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Citrus aurantium L. peel extract mitigates hexavalent chromium-induced oxidative stress and cardiotoxicity in adult rats

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Abstract

In the present study, we aimed to examine the potential protective effect of *C. aurantinum L.* peel extract against oxidative damage induced by hexavalent chromium in the heart of adult rats. Rats were divided into six groups. Group I served as controls and received standard diet. Group II received via drinking water potassium dichromate ($K_2Cr_2O_7$) alone (700 ppm) during 3 weeks. Groups III and IV were pre-treated for 10 days by gavage with the ethanolic extract of *C. aurantium* peels at doses of 100 and 300 mg/kg body weight/day, respectively, and then $K_2Cr_2O_7$ was administrated during 3 weeks. Groups V and VI received by gavage only *C. aurantium* peel ethanolic extract at doses of 100 and 300 mg/kg body weight/day, respectively, during 10 days. $K_2Cr_2O_7$ treatment increased the cardiac levels of malondialdehyde (MDA), protein carbonyls (PCO), advanced oxidation protein products (AOPP), non-protein thiols, glutathione and vitamin C, as well as the activities of catalase, superoxide dismutase and glutathione peroxidase. Cardiac histological alterations, manifested by hemorrhage and cytoplasmic vacuolization, were also observed. Pre-treatment with *C. aurantium* peel extract (300 mg/kg) attenuated significantly the biochemical and histopathological changes observed following $K_2Cr_2O_7$ exposure in rat's heart. Our findings indicated that *C. aurantium* peel extract was able to hamper $K_2Cr_2O_7$-induced myocardial injury, which could be attributed to its antioxidant activity.

Keywords: Chromium, rats, heart, oxidative stress, *C. aurantium* peel

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Introduction

Cardiovascular diseases (CVDs) remain one of the most important causes of mortality in the world. Commonly known risk factors, such as hypertension and hypercholesterolemia, can be responsible for only about 50-75% of all CVDs. Therefore, there is an urgent need to identify new CVD risk factors. A great body of evidence indicates that heavy metal exposure is linked to increased incidence of heart diseases (1).

Hexavalent chromium (Cr(VI)) is one of the valence states (+6) of the element chromium. It is usually produced by industrial processes and is a source of exposure in the workplace (2). The industrial applications of this metal include painting, electroplating, leather tanning and welding. Workers in Cr(VI)-based industries are highly exposed to this metal which is a major cause of concern. Excessive exposure to Cr(VI) can be associated with hepatotoxicity (3), nephrotoxicity (4) and carcinogenicity (5) in humans and experimental animals. Moreover, Cr(VI) has drawn considerable attention as one of the potential cardiotoxic heavy metals. In fact, this element has been demonstrated to accumulate in the heart tissue and to affect the cardiac function both *in vivo* and *in vitro* (6). In addition to that, Soudani *et al.* (7) have shown that Cr(VI) induces cardiotoxicity in adult rats. The excessive generation of reactive oxygen species (ROS) and the subsequent installation of oxidative stress have been reported to be responsible for the toxic effects of Cr(VI) in the heart (7). Herbal remedies have been used for the treatment of various illnesses including heart diseases (8). In fact, these herbs constitute a rich source of bioactive phytochemicals such as polyphenols, flavonoids, and other constituents which have been proven to possess beneficial effects in CVDs (9).

Citrus aurantium L., commonly known as bitter orange, is a flowering plant that belongs to the *Rutaceae* family of the Sapindales order. This species is widely distributed in tropical and subtropical southeast regions of the world. Its flowers and fruits are used for medicinal purposes, mainly as remedies for many disorders of the digestive system, as a cardiac tonic and for anxiety (10). The peel is often used in the formulation of herbal teas due to its flavor profiles, its digestive and carminative effects and for the production of stomachic and laxative products (11). However, citrus fruits

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in general are highly consumed worldwide as fresh produce and juice. The peels, which contain a wide variety of secondary components with substantial antioxidant activity in comparison with other parts of the fruit (12), are most often discarded as waste. In this regard, *C. aurantinum* peels have been demonstrated to be a rich source of phenolic compounds with significant antioxidant activities (13). The phytochemical study of the fruit peels revealed the presence of citral and limonine, in addition to several citrus bioflavonoids, including hesperidin, neohesperidin, naringin and rutin (14). These compounds have been attributed with a wide range of biological activities including cardioprotective properties. For instance, naringin is believed to cross the blood brain barrier and to have antioxidant and antihypertensive effects (15). Naringinin and hesperdin have been shown to exert anti-atherogenic activity (16). Furthermore, antiplatelet properties have been attributed to hesperidin and neohesperidin (17).

Therefore, the present study firstly was planned to evaluate the beneficial effects of *C. aurantinum* peel ethanolic extract in protecting the myocardium against oxidative damage induced in adult rats by potassium dichromate ($K_2Cr_2O_7$).

