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Original research article

Ameliorating effect of *Curculigo orchoides* on chromium(VI) induced oxidative stress via, modulation of cytokines, transcription factors and apoptotic genes

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ABSTRACT

The effect of hydro-alcoholic extract of *Curculigo orchoides* on hexavalent chromium (Cr VI) induced toxicity in rats was investigated. Sub-acute toxicity studies were performed by OECD guidelines. $K_2Cr_2O_7$ (30 mg/kg) was administered to all groups except control group for a period of 28 days by oral gavage. Control group received distilled water; treatment groups received *C. orchoides* (25, 50 and 100 mg/kg). Cr (VI) administration resulted in up-regulation of serum biochemical parameters such as alanine transaminase, aspartate transaminase, alkaline phosphatase, and tissue biochemical markers viz. lipid peroxidation and protein carbonyl content. *C. orchioides* (100 mg/kg) significantly decreased these enzyme levels. The activities of anti-oxidant enzymes like superoxide dismutase, catalase and glutathione S-transferase were significantly decreased by Cr(VI) administration (50.7%, 43.7% and 37.9%, respectively). Further, mRNA expression studies and histopathology studies confirmed Cr(VI) toxicity. In all cases, *C. orchioides* promoted significant restoration of enzyme levels in a dose dependent manner. These results suggest the ameliorating effect of *C. orchoides* on Cr(VI) induced oxidative stress is probably *via*, modulation of cytokines, transcription factors and apoptotic genes.

Introduction

Chromium is a naturally occurring element present in the earth's crust (Callender, 2003). It is released into the environment from natural and anthropogenic sources, with the largest release occurring from industrial releases (Duruibe et al., 2007). In India, more than two lakh mine workers and inhabitants residing in the "Sukinda chromite mine" of Odisha were affected due to hexavalent chromium pollution (Das and Singh, 2011). Despite people suffering from several forms of ill health, physical and mental deformities due to constant exposure to toxic wastes from chromite mining, there is a tragic gap in the availability of 'scientific' studies and data on the health hazards of mining in India (Das and Singh, 2011). Potassium dichromate ($K_2Cr_2O_7$) is a hexavalent form of chromium and has been demonstrated to induce oxidative stress and is carcinogenic in nature (Pedraza et al., 2008). Cr(VI) enters into human/animal cells *via* an active transport process in place of phosphate anions, which it structurally resembles (Tchounwou et al., 2012). Intracellular reduction of Cr(VI) to Cr(III) causes overproduction of reactive oxygen species (ROS), which is an important characteristic of Cr(VI) toxicity (Bagchi et al., 2002). Human exposure to Cr(VI) induces several adverse health effects, such as genotoxicity, nephrotoxicity, carcinogenicity and hepatotoxicity (Garcia et al., 2013). Medicinal herbs contain many bioactive compounds which can chelate metals ions, therefore the effectiveness of herbal extracts serve as an alternative/prophylactic solution in cases of chromium associated occupational hazards. Experimental evidences suggest that most herbs possess a wide range of biological and pharmacological activities including antioxidant properties that may protect tissues against oxidative stress-induced tissue damage caused by metal toxicity (Gurer and Ercal, 2000).

Curculigo orchioides Gaertn (Hypoxidaceae) locally known as Kali musli is an endangered medicinal plant used for many medicinal purposes such as impotency, aphrodisiac, health tonic, hepatoprotection and to treat skin ailments (Soni et al., 2012). Its medicinal potential is due to the presence of several secondary metabolites such as triterpenoids, saponins, flavones and curculigosides (Arun et al., 2013). Methanolic extract of rhizomes of

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C. orchioides is reported for its immune-stimulatory action against cyclophosphamide induced neurotoxicity (Murali and Kuttan, 2015). *In vitro* cell line studies and pre-clinical studies on animals reported several possible benefits of *C. orchioides* such as anti-histaminic, immunomodulatory, anti-oxidant, anti-tumor potential and anti-osteoporotic activity (Jiao et al., 2009). In the present study, we focus on the protective effects of *C. orchioides* against potassium dichromate ($K_2Cr_2O_7$) induced toxicity in rats.

Materials and methods

Chemicals

ABTS – 2, 2-azinobis (3- ethylbenzothiazoline-6-sulfonic acid); BHT- Butylated hydroxytoluene; BSA – Bovine serum albumin; DNPH – Dipenylhydrazine; DPPH – 2, 2-diphenyl-1-picrylhydrazyl; DTNB – 5, 5-dithiobisnitrobenzoic acid; HCl – Hydrochloric acid; EDTA- Ethylenediamine tetraacetate disodium salt; $FeCl_3$ – Ferric chloride; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H_2O_2 – hydrogen peroxide; NaCl – Sodium chloride; SDS – Sodium dodecyl sulphate; TBA – Thiobarbituric acid and $K_2Cr_2O_7$ – Potassium dichromate. All the chemicals used in the experiment were purchased from Sigma Aldrich, USA.

