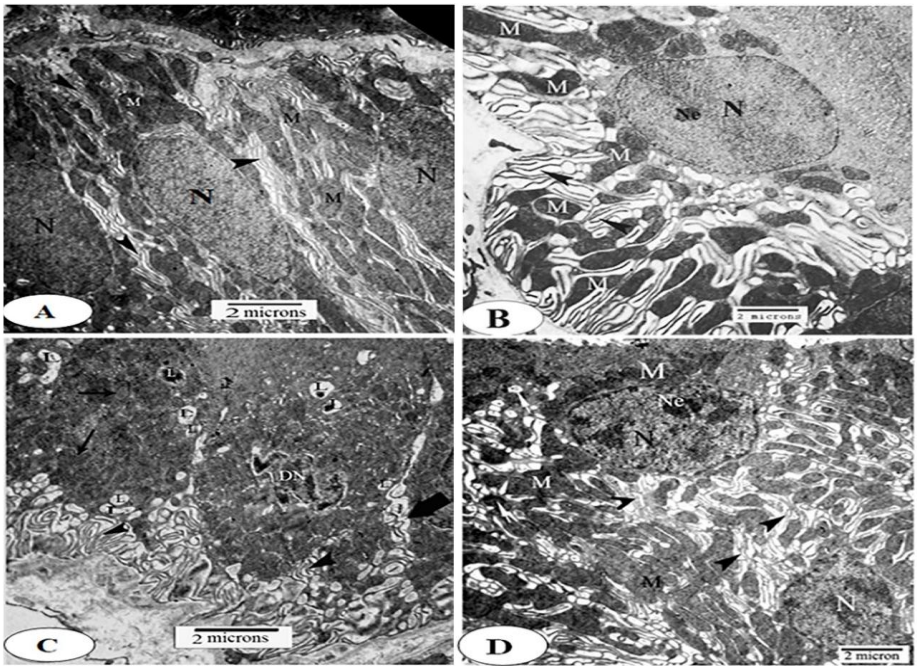

Fig. 3. Electron micrographs of the kidney of *B regularis*: Renal tubule with its lining of epithelial cells, each containing a basally located rounded nucleus (N) and mitochondria (M) located mainly in the compartments found between infoldings of the basal membrane (arrow head) in control and MEM treated groups **A** and **B** respectively. Renal tubule cells with abnormal shape of nuclei (DN) and mitochondria (arrow) beside the presence of lysosomes (L). Clear damage appears in the infoldings of the basal membrane (arrowhead) as well as the lateral cellular interdigitations (thick arrow) is depicted in (**C**). Recovery of renal tubule approximately to its normal shape with nuclei (N), mitochondria (arrow), lysosomes (arrows) and infoldings of the basal membrane (arrow head) as shown in (**D**). (Original magnification: 5800X=2 µm).

Effect of *Myriophyllum spicatum* extract on lipid peroxidation

Oxidative stress is the most common destructive agent in toxic metals induced cellular damage. Its destructive ability comes from lipid peroxidation and release of free radicals, in this context; concentrations of MDA in all groups were assessed. Levels of MDA in the CdMEM and MEM groups showed no difference in comparison to the recorded basal levels of MDA in the control group ($P>0.05$). While, MDA level was increased 20% in the Cd group when compared to ND and MEM group ($P<0.01$). Treatment of Cd group with MEM extract showed approximately 17% decrease in the level of MDA which almost reestablished to the basal state as in ND group ($P>0.05$) as shown in Figure 4.

***Myriophyllum spicatum* reduces gene expression of proinflammatory cytokines**

Tumor necrosis factor- alpha (TNFα) induced apoptosis in response to reactive oxygen species via activation of NF-kB, which may be induced by Cd (Ju Kim et al. 2002, Zhang et al. 2016). We examined the effects of Cd on TNF-α gene expression signaling in *Bufo regularis*. Analysis of qRT-PCR data revealed 1.4-fold increase of TNF-α in the Cd group when compared to ND group ($P < 0.0001$). This increment in Cd group was declined 65% by treatment with the plant extract (Cd MEM), while

TNF-α in the MEM group remained unaffected in comparison to ND (Fig. 5a). These results denoted that Cd activated TNF-α in the kidney of frogs.