Materials and methods

Chemicals

$K_2Cr_2O_7$ was purchased from Merck (Darmstadt, Germany). Glutathione (GSH), 5,5'-Dithiobis-2-Nitrobenzic acid (DTNB), thiobarbituric acid (TBA), 1,1,3,3-tetrathoxypropane (TEP), 2,4-dinitrophenylhydrazine (DNPH) and nitro blue tetrazolium (NBT) were purchased from Sigma-Aldrich Co. (MO, USA). The other chemicals used in the present study were provided from the standard commercial suppliers Acros Organics and Carlo Erba (USA).

Plant material

C. aurantium fruits, cultivar laringe, were harvested in the spring from Mars to April from our local garden in the South Suburb of Sfax City (Tunisia). This plant was botanically identified by Dr. Abdelhamid Nabli, Professor Emeritus of Vegetal Biology in Science Faculty of Tunis, Tunisia. A voucher specimen (N-99) was deposited in the herbarium of Pharmacology Laboratory, Faculty of Pharmacy, Monastir, Tunisia.

Preparation of C. aurantium peel extract

C. aurantium peel extract preparation was performed according to the method described by Soudani et al. (18). Briefly, an amount of 25 mg of *C. aurantium* fruits peel was extracted by refluxing for 5 h with 500 mL of ethanol/water (4V/1V). The obtained aqueous ethanol extract was filtered through Whatman No1 filter paper. The filtrate was concentrated to dryness in a rotary evaporator at 50 ± 1 °C to give solid residues and then kept at 4°C until further use.

Determination of total phenolic content in C. aurantium peel extract

The total phenolic content of *C. aurantium* peel extract was determined using the Folin-Ciocalteu method of Julkunen-Titto (19). Results were expressed as mg of caffeic acid equivalents per g of extract (mg CAE/g extract).

Determination of total flavonoid content in C. aurantium peel extract

Determination of *C. aurantium* extract total flavonoid content was carried out by the aluminium chloride colorimetric method as described by Chang et al. (20). Results were expressed as mg of quercetin equivalents per g of extract (mg QE/g extract).

Determination of vitamin C content in C. aurantium peel extract

Vitamin C content of *C. aurantium* extract was determined by using the titration method involving 2, 6 - dichloroindophenol (21). Results were expressed as mg/g extract.

DPPH radical scavenging assay

The capacity of *C. aurantium* extract to scavenge the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined according to the method of Bersuder et al. (22). Various concentrations of sample extract (0.5 mL) were mixed with 0.5 mL of 99.5% ethanol and 0.125 mL of 0.5 mM DPPH solution (dissolved in 99.5% ethanol). The mixture was shaken and incubated in the dark for 1 h at room temperature. Then the absorbance was measured at 517 nm. The scavenging activity was calculated based on the percentage of DPPH radical scavenged using the following equation:

$$DPPH\ radical - scavenging\ activity\ \% = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Vitamin C was used as positive control.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of *C. aurantium* extract was measured by replacement titration according to

the method of Zhao et al. (23). 1 mL of H_2O_2 (0.1 mM) and an aliquot of 1 mL of the peel extract at different concentrations were mixed, followed by 2 drops of 3% ammonium molybdate, 10 mL of H_2SO_4 (2M) and 7 mL of KI (1.8 M). The mixed solution was titrated against $Na_2S_2O_3$ (5 mM) until disappearance of yellow color. The relative activity of the test extract to scavenge H_2O_2 was expressed as percentage of the titer volume change as follow:

% inhibition = ((V control-V sample)/V control) × 100
Vitamin C was used as standard.

Reducing power assay

The reducing power, which reflects the electron donation capacity of bioactive compounds, was monitored by using the method of Oyaizu (24). *C. aurantium* extract (0.5 mL) at different concentrations was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide (1%). The mixture was incubated in a water bath at 50°C for 20 min. After that, the reaction was stopped by adding 1 mL of 10 % trichloroacetic acid and the mixture was centrifuged at 20 ×g for 10 min. To 1.5 mL of the supernatant, 1.5 mL of deionised water and 0.1 mL of 0.1 % ferric chloride ($FeCl_3$) were added. After allowing the solution to stand for 10 min, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated stronger reducing power. Vitamin C was used as standard.

Animals

A total of 36 female Wistar rats with an initial mean body weight of 140 ± 10 g, purchased from the Central Pharmacy (SIPHAT, Tunis, Tunisia), were used in the present work. They were housed under standard laboratory conditions of temperature (22 ± 2 °C), relative humidity (40%) and 12h light–dark cycle. Water and commercial standard pellet diet (SICO, Sfax. Tunisia) were provided *ad libitum*. The experimental study was carried out according to the National Guidelines for Animal Care (25) and approved by the Ethical Committee of Sfax Faculty of Sciences with ethics approval number 1204.