Plant material and extraction

Curculigo orchoides Gaertn. rhizomes were collected from Mysore and properly authenticated by the Department of Botany, University of Mysore, Mysore, India (Voucher specimen no. AND8024). Rhizomes were shade dried and powdered using lab mill grinder and screened with sieve no 40. The rhizome powder (100 g) was defatted with diethyl ether for 24 h and the filtrate was suspended in 500 ml of 50% ethanol for 2 days on shaker at 200 rpm. This mixture was filtered using Whatman no.1 filter paper, and then concentrated under reduced pressure then, lyophilized and stored at −20 °C for further analysis. The yield of the *Curculigo orchoides* CO extract was 6.12% of dry rhizome.

Determination of in vitro *antioxidant activities*

In vitro antioxidant activity of CO was evaluated for DPPH, nitric oxide, hydrogen peroxide and ABTS radical scavenging activities. Free radical scavenging activities were determined by spectrophotometric method (Kumar et al., 2013a,b). Percent inhibition was calculated as follows: $[A_{cont} - A_{sample}]/A_{cont} \times 100$, where A_{cont} is the absorbance of the control and A_{sample} is the absorbance of sample. Ferrous reducing antioxidant power assay (FRAP) was measured for the colored product of ferrous tripyridyltriazine complex at an absorption maximum of 593 nm and expressed in units of μmol Fe(II) (Kumar et al., 2013a,b).

Experimental procedure

Animal studies were conducted according to the institute animal ethical committee regulations approved by the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA) with the IAEC approval no.28/1999/CPCSEA. Male Wistar albino rats (150–200 g) were selected from the stockpile colony, Defence Food Research Laboratory (DFRL), Mysore, India and were housed in an acryl fibre cage in a temperature controlled room (23 ± 2 °C). Animals were maintained at 12 h light/dark cycle with standard pellet diet and water was provided *ad-libitum*.

Experimental design

Thirty six animals were randomly divided into 6 groups: control group received distilled water (Con); positive control group received silymarin (Syl, 60 mg/kg) (Pradhan and Girish, 2006); treatment groups received 25, 50 and 100 mg of *C. orchoides* (CO 25, CO 50, and CO 100 mg/kg, respectively). $K_2Cr_2O_7$ (30 mg/kg) was administered by oral gavage to all animals except control group for a period of 28 days. Negative control group received only $K_2Cr_2O_7$ (Cr VI). Dosage selection and mode of administration of both $K_2Cr_2O_7$ and CO were based on previous studies (Susa et al., 1996). Animals were sacrificed under mild anaesthesia; serum was separated and stored at −20 °C for further biochemical assays. The tissue samples were excised, washed with normal saline and stored in −80 °C for further biochemical assays. Washed tissues were fixed immediately in 10% formalin solution for histopathology studies.

Assessment of serum biochemical markers

Serum analyses of various liver marker enzymes like alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were estimated using commercially available kits (AGAPEE, India).

Estimation of TBARS

Lipid peroxidation was determined by thiobarbutric acid reactive species (TBARS) and expressed as malondialdehyde (MDA mmol/cm/g) (Garcia et al., 2005). Liver tissues were homogenized in phosphate buffer (pH 7.0); Trichloroacetic acid (TCA) (10%), 0.5 ml and 2 ml of TBA mixture were added to tissue homogenate (0.5 ml). The TBA mixture contained TBA (0.35%), SDS (0.2%), $FeCl_3$ (0.05 mM) and BHT in glycine- HCl buffer (100 mM, pH 3.6). The above reaction mixture was boiled at 100 °C for 30 min and then allowed to cool. The mixture was centrifuged at 8000 rpm for 10 min and the absorbance was measured at 532 nm.

Protein carbonyl assay

Protein carbonyl content was estimated by Dalle et al. (2003) with slight modifications. Samples (10 μl) were diluted 1:40 with phosphate-buffered saline (PBS) containing sodium phosphate (10 mM; pH 7.4) and NaCl (0.14 M), and centrifuged twice at 14,000 rpm. Cold trichloroacetic acid (20%; w/v) was used to precipitate the protein portion. A solution of 10 mM DNPH in 2 N HCl was added to the protein pellet to get a final protein concentration of 1–2 mg/ml. Samples were allowed to stand in the dark at room temperature for 1 h by vortexing every 10 min and then precipitated with 10% TCA. The protein pellets were washed with 20% TCA (two times) and ethanol/ethyl acetate (1:1, v/v; three times) to remove free DNPH. Samples were then suspended in 6 M guanidine hydrochloride at 37 °C for 15 min. Carbonyl content was determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

Assay of SOD, CAT and GST activities

Liver tissues (100 mg) were homogenized in 50 mM phosphate buffer saline (1 ml) (pH 7.4) and 0.1 ml of tissue homogenate was used for each assay. Superoxide dismutase (SOD) activity was determined with commercially available kit (Randox, Cat no. SD. 125; Canada). The catalase activity (CAT) was determined by measuring the decay of 6 mM H_2O_2 solution at 240 nm by spectrophotometric degradation method (Kumar et al., 2013a,b).

