# An investigation of the effect of chlorogenic acid on potassium dichromate- induced oxidative stress in rats

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# Abstract

Hexavalent chromium (CrVI) is known to be a potential hepatotoxic and nephrotoxic contaminant in humans and animals, with toxicity associated with oxidative stress and inflammation. The aim of this study was to evaluate the potential protective effect of chlorogenic acid (CGA), which has known anti-inflammatory and antioxidant effects, on potassium dichromate (K2Cr2O7)-induced acute hepatotoxicity and nephrotoxicity in rats. A total of 36 male Wistar albino rats were separated into 6 groups. The rats were first administered CGA (10, 20, or 40 mg/kg) and 6 hours later a single dose of K2Cr2O7 (15mg/kg) was administtered intraperitoneally. All the rats were sacrificed 24 hours after the final drug administration, then serum, liver and kidney tissues were examined biochemically (MDA, GSH, SOD, CAT, GPx, TNF-α, IL-1β, IL-6), histopathologically, and immunohistochemically (NFKB, VEGF iNOS, eNOS). The serum GSH level and the liver CAT, TNF-α, and IL-1β levels were determined to be lower in the group given K2Cr2O7 than in the control group, and the kidney GSH and serum IL-6 levels were found to be increased. The application of K2Cr2O7 was determined to have led to histopathological and immunohistochemical changes in rat liver and kidney tissues. With the application of chlorogenic acid, especially at the 10mg/kg dosage, the above-mentioned parameters approached normal levels. It was concluded that oxidative stress and inflammation played a key role in acute hepato and nephrotoxicity caused by $K_2Cr_2O_7$ and these harmful effects were prevented by chlorogenic acid (especially at the 10mg/kg dosage) suppressing inflammation.


# Introduction

With an abundance found in the environment, Chromium (Cr) has become a threat to human society because of its toxicity (Salama et al.,2021). The toxicity of Cr varies depending on the chemical form and dose and duration of exposure. Although it is found at several values (between -2 and +6), Cr mostly occurs in the environment in trivalent [Cr(III)] and hexavalent forms (Cr[VI]) (Balali-Mood et al.,2021). The hexavalent form is generally associated with oxygen (chromate $CrO_4^{2-}$; dichromate $Cr_2O_7^{2-}$) and the toxic, carcinogenic, and mutagenic effects on humans and other living organisms are well known (Holmes et al.,2008). According to the International Agency for Research on Cancer (IARC) report, hexavalent chromium is classified as a Group I occupational carcinogenic (Loomis et al., 2018).

After penetrating the cell membrane, hexavalent chromium binds in the form of oxygen chromate ($CrO_4^{2-}$) or dichromate ($Cr_2O_7^{2-}$) with very high oxidative capacity. In this form, Cr passes through biological membranes very well. By entering into a reaction with intracellular protein components and nucleic acids, it loses oxygen and reverts to $Cr^{3+}$ form. As a result of this reaction with genetic material, reactive oxygen species (ROS) emerge (Mattia et al.,2004). Previous studies have shown that in experimental animal models, $K_2Cr_2O_7$ (CrVI) leads to hepatotoxicity by inducing oxidative stress (Navya et al.,2017, Navya et al., 2018). In a hepatotoxicity model created in rats with the administration of 2mg/kg hexavalent chromium for 3 weeks, El-Demerdash et al (2021a) reported that with oxidative stress there was an increase in liver function enymes and a decrease in antioxidant enzyme activities.

The kidneys have become the focus of research in studies of potassium dichromate toxicity as the kidneys are the primary excretory organ of potassium dichromate and there is a tendency for a high level of accumulation in this tissue (Avila-Rojas et al 2018, Feng et al 2021). The application of potassium dichromate ($K_2Cr_2O_7$) can also be used as an acute kidney damage model (Gumbleton et al 1988, Avila-Rojas et al 2020). Kidney damage has been determined with biochemical, histopathological, and immunohistochemical methods in rats exposed to potassium dichromate, and this damage has been associated with apoptosis, inflammation, and over-production of free radicals (Awoyomi et al 2021).