Experimental design and animal grouping

Rats were randomly divided into six groups of six animals each. Group I served as controls and received standard diet. Group II received via drinking water $K_2Cr_2O_7$ alone (700 ppm) during 3 weeks. Groups III and IV were pre-treated for 10 days by gavage with the ethanolic extract of *C. aurantium* peels at doses of 100 and 300 mg/kg body weight/day, respectively, and then $K_2Cr_2O_7$ was administrated during 3 weeks. Groups V and VI received by gavage only the ethanolic extract of *C. aurantium* peels at doses of 100 and 300 mg/kg body weight/day, respectively, during 10 days. The $K_2Cr_2O_7$ dose and the treatment period used in our study were selected on the basis of the previous works of Junaid et al. (26) and Kanojia et al. (27). Regarding *C. aurantium* peel extract doses, Soudani et al. (18) have previously shown that administration of this plant extract, at 100 and 300 mg/kg, was found to alleviate oxidative stress induced by Cr (VI) in the lung of adult rats.

At the end of the experimental period, the animals of the different groups were sacrificed by cervical dislocation to avoid stress. Hearts were dissected out and cleaned. Some samples were rinsed and homogenized (10% w/v) by an Ultra-Turrax T25 (IKA, Staufen, Breisgau, Germany) in an appropriate buffer (pH = 7.4) and then centrifuged. The resulting supernatants were used for biochemical assays. Other samples of heart tissues were immediately removed, cleaned and fixed in 10% formalin solution for histological examination.

Biochemical assays

Protein estimation in heart

Protein content in the heart homogenates was determined by following Lowry et al. (28) method, using bovine serum albumin as a standard.

Evaluation of lipid peroxidation in heart

According to the method of Draper and Hadley (29), the concentration of malondialdehyde (MDA), an endpoint of lipid peroxidation, was measured spectrophotometrically in heart tissues using TEP as a standard. Results were expressed as nmoles/g tissue.

Determination of heart protein carbonyl (PCO) content

Heart PCO content was measured using the DNPH method described by Reznick and Packer (30). Results were expressed as nmoles/mg protein.

Determination of heart advanced oxidation protein product (AOPP) levels

The determination of AOPP levels was carried out according to the method of Kayali et al. (31). AOPP concentration was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and results were expressed as µmoles/mg protein.

Determination of heart antioxidant enzyme activities

Catalase (CAT) activity in heart tissue was measured spectorphotometrically at 240 nm by calculating the rate of H_2O_2 degradation (32). CAT activity was expressed as µmoles H_2O_2 consumed/min/mg of protein.

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich (33), by monitoring the photochemical reduction of NBT. SOD activity was expressed as units/mg protein, where one unit corresponded to the amount of this enzyme required to cause 50% inhibition of NBT reduction at 560 nm.

Glutathione peroxidase (GPx) activity was measured according to the protocol of Flohe and Gunzler (34) and results were expressed as nmoles of GSH oxidized/min/mg protein.

Heart GSH content

The heart GSH content was measured according to the method of Ellman (35) modified by Jollow et al. (36). The principle of this method is based on the development of a yellow color when DTNB is added to compounds containing sulfhydryl groups. Results were expressed as µg/g tissue.

Heart non-protein thiol (NPSH) content

Heart NPSH levels were determined according to the method of Ellman (35) and results were expressed as µmoles/g tissue.

Heart vitamin C content

Vitamin C determination was performed as described by Jacques-Silva et al. (37) and results were expressed as µg/g tissue.

Histopathological studies

Some heart samples, intended for histological examination, were fixed in 10% buffered formalin solution, embedded in paraffin blocks and then sectioned at 5µm. The obtained sections were deparaffinized, stained with hematoxylin-eosin (H&E) and examined for histopathological changes under light microscopy.

Statistical analysis

All data were presented as means ± standard deviation (SD). They were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Statistical comparisons between groups were made by means of one-way analysis of variance (ANOVA) followed by Fisher protected least significant difference (PLSD) test as a post hoc test. Student unpaired t test, used for comparison between two groups, was also used when required. Differences were considered significant if $p < 0.05$.

Results

In vitro study

Antioxidant components in the ethanolic extract of C. aurantium

The phytochemical analysis of the antioxidant components of *C. aurantium* extract showed the presence of phenolic compounds, flavonoids and vitamin C (Table 1).

Table 1 Content of total phenolics, flavonoids and vitamin C in *C. aurantium* peel extract

	Total phenolics mg CAE/g extract	Total flavonoids mg QE/g extract	Vitamin C mg/g extract
C. aurantium	92.82 ± 4.42	35.96 ± 2.68	55.55 ± 15.71

Values represent the means of three replicates ± SD.
CAE: Caffeic acid equivalents; QE: Quercetin equivalents.