Glutathione *S*-transferase (GST) activity was assayed using the CDNB conjugate method (Habig et al., 1974).

Gene expression studies

RNA isolation and cDNA conversion

Liver tissue samples were placed in a vial containing RNA later and stored at −80 °C. Total RNA was extracted from ∼100 mg of tissue by TRIzol reagent (Sigma Aldrich), as described the manufacturer's protocol. RNA samples were treated with RQ1 RNase-free DNase (Applied Bio-systems) to avoid DNA contamination. The concentration of total RNA was measured with spectrophotometer (NanoDrop) and the OD_{260}/OD_{280} ratio was tested for the purity of RNA and the purity of RNA yield was also checked by electrophoresis. The first cDNA strand was synthesized by using high capacity cDNA converting kit (Applied Bio systems) in one vial procedure containing a total reaction of 20 μl having 3.2 μl of distilled H_2O, 0.8 μl of dNTP's, 2 μl of the 10 × buffer, 2 μl of the random primers, 1 μl of RNAse inhibitor and 10 μl of RNA sample. Further the sample was incubated as per manufacturer's protocol (Applied Bio systems).

Semi-quantative polymerase chain reaction (PCR)

Ten different genes were targeted to check the modulation of gene expressions in response to Cr(VI) induced toxicity, such as: GAPDH (glyceraldehyde 3-phosphate dehydrogenase, housekeeping gene), TNF-α (tumor necrosis factor), IL-1 (interlukin), Mapk1 (mitochondria associated protein kinase), Atf-1(cyclic AMP dependent transcription factor), Gadd45 (growth arrest and DNA damage inducible protein), OGG-1 (oxoguanine glycosylase), Bcl-2 (B-cell lymphoma), Bax (Bcl-2 associated protein) and Casp-1 (caspase). Primers were designed on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nucleotide/) using the *Rattus norvegicus* gene sequences. Primers were supplied by Sigma Aldrich (India), and stored at −20 °C prior to using them for the gene expression study. Forward and reverse primers of targeted genes were provided in Table 1. PCR amplification was performed as a 20 μl reaction with 6 μmol l^{-1} of each forward and reverse primer, 2 μl of 10 × PCR buffer, 1.5 mmol l^{-1} of $MgCl_2$, 80 μmol l^{-1} of each dNTP's and 1 unit of Taq polymerase (Sigma, India) in a thermal cycler (Eppendorf, Germany). The PCR programme consisted of initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C to 60 °C (subject to primer; Table 1) for 30 s and extension at 72 °C for 8 min. PCR amplicons were resolved on 1.5% agarose gel and visualized under UV transilluminator.

Western blot analysis

Liver tissue samples were processed for SDS-PAGE followed by western blot for the analysis of antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) expression. Tissue samples were homogenized with HEPES buffer (pH 7.4) and were centrifuged at 10,000 rpm for 20 min. Total protein content was estimated from the supernatant (Lowry et al., 1951). Protein from each sample (50 μg) was separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Bio-Rad, USA). The membrane was blocked in 5% defatted milk solution at 4 °C for 24 h, later probed by respective primary antibody (1: 1000) and incubated at room temperature for 3 h. The membrane was washed four times in PBST and one time in PBS for 15 min followed by incubation for 2 h in HRP conjugated rabbit anti-mouse secondary antibody (Sigma, India; 1: 1000). The membranes were washed again and developed using an enhanced chemiluminescent method (luminal and p-coumaric acid, Sigma). The band intensity was captured by exposing the membranes onto the X- ray film (Anand et al., 2012).

Histopathological studies

Sections of brain, lungs, liver, kidney, spleen and intestine were carried out for histopathology to know tissue architecture. Fixed tissues were embedded in paraffin, cut into 5-mm thin sections, and stained with hematoxylin and eosin for light microscopic examination.

Table 1
Forward and reverse sequence of primers.

Primer	Sequence	Product Size (bp)	Annealing temp (°C)
TNFα	F-5′CTCACACTCAGATCATCTTC 3′ R-5′GAGAACCTGGGAGTAGATAAG3′	195	55
IL-1	F-5′ACAGCCTTATTTAGGAGTCTA3′ R-5′CAAACAGTATCATATGTCGG3′	190	57
MAPK1	F-5′CCATTGATATTTGGTCTGTGG 3′ R-5′ATCCAAGAATACCCAGGATG 3′	114	60
ATF-1	F-5′AGACTGCATCAGGAGATATG3′ R-5′CTTTGTTGTCTGAGATGTAAGG 3′	116	55
GADD45	F-5′GAAGATCGAAAGGATGGAC3′ R-5′TGAGGGTGAAATGGATCTG 3′	200	59
OGG-1	F-5′GATGATGTCACTTATCATGGC3′ R-5′TAGCACTGGCACATACATAG3′	118	57
BCL-2	F-5′AAAGCGCTGGATATAACTTC3′ R-5′GATGTACTTCATCACGATCTC3′	156	56
BAX	F-5′ATATTGCTGTCCAGTTCATC3′ R-5′ATAATATGGAGCTGCAGAGG3′	111	54
CASP-1	F-5′CCAGGACATTAAAGAATCCAAG3′ R-5′TCATTTTGGGGATTATTGGC3′	86	60
GAPDH	F-5′-CAACTCCCTCAAGATTGTCAGCAA-3′ R- 5′-GGCATGGACTGTGGTCATGA-3′	118	58