Reactive oxygen species (ROS) are important signal molecules which play a role in the prognosis of most inflammatory diseases. ROS produced by polymorphonuclear neutrophils, which are a prominent component in immunological defence in the region of inflammation, cause endothelial dysfunction and tissue damage (Mittal et al 2014). Cytokines are low-molecular weight, soluble proteins, which provide cell-cell interaction and communication. These proteins function in the regulation of the immune system responses, inflammatory response, and tissue healing. The main cytokine initiating the inflammatory response is tumour necrosis factor- alpha (TNF-α), which has the effect of inducing other cytokines such as interleukin 1β (IL-1β) and interleukin-6 (IL-6). Therefore, a decrease in TNF-α synthesis/expression or in the amount of other proinflammatory cytokines is important for the anti-inflammatory effect (Nantel et al 1999). In addition, various inflammatory stimuli such as heavy metals and inflammatory cells such as macrophages can affect the nuclear factor κB (NFκB) signal pathway. NF-κB activation also induces inducible nitric oxide synthesis transcription leading to nitric oxide (NO) production. NO is a proinflammatory mediator that contributes to the pathogenesis of inflammatory diseases. Over-production of these proinflammatory mediators leads to inflammation, as does the activation of NF-κB. Consequently, it is important to evaluate proinflammatory mediator levels and NF-κB activity as a substance for anti-inflammatory activity.

Polyphenols in medical and dietary plants are antioxidants which have therapeutic and preventative roles in different pathological conditions such as oxidative stress and inflammation. One of the polyphenols most studied in the last ten years is chlorogenic acid (CGA), which is a potent antioxidant and anti-inflammatory found in some food and drinks such as apples, coffee beans, tomatoes, potatoes and apricots (Bagdas et al 2020). CGA has protective effects against various metabolic diseases (Zamani-Garmsiri et al 2021). Yun et al (2012) showed that CGA ameliorated hepatic ischaemia and reperfusion damage. Zhao et al (2008) reported that IL-8 and mRNA expression due to oxidative stress was significantly inhibited by CGA. The efficacy of CGA in several heavy metal toxicities, such as cadmium (Shi et al 2021), lead (Ji et al 2021), and arsenic (Dkhil et al 2020) has also been shown.

However, to date there is no information in literature about the potential benefits of CGA against potassium dichromate-related toxicity. The aim of this study was to investigate potassium dichromate-induced toxicity in rats and to evaluate the potential protective effect of chlorogenic acid against oxidative stress and inflammatory response to potassium dichromate.

# Material And Method

## Chemicals and Kits

Chlorogenic acid (C3878) and potassium dichromate (207802) were purchased from Sigma. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), tumour necrosis factor-alpha(TNF-α), interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) kits were obtained from Bioassay Technology Laboratory (Shanghai, China). NFKB (SC-8008), VEGF (SC-7269), eNOS (SC-376751), iNOS (SC-7269).

## Animals and Experiment Set-Up

The study sample comprised 36 male albino Wistar rats, 8-10 weeks old, each weighing 200-250gr. The animals were obtained from Afyon Kocatepe University Experimental Animal Research and Application Centre (Afyonkarahisar, Turkey). The care of the rats and all the experimental procedures were applied in the framework of the ethical rules and regulations of the Experimental Animals Local Ethics Committee of Afyon Kocatepe University. The study was approved by the Experimental Animals Local Ethics Committee of Afyon Kocatepe University (protocol no: 2016/54).

The animals were given a week of acclimatisation to the laboratory environment (temperature: 22±2°C, humidity: 55-60%, 12-hour light-dark cycle), with free access to commercial rodent food and tap water. The 36 male albino Wistar rats were then randomly separated into 6 groups of 6. The rats were weighed then the experimental applications were performed as follows with a single intraperitoneal injection at 0.5ml volume: Group 1 was defined as the control group and was injected with physiological saline. Group II received 15mg/kg potassium dichromate, Groups III, IV, and V were administered 10, 20, and 40 mg/kg CGA 6 hours before the potassium dichromate dose (15mg/kg), and Group VI was injected with CGA only (40mg/kg). The doses were selected according to literature data based on inducing oxidative damage and the possibility of protection (El-Guendouz et al 2020, Wang et al 2018).

## Preparation of serum and tissue homogenates for biochemical measurements

At 24 hours after the final drug administration, under anaesthesia (ketamine-xylazine), the rib cages of the rats were opened with an appropriate technique and 3-5ml blood was collected from the heart in the working position. The blood samples were withdrawn into tubes without anticoagulant and centrifuged at 3500rpm for 10 mins in a Nuve NF 1000 R model centrifuge device for separation of the serum. The serum samples were stored at -70°C until the biochemical measurements were performed.