Antioxidant activity

Since a single method cannot accurately reflect the antioxidant capacity of plant extracts, different techniques (DPPH radical scavenging activity, H_2O_2 scavenging activity, reducing power) were used in our study to evaluate the antioxidant activity of *C. aurantium*. Since it is present in our tested plant extract, vitamin C was used as a standard for the comparison of the antioxidant power.

DPPH radical scavenging activity

The scavenging capacity of *C. aurantium* extract on DPPH radical is shown in figure 1. The results indicated that this extract exhibited an antiradical activity which was enhanced with the increase of the extract concentration. The data obtained showed also that the IC_{50}, the concentration of the tested extract required to decrease 50% of DPPH initial concentration, was about 0.275 ± 0.07 mg/mL. However vitamin C, used as a positive control, showed higher antiradical activity than *C. aurantium* extract.

H_2O_2 scavenging activity

The H_2O_2 scavenging activity is illustrated in figure 2. *C. aurantium* extract displayed a scavenging capacity against H_2O_2 in a concentration dependent manner, but it remained weaker than that of vitamin C used as a standard reference.

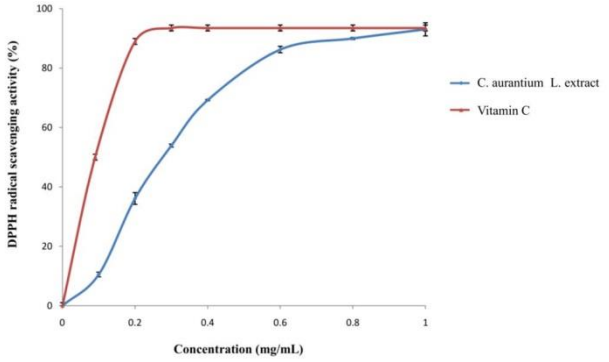

Figure 1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *C. aurantium* extract in comparison

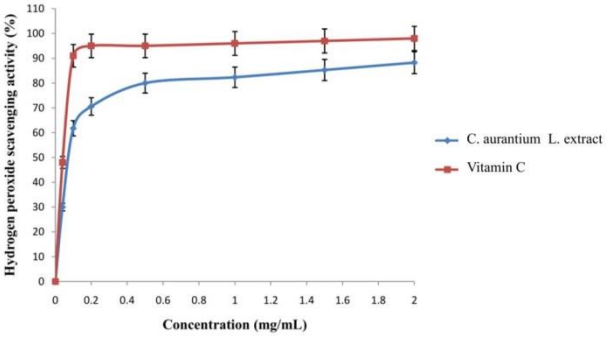

Figure 2 Hydrogen peroxide scavenging activity of *C. aurantium* extract in comparison with vitamin C.

Reducing power

In our study, *C. aurantium* extract revealed the ability to reduce Fe^{3+} to Fe^{2+} in a concentration dependent manner. However, the reducing power of this extract was found to be significantly lower than that of vitamin C used as a standard (Figure 3).

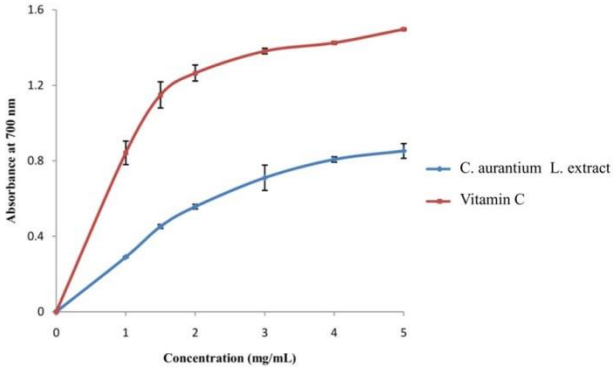

Figure 3 Reducing power of *C. aurantium* extract in comparison with vitamin C.

In vivo study

Lipid peroxidation

The levels of MDA were significantly increased by 55% in the heart tissue of $K_2Cr_2O_7$-treated rats, when compared to controls rats. Pre-treatment with *C. aurantium* extract at both doses 100 and 300 mg/kg bw lowered significantly the MDA cardiac levels by 22 and 31% respectively, as compared to $K_2Cr_2O_7$-treated rats, without reaching normal values (Table 2).

Protein oxidation

A significant increase in the cardiac levels of PCO (+129%) and AOPP (+58%) was observed in $K_2Cr_2O_7$-treated rats, as compared to the controls. Pre-treatment with *C. aurantium* extract at the doses of 100 or 300 mg/kg bw resulted in a marked decrease in the levels of PCO (-20 and -46%, respectively) and AOPP (-17 and -28%, respectively), compared to $K_2Cr_2O_7$-treated rats, without reaching control values (Table 2).