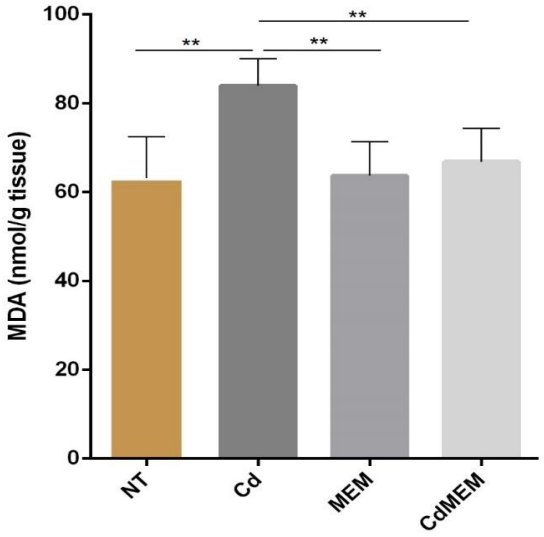

Fig. 4: Levels of lipid peroxidation (MDA) in the kidney homogenates. Levels of MDA were expressed as Mean ± SE differ, ***P* <0.001.

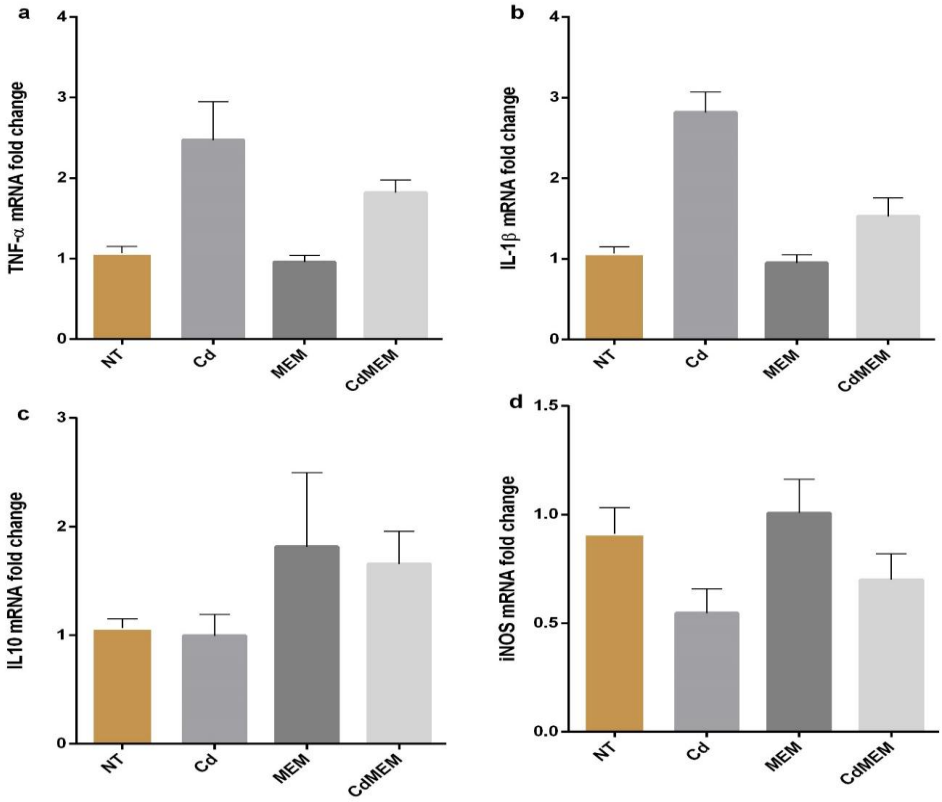

Fig. 5. Profile of gene expression of cytokines and iNOS. Relative gene expression of TNF-α (a); IL-1β (B); IL-10 (C) and iNOS (D) in the kidney. Data were represented as mean ± SD.

