Hexavalent chromium toxicity induced biochemical perturbation in Tilapia nilotica: role of Phoenix

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Abstract. The current study was designed to investigate the protective role of Phoenix dactylifera (date palm) water extract (DPE) against oxidative injury induced by different concentrations of hexavalent chromium (CrVI) in liver and muscles of Tilapia nilotica (Oreochromis niloticus) fish in vitro. Results demonstrated that CrVI caused a significant concentration-dependent inhibition in glutathione S-transferase, superoxide dismutase and catalase activities as well as glutathione content. In addition, inhibition in transaminases and alkaline phosphatase activities were observed. While, thiobarbituric acid reactive substances levels in liver and muscles homogenate were increased. On the other hand, homogenates treated with DPE alone improve the antioxidant status and liver function biomarkers. Moreover, homogenate pretreated with DPE then exposed to CrVI showed marked modulation in lipid peroxidation, enzyme activities and protein content as compared to their respective CrVI treated ones. Conclusively, Phoenix dactylifera might have a potential protective role against CrVI toxicity.

1. Introduction:
Aquatic ecosystems have become continually polluted with toxic effluents derived from different sources. Industrial and agricultural discharges are the main sources of heavy metals in water bodies that are easily taken into the food chain. One of these heavy metals is hexavalent chromium, which is widely used in painting, electroplating, dyes and various other things. The discharge of chromium-containing solutions may lead to water and soil pollution [1]. Also, chromium enters the food chain and presents a potential threat to human's health through oxidative stress. Oxidative injury primarily occurs through production of oxygen and nitrogen radicals and can damage cellular components leading to loss of enzyme activities and various diseases [2]. Excess concentrations of chromium hexavalent compounds induced carcinogenic, cytotoxic, immunotoxic, neurotoxic and genotoxic effects as well as general environmental toxicity [2]. Moreover, Cr compounds cause DNA single-strand breaks and DNA–protein crosslinks as
well as oxidative damage in male reproductive system [3, 4,5]. Tilapia nilotica, Oreochromis niloticus (L.) is popular freshwater fish in Egypt and worldwide due to its high growth rate, easily adaptation, and high tolerance against diseases and environmental stressors [6].

Considering the adverse effects of heavy metals, most of the world’s population is looking for natural remedies, which are safe and effective. Phoenix dactylifera or date palm is a member of Arecaceae family that have been suggested to have several benefits in protecting against several diseases such as cancer, diabetes, blood pressure, ulcer, diarrhea as well as acting as antioxidant, anti-inflammatory, antiproliferative and antimutagenic. Also, it can be used to cure several pathogens [7]. The antioxidant effects of date fruits are attributed to their components such as coumaric and ferulic acids [8]. Moreover, date fruits are rich in flavonoids, sterols, procyanidins, carotenoids, anthocyanins as well as vitamins and minerals [9]. So, this study was aimed at evaluating the toxic effects of chromium and the possible protective role of Phoenix dactylifera on oxidative stress, antioxidant defense system, liver function biomarkers and protein content in Tilapia nilotica fish in vitro.

2. Materials and methods

Potassium dichromate (K2Cr2O7) was obtained from Sigma-Aldrich Chemical Co., St. Louis, U.S.A. All other reagents used were of analytical grade. Fresh dates were collected from the local market. Fruit flesh was extracted two times with distilled water (1/10 w/v) by grinding with a mortar and pestle then centrifuged at 4000 g for 20 min at 4 °C and the supernatant was taken. Aqueous extract of date was chosen according to the research of [9] because most of their antioxidant components are extracted in water.

Fish Tilapia nilotica (Orechromis niloticus), used in this study were captured from the Nozha Hydrodrome farm (fish farm), Alexandria, Egypt. Ten fish samples (130- 150 mm length and 120-140 g weight) were obtained monthly between May-July 2017. The fish samples were placed on ice and transported to the laboratory within an hour. Fish were anesthetized with 0.02% benzocaine solution, dissected and liver and muscles were removed and washed using chilled saline solution and homogenized in 0.1 M phosphate buffer, pH 8 in homogenizer (10% w/v). Homogenates were centrifuged at 10000 g for 30 min at 4°C to remove cell debris. The supernatants were used for estimation of enzymatic and non-enzymatic antioxidant, liver function enzymes and protein contents. Also, hexavalent chromium was measured in different environmental compartments using inductively coupled plasma optical emission spectrometry (ICP-OES) with LOD=3 ppb and CRM N/A, according to the method described in [10] and [11].