Table 2 Malondialdehyde (MDA), protein carbonyls (PCO) and advanced oxidation protein product (AOPP) levels in the heart of control and treated rats with $K_2Cr_2O_7$, *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) or their combination $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw).

Parameter and treatment	MDA[a]	PCO[b]	AOPP[c]
Control	185.78 ± 5.63	78.56 ± 9.80	4.48 ± 0.56
$K_2Cr_2O_7$	287.78 ± 38.67***	179.85 ± 26.69***	7.07 ± 0.83***
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw)	223.61 ± 26.09^{**+}	144.70 ± 18.33^{***+}	5.90 ± 0.96^{**+}
$K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw)	198.71 ± 10.05^{*+++}	97.93 ± 11.35^{**+++}	5.10 ± 0.55^{*+++}
***C. aurantium* (100 mg/Kg bw)**	179.96 ± 7.29	81.65 ± 8.92	4.20 ± 0.37
***C. aurantium* (300 mg/Kg bw)**	175.81 ± 15.10	88.75 ± 18.80	4.02 ± 0.83

[a]: nmoles of MDA/g tissue
[b]: nmoles/mg protein.
[c]: µmoles/mg protein.
Values are expressed as means ± SD for six animals in each group. $K_2Cr_2O_7$, $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw), *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) groups vs control group: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw) and $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw) groups vs $K_2Cr_2O_7$ group: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$.

Enzymatic antioxidant status

Rats exposure to $K_2Cr_2O_7$ resulted in significantly enhanced activities of SOD, CAT and GPx (+24, +133 and +65%,

respectively) in the heart tissue, as compared to the controls (Table 3). Pre-treatment with *C. aurantium* extract at the dose of 100 mg/kg bw led to a no significant decrease of CAT (-14%), SOD (-6%) and GPx (-4%) cardiac activities, as compared to $K_2Cr_2O_7$-treated rats. Pre-treatment with this plant extract at 300 mg/kg body weight was more effective in decreasing CAT (-44%) and GPx (-30%) activities, without reaching the control values, and reverted back SOD activity (-17%) to near normal value.

Non-enzymatic antioxidant status

Following $K_2Cr_2O_7$ exposure, heart levels of GSH, NPSH and vitamin C were enhanced respectively by 30, 94 and 37%, when compared to the controls (Table 4). Pre-treatment with *C. aurantium* extract at the dose of 100 mg/kg produced a significant decrease in NPSH level by 11%, as compared to $K_2Cr_2O_7$-treated rats, without reaching normal values, while it failed to reverse GSH and vitamin C levels. Compared to $K_2Cr_2O_7$-treated rats, pre-treatment with this extract at the dose of 300 mg/kg reduced significantly GSH, NPSH and vitamin C levels by 14, 37 and 18% respectively, without reaching the control values (Table 4).

Histological findings

Light microscopic examination of heart sections from control rats revealed normal histological structures (Figure 4(A)). Nevertheless, heart sections of $K_2Cr_2O_7$-treated rats showed structural changes in this tissue characterized by hemorrhage and cytoplasmic vacuolization of cardiac muscle cells (Figure 4 (B)). These histological alterations were markedly reduced by pre-treatment with *C. aurantium* at the two tested doses (Figure 4(C1, C2)). Normal cardiac architecture was observed

Table 3 Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in the heart of control and treated rats with $K_2Cr_2O_7$, *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) or their combination $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw).

Parameter and treatment	CAT[a]	SOD[b]	GPx[c]
Control	56.57 ± 6.03	740.87 ± 90.34	20.99 ± 1.29
$K_2Cr_2O_7$	131.75 ± 27.47***	916.66 ± 65.69**	34.64 ± 1.88***
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw)	112.73 ± 11.23***	863.92 ± 62.71**	33.31 ± 1.77***
$K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw)	74.05 ± 8.64^{**++}	763.38 ± 58.01^{++}	24.32 ± 1.99^{**+++}
***C. aurantium* (100 mg/Kg bw)**	54.01 ± 14.87	689.25 ± 90.92	23.08 ± 2.58
***C. aurantium* (300 mg/Kg bw)**	55.28 ± 13.52	730.44 ± 82.68	24.88 ± 5.13

[a]: µmoles H_2O_2 degraded/min/mg protein.
[b]: units/mg protein.
[c]: nmoles of GSH/min/mg protein.
Values are expressed as means ± SD for six animals in each group.
$K_2Cr_2O_7$, $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw), *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) groups vs control group: $^{**}p < 0.01$; $^{***}p < 0.001$.
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw) and $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw) groups vs $K_2Cr_2O_7$ group: $^{++}p < 0.01$; $^{+++}p < 0.001$.

Table 4 Glutathione (GSH), non-protein thiols (NPSH), and vitamin C levels in the heart of control and treated rats with $K_2Cr_2O_7$, *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) or their combination $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw).