In order to get deep insights about the immunomodulatory effects of Cd, the gene expression of proinflammatory (IL-1*β* besides TNF-*α*) and anti-inflammatory cytokines (IL-10) in response to Cd administration were quantified using the qRT-PCR. The results revealed that in case of IL-1β, animals treated with cadmium and

MEM extract (Cd MEM) showed a significant reduction approximately 1.3 fold compared to Cd treated group ($P<0.0001$), while IL-1β gene expression level was increased 1.7 fold in case of Cd group in comparison to ND group ($P<0.0001$) as shown in Figure 5b. Level of the anti-inflammatory cytokine IL-10 was comparable between Cd and ND group ($P>0.05$), while was higher about 0.6 and 0.8 compared to Cd group in case of Cd MEM and MEM respectively (Fig. 5c).

In contrast, NO production was found to be reduced by approximately 37% in Cd group in comparison to ND group ($P\leq0.003$); while, Cd treated MEM group did not show any ameliorative effects comparable to Cd group ($P>0.05$) as shown in figure 5d.

DISCUSSION

Anthropogenic activities directly influence the environment and the ecosystem, resulting in risk for extinction of some modern species. An important activity is the chemical pollution, which is hypothesized as the most liable hazard factor. Particularly, Amphibians are exposed to higher levels of such activities, which are discharged into the aquatic and terrestrial environments in different forms. Heavy metals including cadmium (Cd) have a very long decomposition lifetime. They affect all habitants and may cause behavioral, developmental, physiological, reproductive and histopathological alterations and also mortality (James and Little 2003). The mechanism by which Cd causes toxicity is far from being completely understood. However, lipid peroxidation has long been considered to be the primary mechanism for Cd toxicity (Jamall and Crispin Smith 1985, Manca et al. 1991, Dickinson et al. 2002).

Cadmium continuously accumulates in vertebrates, particularly in freshwater organisms, even at low molecular concentrations, damaging tissue and cause organ dysfunction (Burger 2008). Kidney is the excretory organ and is the primary organ to exhibit signs of toxicity (Vogiatzis and Loumbourdis 1998, Burger 2008). Current data showed that administration of Cd induced morphopathological changes in the kidneys and severe deleterious renal damage including proximal tubular cells damage accompanied with an increase in the glomerular cellularity and a degeneration of the renal tubules along with mononuclear infiltration. Moreover, the present study indicated that the Cd group showed epithelial cell deformations in the renal tubules and progressive glomerular and nephropathy because of cadmium exposure. These observations are in harmony with the findings of experimental studies on the Cd treated amphibians which includes pathological changes in the renal tubules, tubular necrosis, and decrease cellularity of the epithelial cells of the renal tubule, karyolysis and karyorrhexis (Capaldo et al. 2016).

In our study, the histopathological alterations were correlated with a substantial increase in MDA levels in the kidney tissue compared to control, indicating high lipid peroxidation and ROS hyperactivity in the kidney, which provoke a massive damage in that organ. Concomitantly, these data confirm ROS as the primary mechanism(s) behind heavy metal induced kidney toxicity. Our results are in consensus with others who showed that exposure of *R. arenarum* to Cd can cause biochemical and histological alterations in both liver and kidney (Leonard et al. 2004, Medina et al. 2016).

Furthermore, we examined the effects of Cd toxicity on the proinflammatory (IL-1β; TNF-α) and the anti-inflammatory cytokines (IL-10) alongside with the cytotoxic killing molecules iNOS gene expression in *Bufo regularis.* Our data

revealed activation of proinflammatory cytokines, inhibition of anti-inflammatory cytokines and decreases of iNOS in Cd group compared to control, indicating a strong inflammatory response in the kidney tissue by Cd treatment. In addition, Inhibition of iNOS may be due to the high capacity of toxic molecules that intend to be removed by iNOS. Particularly, iNOS data are controversy for previous published data which mentioned that Cd toxicity increased levels of NOS isoenzymes in the renal tissues (Soyupek et al. 2012).