TNF-α – tumor necrosis factor, IL-1–interlukin1, MAPK1 – mitochondria associated protein kinase1, ATF-1 – cyclic AMP dependent transcription factor1, GADD45 – growth arrest and DNA damage inducible alpha, OGG-1 – oxoguanine glycosylase, BCL-2 – B cell lymphoma2, BAX – Bcl2 associated protein, CASP-1 – Caspase1, GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

Table 2
Serum biochemical parameters.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Con	40.66 ± 1.15	49.67 ± 3.80	104.67 ± 1.15
Syl	45.33 ± 1.14	56.33 ± 4.85	116.67 ± 3.15
Cr(VI)	72 ± 4.55*	103.67 ± 5.45*	166 ± 5.70*
CO 25	63.33 ± 2.01^	87 ± 5.72^	157 ± 3.77^
CO 50	58.67 ± 1.15^	80.67 ± 4.91^	150.33 ± 1.57^
CO 100	46.67 ± 1.90^	75.33 ± 1.90^	142.33 ± 3.40^

Significance was determined by one way ANOVA followed by Tukey's test, * $p \leq 0.05$ *vs* control, ^ $p \leq 0.05$ *vs* Cr(VI). Values represent the mean ± SD; n = 6.
Con – control group, Syl – positive control (Silymarin 60 mg/kg), Cr(VI) – negative control ($K_2Cr_2O_7$ 30 mg/kg), CO 25, CO 50 and CO 100 treatment groups (25, 50 and 100 mg/kg of *C. orchoides*, respectively).

Statistical analysis

The data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA. Differences at $p < 0.05$ were considered to be significant.

Results

In vitro *free radical scavenging activity of CO*

CO has showed *in vitro* anti-oxidant activity against free radicals such as, DPPH•, ABTS• and H_2O_2•. The half maximal inhibitory concentration IC_{50} values were observed as 82.6 ± 0.2, 25.0 ± 3.2 and 27.2 ± 0.1 μg/ml for the free radicals DPPH•, ABTS•· H_2O_2• and 24.89 ± 5 units of μmol Fe (II)/g for FRAP activity, respectively.

Effect of CO on serum biochemical parameters

$K_2Cr_2O_7$ had significantly increased the levels of ALP, AST and ALT (72 ± 4.5, 103.7 ± 5.5 and 166.0 ± 5.7 U/l, respectively) in serum when compared to control group (40.7 ± 1.6, 49.7 ± 3.8 and 104.7 ± 1.2 U/l, respectively) ($p < 0.01$). However, supplementation of CO significantly decreased the serum ALP, AST and ALT levels in a dose dependent manner as mentioned in (Table 2).

Effect of CO on lipid peroxidation, protein carbonyls and antioxidant enzymes

Cr(VI) treatment increased the levels of lipid peroxidation by tenfold (Table 3). Protein carbonyl content was doubled with the effect of Cr(VI) treatment when compared with control animals. The activities of anti oxidant enzymes like SOD, CAT and GST were significantly decreased by Cr(VI) administration (50.7%, 43.7% and 37.9%, respectively). All these enzyme activities were altered by CO in a dose dependent manner (Table 3). Further, western blot analysis confirmed the anti-oxidant role of CO supplementation on Cr(VI) induced oxidative stress by increased levels of SOD, CAT and GR (Fig. 1).

Effect of CO on gene expression studies

As shown in Fig. 2, Cr(VI) stimulated mRNA expression of several genes that are involved in inflammatory, transcription and apoptotic pathways. Relative expression was determined by densitometric analysis and normalized to the respective expression of housekeeping gene GAPDH. The expression levels of following genes were up-regulated viz., TNF-α, IL-1, Atf-1, MAPK1, Gadd45, Bax and Casp-1, and the expression of OGG-1 and Bcl-2 were down regulated by Cr(VI) treatment. These expression levels were positively modulated by CO (Fig. 2).

Effect of CO on tissue architecture

Histopathological studies were carried out to know the morphological changes in the organs. Cr(VI) administration caused several architectural changes such as: Gliosis in the brain; kidney showed occasional appearance of chronic inflammatory cells; liver appeared like hepatitis features with distorted lobules and central veins, individual hepatocytes showed feathery degenerative morphology; intestine showed mild sloughing of mucosa; lungs showed patchy chronic inflammatory foci. However, CO at 100 mg/kg showed normal histology as equivalent to the control group (Fig. 3).

Discussion

The present study demonstrates that oral administration of CO could ameliorate oxidative stress and attenuate the damaging effect induced by Cr(VI) in Wistar albino rats. It also explains the protective mechanism of CO against Cr(VI) induced toxicity. *In vitro* antioxidant activity results of the CO rhizome extract confirmed that it is having potential to scavenge free radicals in presence of many bioactive compounds, which may help in chelating the metal ions like chromium.