The rats were sacrificed using the exsanguination method, then liver and kidney tissues were excised and washed in cold physiological saline. These tissues were separated into two parts, one part for biochemical analyses and the other part for histopathological analyses. The tissue samples for biochemical analysis were placed in a tube with a thick grinder in a solution of 1 part tissue to 9 parts 0.15 M KCl (pH: 7.4) and homogenised over ice in a Teflon homogenisator. The homogenised tissues were centrifuged at 4000 rpm for 10 mins at 4°C, and the supernatant was obtained. The supernatant was stored at -70°C until the biochemical measurements were performed.

MDA and GSH levels, SOD, CAT, and GPx activities, and TNF-α, IL-1β and IL-6 levels in the serum and liver and kidney homogenates were determined using commercial rat ELISA kits (Bioassay Technology Laboratory, Shanghai, China).

### Preparation of tissues for histopathological analyses

For histopathological examination, the tissue samples were fixed in 10% formaldehyde, then passed through graded alcohol series and xylene, after which they were embedded in paraffin blocks and slices 5μm in thickness were cut and placed on polylysinized slides. The prepared slices were stained for 8 mins with hematoxylin, washed and then stained with eosine, and then covered with a xylene-based medium. For the histomorphometric evaluation, the slides were visualised under an Olympus BX51 microscope. Liver and kidney lesions were graded semi-quantatively according to the Gibson-Corley et al (2013) classification as -:no lesion, +: mild, ++: moderate, and +++: severe.

### Immunohistochemical staining

The 5μm thick slices from the tissues were boiled for 28 mins in a microwave with citrate buffer (pH 6.0) for the antigen retrieval procedure. After washing, 3% H2O2 was applied for 5 mins. The slices were washed 3 times with PBS for 5 mins, then left in blocking solution for protein blocking. The slices were incubated overnight at +4°C with primary antibodies anti-VEGF, anti-NF kappa B, anti-NOS2, and anti-NOS3. The washed slices were then incubated for 30 mins with anti-mouse biotin streptavidin hydrogen peroxidase secondary antibody. Staining was applied with AEC. After nuclear staining with Mayer's hematoxylin, the slices were covered with water-based coverage medium, and immunohistochemical evaluation was made under a light microscope.

### Histopathological evaluation method

The histopathological evaluation of the liver and kidney tissues was made with magnification under a light microscope. On the kidney tissue, glomerular areas and tubulointerstitial areas were evaluated separately. All the glomerular areas in each section of the kidneys were graded according to the severity of glomerular damage as 0:normal areas, 1: mild glomerular damage with focal adhesion hyalinosis involving 25% of the glomerulus and/or mesangial matrix, 2: 25%-50% sclerosis, 3: 50%-75% sclerosis, and 4: 75% sclerosis of the glomerulus.

The tubulointerstitial areas of the cortex were graded as 0: normal, 1:tubulointerstitial inflammation and fibrosis covering 25% of the area and expanded areas with tubular atrophy and shedding, 2: lesion and bleeding in 25%-50% of the area, 3: lesions and bleeding covering 50%. The glomerular damage or tublointerstitial injury indexes and the grades of all the glomerular or tubular areas were determined for each sample and the mean values were calculated (Cao et al 2002, Hassanen et al 2019).

To determine the liver tissue damage, hepatocellular degeneration, necrosis, fibrosis, and various focus criteria were used. Pathological changes were graded on a scale of 0-4 as certain-uncertain, mild,

moderate, or severe, and were scored as 0: normal histology, 1: tissue damage <25%, 2: 25-50%, 3:50-75%, 4: >75% (Hassanen et al 2019).

### Immunohistochemical evaluations of liver and kidney tissues:

A semiquantitative method was used in the immunohistochemical evaluation and H-score evaluation was made according to the extent of the area of stained cells and staining severity. The mean ratio of the area stained of the stained cells was graded as 0:<1%, 1:1-25%, 2: 26-50%, 3:51-75%, and 4:>75%. The severity of staining was graded as 0: negative staining, 1:weak staining, 2: moderate staining, and 3: strong staining. The histological score (H-score) for each sample was calculated as:

H-score = the grade of the stained cell area x mean staining severity

The total score was calculated in the range of 0-12, evaluated as negative (-, 0 points), weak (+, 1-4 points), moderate (++, 5-8 points), or strong (+++, 9-12 points) (Wang et al 2014).