Parameter and treatment	GSH[a]	NPSH[b]	Vitamin C[c]
Control	147.90 ± 14.09	6.21 ± 0.46	96.34 ± 8.24
$K_2Cr_2O_7$	192.41 ± 13.22***	12.07 ± 0.51***	131.73 ± 8.59***
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw)	191.31 ± 10.61***	10.71 ± 0.62^{***++}	124.20 ± 11.61***
$K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw)	164.92 ± 9.02^{*+++}	7.62 ± 0.71^{**+++}	107.53 ± 5.73^{*+++}
***C. aurantium* (100 mg/Kg bw)**	145.15 ± 5.36	6.63 ± 0.41	99.08 ± 8.34
***C. aurantium* (300 mg/Kg bw)**	144.91 ± 12.58	6.22 ± 0.55	104.33 ± 10.69

[a]: µg/g tissue
[b]: nmoles/ g tissue
[c]: µg/g tissue
Values are expressed as means ± SD for six animals in each group.
$K_2Cr_2O_7$, $K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw), $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw), *C. aurantium* (100 mg/Kg bw) and *C. aurantium* (300 mg/Kg bw) groups vs control group: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
$K_2Cr_2O_7$ + *C. aurantium* (100 mg/Kg bw) and $K_2Cr_2O_7$ + *C. aurantium* (300 mg/Kg bw) groups vs $K_2Cr_2O_7$ group: $^{++}p < 0.01$; $^{+++}p < 0.001$.

in heart sections of rats treated only with *C. aurantium* at both doses (Figure 4(D1, D2)).

Discussion

The present study provides the first evidence on the preventive effect of the ethanolic extract prepared from *C. aurantium* peels against myocardial oxidative injury induced in the heart of Cr(VI) exposed adult rats.

Cr(VI) is one of the most ubiquitous and toxic heavy metals. In recent years, there has been an increasing ecological and public health concern associated with environmental contamination by this metal. In fact, Cr(VI) has been reported to cause a number of clinical complications including cardiotoxic effects. Oxidative stress has been reported among the main contributing factors to the cardiotoxicity induced by this metallic element.

Lipid peroxidation is one of the major manifestations of Cr(VI) induced cardiac oxidative damage (7). This process is activated by the highly reactive hydroxyl radical through extracting an allylic hydrogen from polyunsaturated fatty acids of cell membranes. In the present study, increased levels of MDA, the end product of lipid peroxidation, were observed in the heart tissue of K_2Cr_2O-treated rats, confirming the earlier findings of Soudani *et al.* (7). This result reflected oxidative damage of the myocardial membrane lipids due to an increased generation of hydroxyl radicals associated with chromium reduction. In fact, Cr(VI) can easily pass through cell membranes via non-specific anion transporters. Inside the cell, Cr(VI) is metabolically reduced to reactive intermediates such as CrV, CrIV and CrIII. During this process, molecular oxygen is reduced to superoxide anion, which is further converted, by dismutation, to hydrogen peroxide (H_2O_2). The resultant intermediates react with H_2O_2 to generate, via Haber–Weiss- or Fenton-like reaction, hydroxyl radicals (38), causing diverse cytotoxic effects.

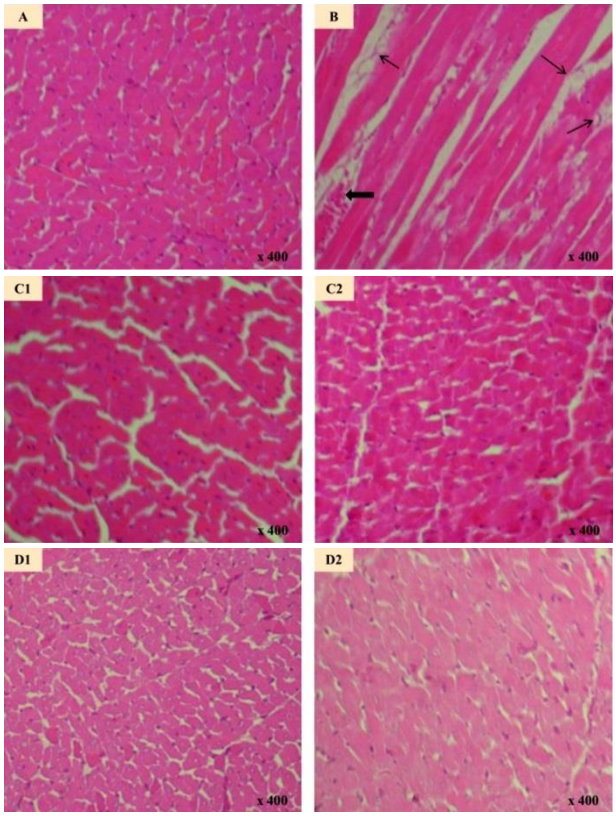

Figure 4 Heart histological sections of control (A) and treated rats with $K_2Cr_2O_7$ (B), $K_2Cr_2O_7$ + C. aurantium at 100 mg/kg bw (C1), $K_2Cr_2O_7$ + C. aurantium at 300 mg/kg bw (C2), C. aurantium at 100 mg/kg bw (D1) or C. aurantium at 300 mg/kg bw (D2). Optic microscopy: Hematoxylin & Eosin (x400).
The arrows indicate :
Hemorrhage Vacuolization