In the present study we observed a significant increase in peroxidation markers i.e., TBARS, protein carbonyls, serum markers viz., ALP, AST, ALT and also decreased levels of antioxidant enzymes such as SOD, CAT and GST. The increased levels of protein carbonyl and TBARS could be due to formation of hydroxyl radical (•OH) through a Haber–Weiss type reaction catalyzed by Cr(VI) (Henkler et al., 2010). These results are in agreement with earlier findings where increase in peroxidation markers and liver markers reduces the antioxidant enzyme status in rats under Cr(VI) influence (Balakrishnan et al., 2013). The supplementation of CO extract significantly reduced the serum biomarkers, TBARS and protein carbonyl levels showing its protective effect against chromium.

Table 3
Antioxidant defenses and Peroxidation biomarkers in liver homogenates.

Groups	Tissue antioxidant markers			Peroxidation biomarkers	
	SOD (U/mg protein)	CAT (U/mg protein)	GST (μM/mg protein)	TBARS (nmole MDA/mg protein)	Protein carbonyl (nmole/mg protein)
Con	10.16 ± 0.64	53.29 ± 4.36	73.56 ± 6.23	0.12 ± 0.005	0.43 ± 0.009
Syl	9.23 ± 0.76	49.40 ± 3.98	68.10 ± 5.03	0.14 ± 0.02	0.51 ± 0.02
Cr(VI)	5.00 ± 0.42*	30.00 ± 5.42*	45.67 ± 3.24*	1.16 ± 0.04*	0.86 ± 0.03*
CO 25	5.05 ± 0.44	35.42 ± 2.94	48.67 ± 3.94	1.03 ± 0.02^	0.64 ± 0.03^
CO 50	7.12 ± 0.56^	37.18 ± 3.16	53.08 ± 4.38^	0.60 ± 0.02^	0.57 ± 0.01^
CO 100	9.56 ± 0.86^	50.43 ± 4.01^	69.27 ± 5.47^	0.40 ± 0.03^	0.34 ± 0.02^

Significance was determined by one way ANOVA followed by Tukey's test, * $p \leq 0.05$ *vs* control, ^ $p \leq 0.05$ *vs* Cr(VI). Values represent the mean ± SD; $n = 6$.
Con – control group, Syl – positive control (Silymarin 60 mg/kg), Cr(VI) – negative control ($K_2Cr_2O_7$ 30 mg/kg), CO 25, CO 50 and CO 100 treatment groups (25, 50 and 100 mg/kg of *C. orchoides*, respectively).