### Statistical Analysis:

Data obtained in the study were analyzed statistically using SPSS vn. 20.0 software. Descriptive statistics were stated as number (n) and percentage (%) for qualitative data and as median and interquartile range values for quantitative data. The Chi-square test was used in the evaluation of categorical data and the Kruskal-Wallis test for continuous data. When the hypothesis was accepted as a result of the Kruskal Wallis test, the multiple comparison Dunn test was used to determine from which group or groups the difference originated. The level of statistical significance was set at p=0.05.

# Results

### The effects of chlorogenic acid on serum oxidative stress/antioxidant parameters changed by potassium dichromate

The effects of potassium dichromate, chlorogenic acid, and the combination of these on serum oxidative stress/antioxidant parameters are presented in Table 1. No significant difference was found between the control group (Group I) and all the other groups in respect of serum MDA levels and SOD and GPx activity (p>0.05).

Table 1
The serum oxidant-antioxidant parameters of all the groups.

| | Group 1 (n=6) | Group 2 (n=6) | Group 3 (n=6) | Group 4 (n=6) | Group 5 (n=6) | Group 6 (n=6) | *P value* |
|---|---|---|---|---|---|---|---|
| MDA nmol/ml Median(IQR) | 1,175± 0,13 | 1,200±0,12 | 1,060± 0,220 | 1,085± 0,150 | 1,125± 0,16 | 1,130± 0,110 | 0,144 |
| GSH mg/L Median(IQR) | 384,830± 141,08$^{bcd}$ | 185,375 ±70,47$^{a}$ | 168,040± 376,300$^{ad}$ | 299,485± 170,700$^{ac}$ | 396,195± 137,85$^{bcd}$ | 458,240± 219,39$^{b}$ | 0,014 |
| SOD ng/ml Median(IQR) | 2,075± 0,28 | 2,160 ±0,130 | 2,030± 0,120 | 2,140± 0,170 | 2,065± 0,37 | 1,915± 0,10 | 0,091 |
| CAT ng/ml Median(IQR) | 29,005± 6,84$^{b}$ | 34,290 ±2,39$^{bc}$ | 33,690± 5,730$^{bc}$ | 37,825± 2,110$^{ad}$ | 35,265± 7,90$^{bd}$ | 35,580± 7,20$^{acd}$ | 0,023 |
| GPX ng/ml Median(IQR) | 17,35± 11,90 | 16,875 ±12,13 | 16,070± 3,330 | 15,405± 6,200 | 13,735± 2,93 | 13,28± 0,53 | 0,067 |

MDA: Malondialdehyde, GSH: glutathione, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase,

In the comparison of the GSH levels in all the groups, with the exception of a significant decrease in Group I compared to the potassium dichromate group (Group II) ($p>0.05$), no other statistically significant difference was determined ($p>0.05$). In the comparison of all the treatment groups with the potassium dichromate group, there was a significant increase in GSH levels in the groups given chlorogenic acid at the highest dose alone (Group VI) or before potassium dichromate (Group V), and the GSH levels were observed to have reached those of the control group ($p<0.05$).

In the comparisons of the control group with all the other groups in respeect of CAT activity, with the exception of the group administered 20mg/kg CGA before potassium dichromate (Group IV) and the group given 40mg/kg CGA alone (Group VI), no other significant difference was observed ($p>0.05$). When all the treatment groups were compared with the potassium dichromate group, a significant increase was observed in CAT activity only in the group administered 20 mg/kg CGA before potassium dichromate (Group IV) ($p<0.05$).

Table 1. **The serum oxidant-antioxidant parameters of all the groups.**

**The effects of chlorogenic acid on serum proinflammatory cytokine levels changed by potassium dichromate**

The effects of potassium dichromate, chlorogenic acid, and the combination of these on serum proinflammatory cytokine levels are presented in Figure 1. No significant change was determined in serum TNF-α levels when the control group (Group I) was compared with all the other groups ($p>0.05$) (Figure 1a). No statistically significant difference was determined in the IL-1β levels between Group I and the group given potassium dichromate (Group II) ($p>0.05$).

When all the treatment groups (Groups III-VI) were compared with Group II, there was observed to be a significant decrease in the IL-1β levels of the group given CGA only at the highest dose (Group VI) and of the group administered 40mg/kg CGA before potassium dichromate (Group V) ($p<0.05$) (Figure 1b). Compared with the control group (Group I), there was seen to be a significant increase in the IL-6 level of the potassium dichromate group (Group II) ($p<0.05$). In the comparisons between the potassium dichromate group and all the CGA-administered groups (Groups III-V), no statistically significant difference was determined ($p>0.05$) (Figure 1c).