Potential antioxidative, anti-inflammatory and pharmacological properties of several plants and herbs had been reported (Sewani-Rusike and Marykutty 2014). The present study demonstrated that the flavonoid-rich extract of *Myriophyllum spicatum* have potent ROS scavenging activity as shown by the ameliorative effects on the tissue integrity, reduction of MDA levels and alteration of proinflammatory, anti-inflammatory cytokines and the cytotoxic scavenger molecules as detailed in the results section above. The present results confirm the plant *in vitro* scavenging studies. Interestingly, treatment with MEM extract reversed the cytotoxic effects in the kidney and reestablished levels of MDA to their approximate basal levels. Moreover, it inversed the cytokine changes to realize the immune homeostasis, it is also applicable for the iNOS expression. These results confirm that phenolic compounds and flavonoids have the ability to scavenge free radicals, and protect cells from the damages due to the oxidative stress (Urquiaga and Leighton 2000, Sivaci et al. 2008, Wang et al. 2012, Lou et al. 2014). We found that MEM extract has the ability to protect *B. regularis* against Cd toxicity, which may has the potent effects in man. Thus, this study showed a potential effects of MEM extract as a novel chemo-protective substitute in pathologies associated with excessive ROS production.

CONCLUSION

In conclusion, pollution is a global concern due to its high frequency, particularly heavy metal toxification. Cadmium is a highly toxic metal causing massive organ damage in water habitants. Peculiarly amphibians, which are the most, jeopardize species for the risk of extinction. Watermill foil is a worldwide-distributed aquatic plant is a cheap and an effective source for elimination of cadmium toxicity. Watermilfoil extract can be used as an antioxidative agent to improve the life standards of aquatic amphibians.

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ARABIC SUMMARY

مستخلص النبات المائي *Watermilfoil* يقلل من سمية الكادميوم في كلى الضفادع المصرية

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أصبح التلوث واحداً من أهم المخاوف البيئية في جميع أنحاء العالم، وخاصة التلوث الكيميائي الذي يؤثر على البيئة والصحة العامة للإنسان ويهدد حياة الأنواع الأخرى. كما أن التلوث الكيميائي في كثير من الأحيان يكون نتيجة للأنشطة البشرية اليومية بجانب تصنيع والتخلص من المواد الكيميائية. الضفدع المصري هي مثال على البرمائيات التي في طريقها إلى الانقراض نتيجة للتلوث الكيميائي.

لقد تم تصميم الدراسة الحالية لإيجاد مادة طبيعية لحماية الضفدع المصري ضد التلوث الكيميائي. وعليه تم تقييم النشاط المضاد للأكسدة من المستخلص الميثانولي للنبات المائي *Myriophyllum spicatum* (MEM) باستخدام ثنائي فينيل هيدرات الهيدرازيل (DPPH). كانت قيمة التركيز التثبيطي (IC50) هي ٢٨.٧٧ ± ١.٥٨ ميكروغرام / مل، وكان مجموع النشاط المضاد للأكسدة 7.1 ± 191.17 ملغ مكافئ g /حمض الاسكوربيك.

لدراسة التأثير على الضفادع، تم تقسيم التجربة الى أربعة مجموعات من الضفادع الذكور كالتالي (المجموعة الضابطة، المجموعة المغتذية على خلاصة MEM، المجموعة المغتذية على الكادميوم Cd والمجموعة المغتذية على الكادميوم Cd بالإضافة إلى (MEM).

ولقد أظهرت الدراسة أن مستويات مرتفعة من مشتقات أكسدة الدهون مستويات Malondialdehyde (MDA)، السيتوكينات الالتهابية وحمض النتريك سينثاز في كلى المجموعة المغتذية على Cd فقط وهذه التغيرات الالتهابية كانت مصحوبة بتلف ملحوظ في الأنسجة الكلوية. ولقد لوحظ تحسن معتدل في بنية الكلى عند إعطاء مستخلص MEM كما هو موضح بواسطة القطاعات المأخوذة من المجهر الضوئي والإلكتروني. في الختام، تؤكدت البيانات الحالية أن الخلاصة المستخرجة من النبات المائي *Myriophyllum spicatum* لديها نشاط قوي كمضاد للأكسدة ضد السمية الناجمة عن الكادميوم في كليات الضفدع المصري.