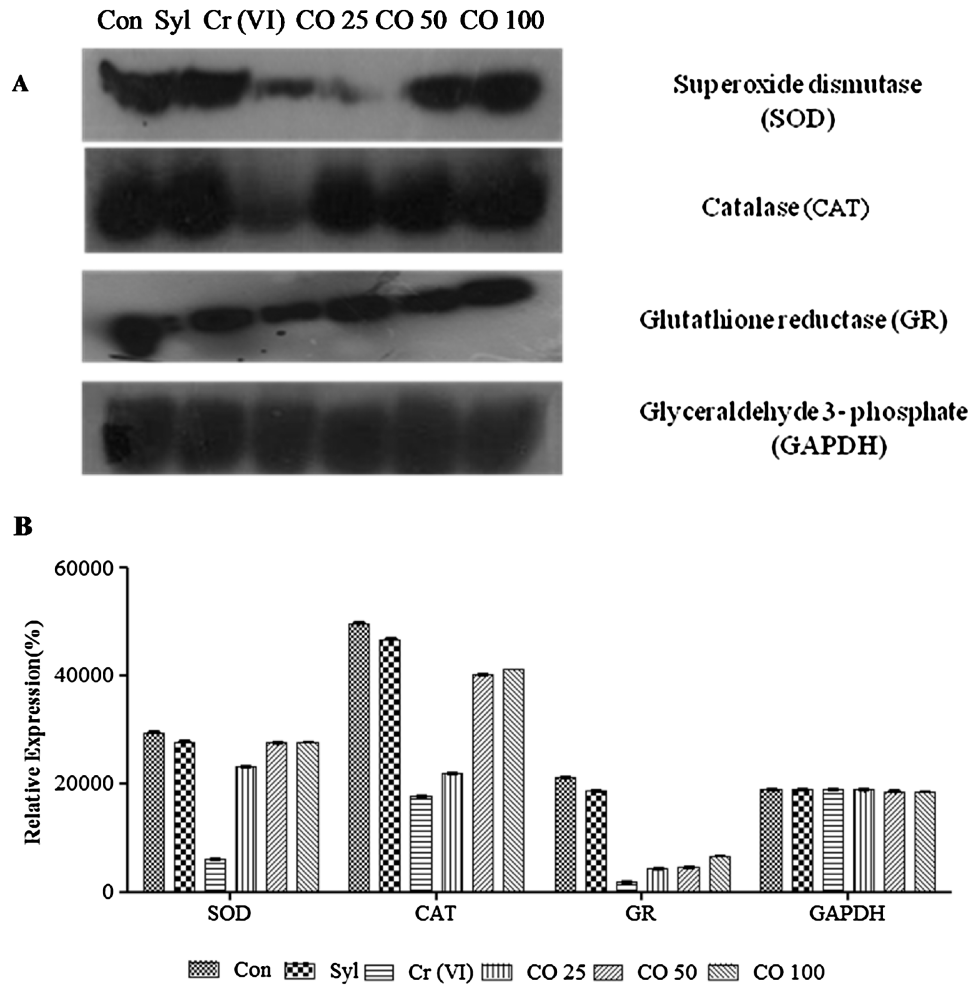

Fig. 1. Effect of *C. orchoides* on antioxidant enzyme levels.
(A) Gel images; (B) Densitometric analysis of gel images.
Con – control group, Syl – positive control (Silymarin 60 mg/kg), Cr(VI) – negative control ($K_2Cr_2O_7$ 30 mg/kg), CO 25, CO 50 and CO 100 treatment groups (25, 50 and 100 mg/kg of *C. orchoides*, respectively).

Chromium(VI) compounds are internalized in cells *via* anionic channels then, reduced and accumulates as trivalent ion. Formation of Cr(III)-DNA adducts is regarded as predominant carcinogenic mechanism (Salnikow and Zhitkovich, 2007). In parallel, chromium ions can engage in Fenton-like reactions and generating hydroxyl radicals. Human body is equipped with a variety of antioxidants that serve to counter balance the effect of oxidants. The major enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX). These antioxidant enzymes are potential targets for heavy metal toxicity (Liu et al., 2010). In the current study, we observed that administration of Cr(VI) has not only altered serum and peroxidation markers but also modified the antioxidant enzyme status in the liver tissue. These results are in agreement with the previous findings where peroxidation of membrane lipids and generation of reactive oxygen species (ROS) are well associated with the initiation of carcinogenesis affecting the normal biochemical process (Barrera, 2012). In the present study the levels of SOD, CAT, GST and GR activities were decreased in Cr(VI) treated group. This decrease might be due to the ROS formation and utilization of antioxidant enzymes by the hydroxyl radicals in the removal of H_2O_2 formed by Cr(VI). Elevated levels of ROS had down regulated ROS scavengers and antioxidant enzymes.The effects on these enzymes were improved by the supplementation of CO extract which clearly suggest the protective role of CO in ameliorating Cr(VI) induced oxidative stress.

The generated ROS are believed to mediate cell injuries and also involves in multiple pathways. These generated reactive oxygen species stimulates inflammatory cytokines, such as interleukins (ILs), interferons (IFNs), tumor necrosis factor (TNF) and other growth factors by activation of NF-B, TAK1 and MAPK pathways. In our study, it is evident that administration of Cr(VI) had stimulated the inflammatory cytokines and elevated the expression of *TNF-α* and *IL-1*. At cellular level interleukins also triggers MAP kinases (Kalia and Flora, 2007) which are a family of protein kinases, that transmit extracellular signals into the nucleus and participate in cellular functions such as cell growth, differentiation and apoptosis (Roux and Blenis, 2004). The MAPK pathway triggered by hexavalent chromium was in line with earlier reports (Rios et al., 2009). MAPK also regulates Atf1, cyclic AMP-dependent transcription factor to elicit stress responses under extreme environmental conditions i.e. heavy metal stress etc. Similarly in the current study, exposure to Cr(VI) elevated the expression of interleukins that might have triggered MAP kinase and Atf-1 which clearly states stress response to heavy metals. Moreover, CO supplementation had regulated the expression levels of these genes in a dose dependent manner.