However, pre-treatment with the two tested doses of the ethanolic extract of *C. aurantium* resulted in a significantly lower degree of lipid peroxidation in the heart of adult rats. These improvements were in a dose related manner. This may be attributed to the inhibitory effect of this extract on lipid peroxidation, resulting in the protection of integrity and functioning of tissues and cells. The anti-lipid peroxidative effect of *C. aurantium* extract could be ascribed to its ROS scavenging activity. In fact, in our present study, the *in vitro* evaluation of the antioxidant capacity of *C. aurantium* extract showed that this extract is endowed with a H_2O_2 scavenging activity. Moreover, this extract was found to display an antiradical activity against DPPH radicals reflecting its potent proton-donating ability. *C. aurantium* extract exerted also a reducing power which could be due to the presence of reductones, being involved in breaking the free radical chain reaction. So, it can be suggested that *C. aurantium* extract decreased the levels of pro-oxidant agents like H_2O_2 and/or neutralized free radicals produced during the metabolic reduction of Cr(VI) in the heart of adult rats which protected lipid membranes from oxidative insult.

Irreversible protein modification such as the generation of carbonyls is another consequence of oxidative stress. The excessive ROS generation can trigger protein auto-oxidation with consequent formation of PCO and AOPP (39), valuable markers of oxidant-mediated protein damage. Protein oxidative modification alters the biological properties of these biomolecules leading consequently to their fragmentation, increased aggregation and enzyme dysfunction (39). Earlier studies showed that chromium exposure induces protein oxidation in several organs of experimental rodents such as uterus and ovaries of female

rats (40), mouse liver (41) and rat lung (18), while no information is available regarding the heart tissue. Results from our study showed for the first time the increased levels of PCO and AOPP in the heart of K_2Cr_2O-treated rats. These findings postulate protein oxidation as a possible mechanism by which this heavy metal mediates its cardiotoxic effects. Increasing evidence suggests that irreversible oxidative modifications of proteins are important in the pathophysiology of CVDs (42).

Notably, pre-treatment with *C. aurantium* extract at the high and low doses reduced the damage to proteins as evidenced by the decreased cardiac levels of PCO and AOPP. However, better results were obtained using the high dose. Attenuation of protein oxidation may be due to the ROS scavenging capacity and the reducing ability of this extract.

The myocardium has different antioxidative mechanisms to alleviate oxidative challenge and repair the damaged macromolecules. The primary defense is offered by enzymatic antioxidants such as SOD, CAT and GPx, which have been shown to scavenge ROS. Non-enzymatic antioxidants constitute a second line of cellular defense against free radicals. Among them, GSH is considered to be the most important antioxidant. This sulfhydryl containing tripeptide is the most abundant non-protein thiol which functions as an antioxidant through donating its reducing equivalents to unstable species like free radicals. GSH is also directly involved in recycling vitamin C, a water soluble antioxidant compound well recognized for its powerful inhibitor effect on lipid peroxidation in biological systems. In agreement with the previous findings of Soudani *et al.* (7), our data demonstrated a marked increase in myocardium SOD, CAT and GPx activities in K_2Cr_2O-treated rats. A similar pattern was observed regarding the cardiac levels of NPSH, GSH and vitamin C. These results reflect probably an adaptive response towards the increased ROS generation following Cr(VI) exposure.

Interestingly, a significant decrease in the cardiac activities of CAT, SOD and GPx, as well as the levels of GSH, NPSH and vitamin C was observed in rats pre-treated with *C. aurantium* extract at the dose of 300 mg/kg bw, as compared to chromium exposed rats. Using the dose of 100 mg/kg bw was found to reduce changes in NPSH level, while no significant improvement was observed regarding CAT, SOD, GPx, GSH and vitamin C. A possible explanation for the observed decrement in the antioxidant status of rats pre-treated with *C. aurantium* as compared to K_2Cr_2O-treated rats, is that this extract through its antioxidant capacity, helped the endogenous enzymatic and non-enzymatic antioxidants in the removal of ROS generated by Cr(VI) which led to the decrease of their levels.