Figure 1. **The serum proinflammatory cytokine levels of all the groups**

## The effects of chlorogenic acid on the oxidative stress/antioxidant parameters of kidney tissue changed by potassium dichromate

The effects of potassium dichromate, chlorogenic acid, and the combination of these on kidney tissue oxidative stress/antioxidant parameters are presented in Table 2. No significant difference was found between the control group (Group I) and all the other groups in respect of kidney tissue MDA levels and CAT activity ($p>0.05$).

Table 2
The kidney oxidant-antioxidant parameters of all the groups

| | Group 1 (n=6) | Group 2 (n=6) | Group 3 (n=6) | Group 4 (n=6) | Group 5 (n=6) | Group 6 (n=6) | *P value* |
|---|---|---|---|---|---|---|---|
| MDA nmol/ml | 1,745± | 1,560 | 1,560± | 1,730± | 1,620± | 1,675± | 0,254 |
| Median(IQR) | 0,23 | ±0,15 | 0,14 | 0,360 | 0,35 | 0,34 | |
| GSH mg/L | 5,500± | 324,645 ±82,0[b] | 7,300± | 5,00± | 5,000± | 29,155± | 0,002 |
| Median(IQR) | 25[a] | | 1,8[a] | 0,5[a] | 0,50[a] | 104,7[a] | |
| SOD ng/ml | 3,270± | 3,280 ±0,67[a] | 2,660± | 2,610± | 2,875± | 2,905± | 0,002 |
| Median(IQR) | 0,95[a] | | 0,210[bc] | 0,140[b] | 0,46[ab] | 0,43[ac] | |
| CAT ng/ml | 45,065± | 44,160 ±3,70 | 42,030± | 44,140± | 40,490± | 42,655± | 0,495 |
| Median(IQR) | 7,99 | | 2,13 | 6,37 | 7,89 | 5,56 | |
| GPX ng/ml | 27,495± | 27,415 ±2,60[a] | 30,260± | 26,815± | 23,300± | 30,670± | 0,025 |
| Median(IQR) | 2,20[a] | | 10,30[a] | 7,80[a] | 2,30[b] | 4,90[a] | |

MDA: Malondialdehyde, GSH: glutathione, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase,

In the comparisons between the control group (Group I) and all the other groups in respect of GSH levels, with the exception of a significant increase in the potassium dichromate group (Group II) ($p<0.05$), no other significant difference was determined ($p>0.05$).

In the comparisons between the control group (Group I) and all the other groups in respect of SOD activity, a significant difference was determined only in the groups administered 10mg/kg CGA (Group III) and 20mg/kg CGA (Group IV) before potassium dichromate ($p<0.05$). With the exception of Groups III and IV, the SOD activity of the other treatment groups (Groups V and VI) was seen to be at the same level as that of the control group (Group I) and the potassium dichromate group (Group II) ($p>0.05$).

In the comparisons between the control group (Group I) and all the other groups in respect of GPx activity, with the exception of a significant decrease in the group administered 40mg/kg CGA before potassium dichromate (Group V) ($p<0.05$), no other significant difference was determined ($p>0.05$).

Table 2. **The kidney oxidant-antioxidant parameters of all the groups**

**The effects of chlorogenic acid on the kidney proinflammatory cytokine levels changed by potassium dichromate**

The effects of potassium dichromate, chlorogenic acid, and the combination of these on the kidney tissue proinflammatory cytokine levels are presented in Figure 2. No significant difference was determined between the control group (Group 1) and all the other groups in respect of kidney tissue TNF-α, IL-1β, and IL-6 levels ($p>0.05$) (Figure 2a, b, c).

Figure 2. **The kidney proinflammatory cytokine levels of all the groups**

## The effects of chlorogenic acid on the oxidative stress/antioxidant parameters of liver tissue changed by potassium dichromate

The effects of potassium dichromate, chlorogenic acid, and the combination of these on the liver tissue oxidative stress/antioxidant parameters are presented in Table 3. No significant difference was found between the control group (Group I) and all the other groups in respect of liver tissue MDA-GSH levels and SOD and GPx activity ($p>0.05$).