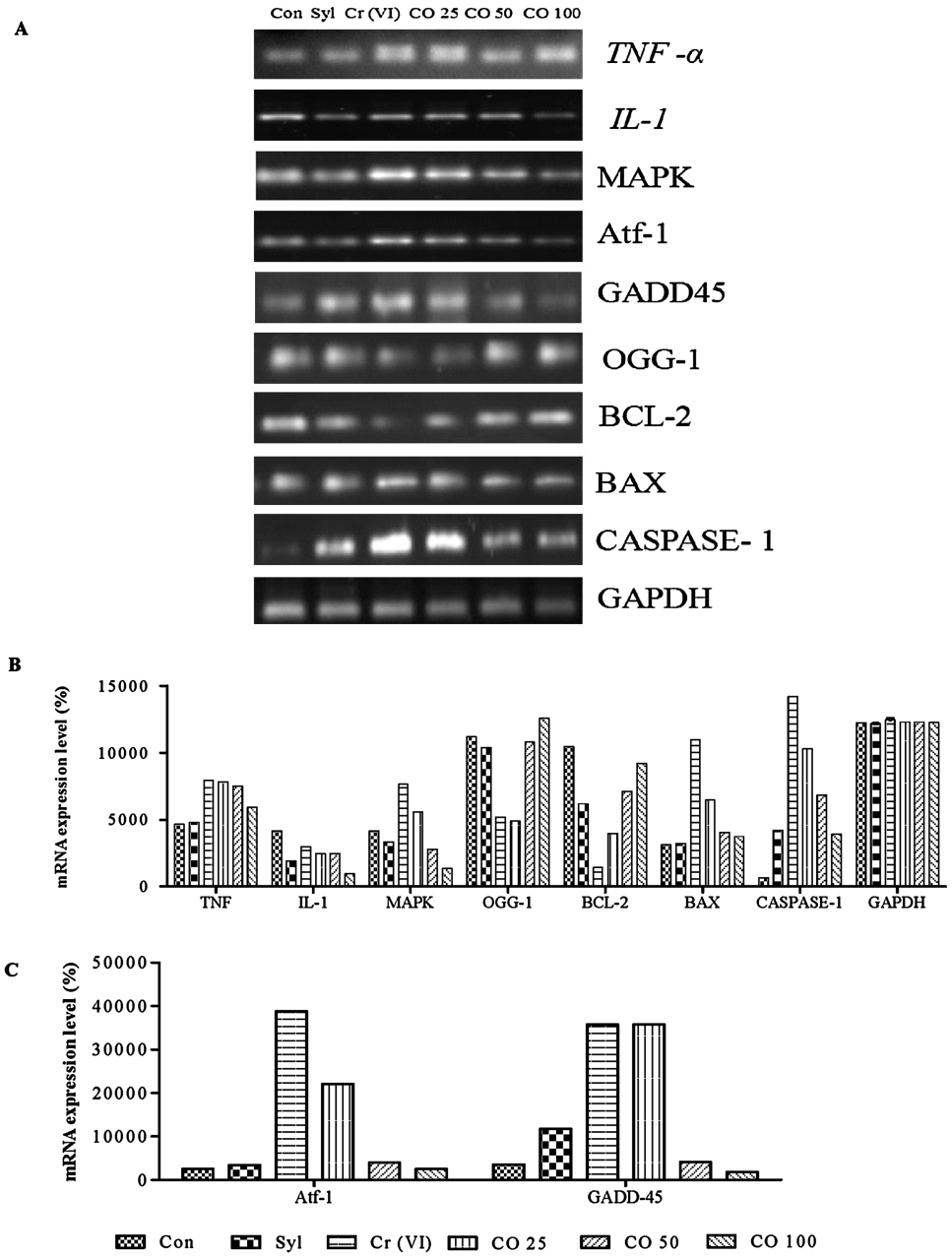

Fig. 2. Effect of *C. orchoides* on modulation of gene expressions.
(A) Gel images; (B) and (C) Densitometric analysis of gel images.
Con – control group, Syl – positive control (Silymarin 60 mg/kg), Cr(VI) – negative control ($K_2Cr_2O_7$ 30 mg/kg), CO 25, CO 50 and CO 100 treatment groups (25, 50 and 100 mg/kg of *C. orchoides*, respectively). Tnf-1–Tumor necrosis factor, IL-1 – Interlukin-1, MAPK – Mitochondria associated protein kinase, ATF-1 – Cyclic AMP dependent transcription factor-1, GADD45 – Growth arrest and DNA-damage-inducible alpha, OGG-1 – Oxoguanine glycosylase, BCL-2 – B-cell lymphoma 2, BAX- Bcl2 associated protein and GAPDH – Glyceraldehyde 3-phosphate dehydrogenase.

One of the most important damage aroused by extraneous Cr (VI) is DNA damage to the cell. Gadd45 and OGG-1 expressions are considered as markers for cellular response to DNA damage as they are known to be involved in growth arrest and base excision repair of oxidative DNA damage (Yoon et al., 2007). In the present study, Gadd45 and OGG-1 mRNA expression levels were altered by Cr(VI) exposure, these results evidenced that administration of chromium might have caused DNA damage. However, administration of CO at higher doses (CO 50 and CO 100 mg/kg) showed down regulation of Gadd45 and up-regulated expression of OGG-1.

Hexavalent chromium Cr(VI) compounds are redox cycling environmental carcinogens that induce apoptosis as a primary mode of cell death (Das et al., 2015). In response to DNA damage, the tumor suppressor gene p53 regulates the expression of pro-apoptotic members of the family (e.g. Bax, Bak) at the outer mitochondrial membrane to induce mitochondrial outer membrane permeabilization (MOMP) (Reisman et al., 2012). In the current study Cr(VI) induced tissues showed down regulation of Bcl-2 and up regulated expression of Bax and Casp-1 genes. It is clearly evident that hexavalent chromium had induced cytotoxicity through apoptotic process. Moreover, supplementation of CO suppressed the cytotoxicity *via* positive modulation of Bcl-2, Bax and Casp-1.