Our biochemical data are in close agreement with the histopathological findings of the cardiac tissue. Indeed, Cr(VI) exposure induced alterations of the normal heart histoarchitecture, manifested by hemorrhage and cytoplasmic vacuolization of cardiac muscle cells, a step known to precede the necrosis process. These morphological pathologies could result from the increased generation of ROS following Cr(VI) exposure. Similar histopathological observations were reported by Soudani *et al.* (7). Pre-administration of *C. aurantium* extract at both doses to K_2Cr_2O-treated rats showed a protective effect with less disruption of the cardiac tissue, confirming its efficacy against the cardiotoxicity of this metal.

The antioxidant and cardioprotective beneficial effect of *C. aurantium* peel extract may be attributed to its bioactive phytochemical constituents. In fact, we have shown in the present study that this extract contains considerable amounts of vitamin C, phenolic compounds and flavonoids. The presence of condensed tannins has been also reported previously by Soudani *et al.* (18).

The usefulness of vitamin C as a cardioprotective agent has been well documented (43). It is also worth mentioning that in our *in vitro* study, vitamin C showed a more efficient antioxidant activity than the tested plant extract. However, in our *in vivo* study, *C. aurantium* peel extract was used for the treatment of cardiotoxicity associated with Cr(VI) exposure. Indeed, our purpose was to valorize the peels of this plant, which are considered as by-products and often discarded as waste, in order to evaluate their potential use as a dietary source of flavonoids, that could be used in protecting persons exposed to Cr(VI) in the workplace. Phenolic compounds, as well, can act as antioxidants through several mechanisms including radical scavenging (44), singlet oxygen quenching (45) and metal chelation (46). Growing evidence from epidemiological studies strongly support that phenolic compounds contribute to the prevention of CVDs (47, 48). The identification of the phenolic compounds present in the ethanolic extract of *C. aurantium* peel has been previously performed by Soudani *et al.* (18) using HPLC/MS. These authors have shown the richness of this extract in naringin, naringenin, quercetin, poncirin, isosinensetin and tetramethyl-o-isoscutellarein. Naringin, naringenin and quercetin are the strong scavengers of free radicals and prevent lipid peroxidation (49, 50). The ameliorative effects of these flavonoids against cardiac toxicity in rats have been reported in several

reports. In fact, pre-treatment with naringin or quercetin was shown to be effective in preventing cardiotoxicity associated with isoproterenol exposure in rats (51, 52). Moreover, the prior administration of naringenin ahead of doxorubicin challenge was found to attenuate cardiac oxidative damage induced by this antibiotic in rats (53). In addition to that, cardioprotective properties such as antihypertensive (15) and antiatherogenic (16) activities have been attributed to naringin and naringinin respectively. Quercetin has been also demonstrated to exert blood pressure lowering effects (54). In our present work, we showed that Cr(VI) treatment induced oxidative stress in the heart tissue. Considerable evidence indicates that the oxidative stress process plays a pivotal role in the progression of CVDs such as atherosclerosis (55), hypertension (56) and heart failure (57). Based on these data, *C. aurantium* peel extract could be suggested in the treatment of pathological conditions related to heart diseases. Future investigations are required to examine these suggestions.

Hence, the mechanisms of the protective effect of *C. aurantium* peel extract against Cr(VI) myocardial injury could be explained by the ROS scavenging properties of its bioactive compounds. Moreover, we cannot exclude the possibility of this extract exerting a metal chelating effect on Cr(VI), probably due to the presence of tannins, which are widely used as chelating agents. In accordance with our findings, the cardioprotective effect of other *Citrus* species, namely *Citrus hystix* (peel ethanolic extract) and *Citrus medica* (fruit ethanolic extract), has been also proven by other researchers (58, 59).

In addition to that, the hepatoprotective effect of *C. aurantium* peel extract was recently evaluated in three different models of hepatic damage. Choi *et al.* (60) have reported that this extract shows antioxidant activity and ameliorates ethanol-induced liver injury through modulating adenosine monophosphate activated protein kinase and nuclear factor erythroid-2-related factor 2 in a bindge drinking mouse model. Likewise, Kim *et al.* (61) and Lim *et al.* (62) have demonstrated that *C. aurantium* peel extract regulates efficiently, with its antioxidant, anti-inflammatory and antiapoptotic activities, carbon tetrachloride and bile duct ligation-induced hepatotoxicity in mice. Taken together, these data underline the therapeutic values of *C. aurantium* peel extract.

Conclusion

In summary, the biochemical and histopathological data obtained from the present study indicated that *C. aurantium* peel extract offered, in a dose dependent manner, a protection to the myocardium of adult rats against $K_2Cr_2O_7$-induced cardiac oxidative damage. The protective effect of *C. aurantium* could be attributed to the combined antioxidant activities of its different bioactive phytochemical constituents. Therefore, *C. aurantium* peel may be valorized for the development of beneficial nutraceuticals to combat cardiotoxicity associated with Cr(VI) exposure.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

All authors contributed in the studies performed and in the preparation of the manuscript.

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