The in vitro inhibitory effects of different concentrations of chromium (VI) was tested alone and in combination with Phoenix dactylifera water extract (date palm extract; DPE) as antioxidant on fish liver and muscles homogenates. Chromium was prepared in deionized water and preincubated with the homogenates for 4 hours at 37oC. The experiment was designed as follows: fish liver and muscles homogenate used as control which is left without any treatment, homogenate incubated with 400µg/ml of Phoenix dactylifera extract (DPE) alone at 37oC for 30 min, homogenates were treated with different concentrations (30, 60, 90, and 120 µM) of hexavalent chromium at 37oC for 4hr and finally, homogenates were pretreated with 400µg/ml of DPE at 37oC for 30 min then exposed to the same different concentrations (30, 60, 90, and 120 µM) of hexavalent chromium at 37oC for 4hr [12; 13].

Thiobarbituric acid-reactive substances (TBARS) were determined in fish liver and muscles homogenates using the method of [14]. Reduced glutathione (GSH) content was measured according to [15]. Superoxide dismutase activity (SOD; EC 1.15.1.1) was examined by [16]. Catalase (CAT; EC 1.11.1.6) activity was distinguished by the technique of. [17]. Glutathione S-transferase (GST; EC 2.5.1.18) activity was estimated utilizing para-nitrobenzyl chloride as a substrate [18]. Aspartate transaminase (AST; EC 2.6.1.1) and alanine transaminase (ALT; EC 2.6.1.2) activities were determined
with kits from SENTINEL CH. (MILAN ITALY). Alkaline phosphatase (ALP; EC 3.1.3.1) activity was determined following the method of [19] and finally, protein content was tested by Lowry et al. (1951). Calibrated UV/VIS spectrophotometer and ICP were used.

Data were analyzed according to [20]. Statistical significance of the difference in values of control and treated samples was calculated by (F) test at 5% significance level. Data of the present study were statistically analyzed by using Duncan’s Multiple Range Test [21].

3. Results and discussion:
Chromium has diverse applications in different industrial processes and inadequate treatment of the industrial effluents leads to water contamination. Concentrations of Cr(VI) in Nozha Hydrodrome farm were 0.049, 0.004, 0.105 and 0.072 ppm in water, fish, plants and sediment, respectively. These values represent the means of 5 samples. It is known that chromium induced its toxicity through oxidative stress in fish [22] because of its capability to enter the oxidation reduction reaction [23, 24]. Lipid peroxidation (LPO) directly decompose double bond of unsaturated fatty acids leading to destruction of membrane structure [25], and loss of membrane functionality or react with the cellular components leading to inactivation of protein and formation of DNA adducts [26]. A significant concentration dependent induction in TBARS levels in the liver and muscles of Tilapia nilotica fish (Table 1 and 2) is in accordance with several previous studies [22, 27, 28]. Reduced glutathione is a powerful antioxidant that acts as enzymes cofactor [29]. Therefore, it is an active player in the metabolism of reactive oxygen species (ROS) and have been previously used to characterize redox processes in fish under treatment with chromium ions [24]. Glutathione is converted into oxidized glutathione which is considered as a marker of oxidation by xenobiotics [30]. The observed decline in GSH concentration fish liver and muscles exposed to different concentrations of CrVI in vitro as compared to control (Table 1 and 2) is in agreement with other studies [31]. The effect of metals on glutathione may be related to the high affinity of metals for this molecule where a sulfhydryl, an amino and two carboxylic acid groups, as well as two peptide linkages, represent reactive sites for metals.
Table 1. In vitro effect of date palm extract (DPE) and different concentrations of hexavalent chromium (Cr) alone and in combination on TBARS, GSH and some antioxidant enzymes in liver of Tilapia nilotica fish.

| Groups      | Parameters | Parameters | Parameters | Parameters | Parameters |
|-------------|------------|------------|------------|------------|------------|
|             |            | TBARS (nmol/g ww) | GSH (µg/g tissue) | SOD (U/mg protein) | CAT (nmol/min/mg protein) | GST (µmol/min/mg protein) |
| Cont.       | 68.52±2.94f | 91.27±4.31b | 10±0.42b | 69.08±3.40b | 16.75±0.74b |
| DPE         | 52.86±2.19g | 103±2.91a  | 11.29±0.41a | 82.84±2.16a | 18.90±0.60a |
| Cr1 (30 µM) | 85.34±3.31cd | 78.56±3.52cde | 8.30±0.23de | 57.74±2.94de | 14.03±0.62cde |
| Cr2 (60 µM) | 90.70±3.70bc | 71.39±3.17e | 7.68±0.26e | 51.07±2.44e | 12.94±0.52ef |
| Cr3 (90 µM) | 98.60±4.36ab | 60.97±2.51f | 6.68±0.25f | 42.94±2.09f | 11.73±0.46f |
| Cr4 (120 µM) | 106±3.78a | 52.19±2.33f | 6.00±0.17f | 38.07±2.06f | 9.60±0.25g |
| DPE+Cr1     | 71.27±2.97ef | 87.82±2.82bc | 9.49±0.32bc | 67.02±3.18bc | 15.73±0.72bc |
| DPE+Cr2     | 77.45±2.87def | 82.54±3.53bcd | 9.02±0.29cd | 60.07±2.39cd | 14.91±0.44cd |
| DPE+Cr3     | 81.13±3.53cde | 76.07±3.28de | 8.52±0.30de | 56.09±2.53de | 14.01±0.56cde |
| DPE+Cr4     | 88.20±3.91c | 70.76±3.17e | 7.92±0.25e | 54.55±2.71de | 13.27±0.15def |