Histopathological observations of the present study showed tissue damage by Cr(VI) toxicity viz., gliosis in brain; feathery degenerative morphology of hepatocytes; sloughing of intestinal

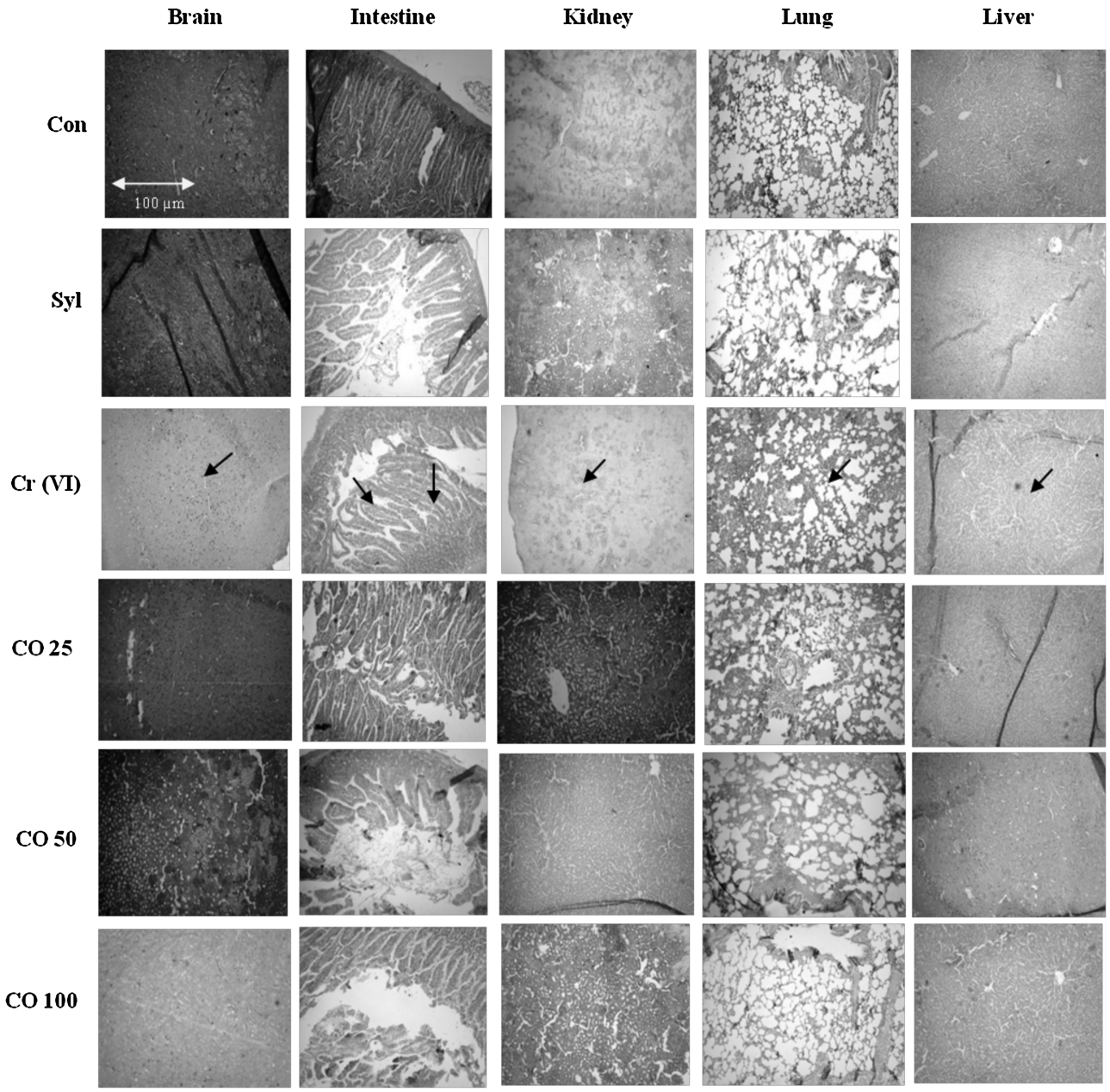

Fig. 3. Histopathological observations.
Con – control group, Syl – positive control (Silymarin 60 mg/kg), Cr(VI) – negative control ($K_2Cr_2O_7$ 30 mg/kg), CO 25, CO 50 and CO 100 treatment groups (25, 50 and 100 mg/kg of *C. orchoides*, respectively). Pointed arrows show abnormal features in the respective tissues compared to the control.

mucosa; appearance of chronic inflammatory cells in kidney and lungs. It is evident that inside the cell, Cr(VI) is reduced to Cr(III), which generates reactive oxygen species (ROS) and induces damage to soft tissues as observed in the present study and confirmed by the earlier workers (Bagchi et al., 2001). In the present study, supplementation of *C. orchoides* extract significantly reduced the tissue damage in a dose dependent manner.

Conclusion

In conclusion, data from this study suggest that CO protects against Cr(VI)-induced toxicity in rat models. Therefore, CO can be used as a potential source of treatment for people who are at risk of chromium toxicity.

Conflict of interests

The authors declare no conflict of interests.

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