*Values are expressed as means±SE; n=5 for each treatment group; means in each column with different superscript letters are significantly different (p<0.05). DPE, Cr1, Cr2, Cr3 and Cr4 groups are compared to control group while DPE+Cr1, DPE+Cr2, DPE+Cr3 and DPE+Cr4 groups are compared to their respective groups (Cr1, Cr2, Cr3 and Cr4) respectively. TBARS; thiobarbituric acid reactive substances, GSH; reduced glutathione, SOD; superoxide dismutase, CAT; catalase and GST, glutathione S-transferase.
Table 2. In vitro effect of date palm extract (DPE) and different concentrations of hexavalent chromium (Cr) alone and in combination on TBARS, GSH and some antioxidant enzymes in muscles of Tilapia nilotica fish

| Groups       | Parameters | TBARS (nmol/g ww) | GSH (µg/g tissue) | SOD (U/mg protein) | CAT (µmol/min/ mg protein) | GST (µmol/min/mg protein) |
|--------------|------------|-------------------|-------------------|-------------------|----------------------------|---------------------------|
| Cont.        |            | 12.92±0.57e       | 96.79±4.06b       | 10.43±0.46b       | 169±7.43b                  | 16.04±0.60b               |
| DPE          |            | 9.08±0.24f        | 118.83±4.88a      | 12.72±0.30a       | 200±8.55a                  | 20.63±0.53a               |
| Cr1(30 µM)   |            | 15.22±0.61bcd     | 76.73±1.18de      | 9.21±0.40cde      | 141±6.07cde                | 13.36±0.42cde             |
| Cr2 (60 µM)  |            | 16.42±0.52b       | 67.17±2.66f       | 8.25±0.34ef       | 135±5.98de                 | 12.06±0.50ef              |
| Cr3 (90 µM)  |            | 17.96±0.32a       | 58.30±1.88g       | 7.33±0.29fg       | 117±5.09fg                 | 10.50±0.46g               |
| Cr4 (120 µM) |            | 18.87±0.78a       | 47.50±1.62h       | 6.47±0.25g        | 103±0.86g                  | 8.25±0.36h                |
| DPE+Cr1      |            | 13.81±0.25de      | 86.00±2.05c       | 9.85±0.20bc       | 157±5.10bc                 | 14.62±0.47c               |
| DPE+Cr2      |            | 14.51±0.52cde     | 81.21±1.29cd      | 9.45±0.35bcd      | 149±5.28cd                 | 14.02±0.44cd              |
| DPE+Cr3      |            | 15.33±0.51bcd     | 75.65±1.37de      | 8.63±0.38de       | 136±4.09de                 | 12.64±0.42de              |
| DPE+Cr4      |            | 16.16±0.72bc      | 69.67±1.80ef      | 8.32±0.24ef       | 128±3.43ef                 | 11.66±0.50fg              |

*Values are expressed as means±SE; n=5 for each treatment group; means in each column with different superscript letters are significantly different (p<0.05)

Antioxidant enzyme activities measured in fish liver and muscles homogenates exposed to different concentrations of CrVI showed a significant reduction in concentration dependent manner (Table 1 and 2). SOD is a metalloenzyme, has essential role in the defense mechanism against free radicals by converting superoxide anions into hydrogen peroxide, which is consequently detoxified by both GPx and CAT enzymes so, when CAT activity is inhibited, more H2O2 is available for production of hydroxide radical leading to lipid peroxidation enhancement [29]. The reduction of antioxidant enzyme activities could explained the increased level of hydroperoxide in addition to the binding of metal to the enzyme active sites [32]. In accordance, fish antioxidant enzymes are influenced by heavy metals [33, 34]. A variety of electrophilic compounds were detoxified by GST producing more hydrophilic and less toxic molecules through the action of GSH resulting in reduction of lipid peroxidation [35, 36]. In consistence, GST inhibition could be attributed to enzyme denaturation or penetration of xenobiotic to the cell membrane causing lipid peroxidation [27].

The observed perturbations in AST, ALT and ALP activities (Table 3) are in congruence with our previous study [37] so, the leakage of enzymes outside the cell indicates cellular damage and membrane instability. Additionally, reduction in protein content is confirmed by the observed inhibition of ALT and AST and indicates excessive protein damage due to general stress response [38].
Table 3. In vitro effect of date palm extract (DPE) and different concentrations of hexavalent chromium alone and in combination on liver and muscles enzyme activities and protein content.

| Groups          | Parameters | Liver          | Muscles         |
|-----------------|------------|----------------|-----------------|
|                 | AST (nmol/g ww) | ALT (nmol/g ww) | ALP (U/mg protein) | Protein (mg/g ww) | ALP (nmol/min/mg protein) | Protein (mg/g ww) |
| Cont.           | 59.22±1.67b   | 44.61±1.73b    | 13.04±0.62b     | 32.84±1.46b      | 18.92±0.82b               | 37.80±1.68b      |
| DPE             | 67.59±2.19a   | 49.57±2.02a    | 14.78±0.44a     | 38.47±0.64a      | 23.86±1.00a               | 44.53±2.00a      |
| Cr1 (30 µM)     | 49.09±1.85def | 36.60±1.34cd   | 10.33±0.34cd    | 26.22±0.43cd     | 14.08±0.61de              | 31.25±1.27cd     |
| Cr2 (60 µM)     | 44.70±1.02f   | 33.76±1.05de   | 8.94±0.39ef     | 22.30±0.86e      | 12.57±0.53ef              | 28.15±1.25cd     |
| Cr3 (90 µM)     | 40.00±1.38g   | 29.0±1.25f     | 8.13±0.26fg     | 17.06±0.70f      | 10.67±0.47fg              | 21.24±0.90e      |
| Cr4 (120 µM)    | 36.59±0.93g   | 22.24±0.39g    | 7.05±0.24g      | 14.5±0.56f       | 9.83±0.44g                | 17.52±0.77f      |
| DPE+Cr1         | 55.22±1.14bc  | 45.65±1.37ab   | 11.42±0.38c     | 31.02±0.83c      | 17.11±0.74bc              | 35.17±1.03b      |
| DPE+Cr2         | 52.88±1.01cd  | 40.12±1.71c    | 10.82±0.47cd    | 29.99±1.36c      | 15.74±0.43cd              | 31.66±1.17c      |
| DPE+Cr3         | 50.59±1.59de  | 34.72±1.21de   | 10.28±0.35cd    | 26.39±1.07de     | 14.11±0.64de              | 27.60±0.83d      |
| DPE+Cr4         | 47.55±1.82ef  | 31.95±1.16ef   | 9.74±0.31de     | 24.00±0.83e      | 11.64±0.54fg              | 23.28±0.72e      |

*Values are expressed as means±SE; n=5 for each treatment group; means in each column with different superscript letters are significantly different (p<0.05)

Phoenix dactylifera fruits are rich in phenolic compounds, flavonoids and antioxidants [9]. Treatment with DPE alone caused a significant improvement in antioxidant status. On the other hand, the presence of DPE with Cr(VI) treated homogenates decreased TBARS and this attributed to their various polyphenolic compounds that play an important role as free radicals scavenger, metal chelator and stimulator for the enzymes involved in lipid peroxidation prevention [39, 40]. Similarly, curcumin [5], rosemary [1] and propolis [41] found to have protective role against xenobiotics toxicity. Antioxidant enzymes protect the cellular membranes against the deleterious effects of ROS and their induction is related to free radicals modulation by DPE. This is because of the antioxidant and chelating features of Phoenix dactylifera that help in scavenging radicals [43, 44] and consequently reduce Cr toxicity. Also, elevation in GSH content detoxifies ROS and protects cellular proteins against oxidation so, natural antioxidants are highly effective in alleviating the toxic effect of xenobiotics.

4. Conclusion
The hexavalent chromium had cytotoxic effects on liver and muscles of Tilapia nilotica fish. It has the capability to induce liver damage, oxidative stress and alterations in antioxidant defense system, its effect was concentration dependent. Besides, Phoenix dactylifera could be useful antioxidant in reducing chromium toxicity. Moreover, enzymatic responses could be used as sensitive biomarkers.
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