Table 3
The liver tissue oxidant-antioxidant parameters of all the groups

| | **Group 1 (n=6)** | **Group 2 (n=6)** | **Group 3 (n=6)** | **Group 4 (n=6)** | **Group 5 (n=6)** | **Group 6 (n=6)** | ***P value*** |
|---|---|---|---|---|---|---|---|
| MDA nmol/ml | 1,485± | 1,32 | 1,060± | 1,30± | 1,38± | 1,39± | 0,376 |
| Median(IQR) | 0,26 | ±0,43 | 0,220 | 0,55 | 0,24 | 0,54 | |
| GSH mg/L | 18,70± | 194,50 ±241 | 154,26± | 144,24± | 199,98± | 21,22± | 0,060 |
| Median(IQR) | 64 | | 129 | 254 | 36 | 238,0 | |
| SOD ng/ml | 2,480± | 2,265 ±0,38 | 2,145± | 2,28± | 2,245± | 2,30± | 0,237 |
| Median(IQR) | 0,46 | | 0,23 | 0,30 | 0,390 | 0,32 | |
| CAT ng/ml | 44,28± | 26,95 | 27,90± | 30,80± | 29,44± | 34,47± | 0,001 |
| Median(IQR) | 5,0$^{b}$ | ±5$^{a}$ | 4,0$^{ac}$ | 9,0$^{cd}$ | 3,0$^{acd}$ | 8,0 $^{bd}$ | |
| GPX ng/ml | 23,165± | 18,69 ±8,73 | 16,62± | 18,60± | 20,09± | 19,29± | 0,153 |
| Median(IQR) | 2,76 | | 3,70 | 6,74 | 8,56 | 6,76 | |

MDA: Malondialdehyde, GSH: glutathione, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase,

In the comparisons related to CAT activity, with the exception of Group VI, a significant decrease was observed in all the other groups compared to the control group (Group I) ($p<0.05$). When all the treatment groups were compared with the potassium dichromate group, a significant increase was determined in CAT activity only in the group administered 20mg/kg CGA before potassium dichromate (Group IV) ($p<0.05$).

Table 3. **The liver tissue oxidant-antioxidant parameters of all the groups**

## The effects of chlorogenic acid on liver tissue proinflammatory cytokine levels changed by potassium dichromate

The effects of potassium dichromate, chlorogenic acid, and the combination of these on the liver tissue proinflammatory cytokine levels are presented in Figure 3.

When the groups were examined in respect of TNF-α levels, a statistically significant difference was only determined between the control group (Group I) and the potassium dichromate group (Group II) ($p<0.05$). No statistically significant difference was determined between Group II and all the CGA-administered groups (Groups III-V) ($p>0.05$) (Figure 3a).

A significant decrease was seen in the IL-1β levels of all the groups compared to the control group (Group I) ($p<0.05$).

No statistically significant difference was determined between the potassium dichromate group and all the CGA-administered groups (Groups III-V) ($p>0.05$) (Figure 3b).

No significant difference was determined was determined between the control group (Group I) and all the other groups in respect of IL-6 levels ($p>0.05$) (Figure 3c).

Figure 3. **The liver tissue proinflammatory cytokine levels of all the groups**

When the histopathological semi-quantitative scores of the kidney and liver tissues were evaluated, statistically high levels of damage in the kidney tubulointerstitial and glomerular areas and in the liver tissue were determined only in the potassium dichromate group (Group II) compared to the control group (Group I) ($p<0.05$) (Table 4). When all the treatment groups were compared with Group II, the damage in these tissues was determined to have been ameliorated at a significant level only in the group administered 10mg/kg CGA before potassium dichromate (Group III) ($p<0.05$). As the CGA dose increased, the damage in these areas was determined to be at a mild and moderate level compared with the control group (Table 4).(Figure 4)

Table 4
Semi-quantitative histopathological scoring of the kidney and liver tissues

| | | Group I (n=6) | Group II (n=6) | Group III (n=6) | Group IV (n=6) | Group V (n=6) | Grup VI (n=6) | *P* |
|---|---|---|---|---|---|---|---|---|
| **Renal tubulointerstitial space** | No damage | 5 | 0 | 1 | 0 | 0 | 0 | <0,001 |
| | Damage is light | 1 | 0 | 4 | 0 | 0 | 4 | |
| | Damage moderate | 0 | 1 | 0 | 2 | 2 | 1 | |
| | Damage is high | 0 | 5 | 1 | 4 | 4 | 1 | |
| **Renal glomerular area** | No damage | 6 | 0 | 2 | 0 | 0 | 0 | <0,001 |
| | Damage is light | 0 | 0 | 3 | 2 | 0 | 4 | |
| | Damage moderate | 0 | 1 | 1 | 3 | 3 | 2 | |
| | Damage is high | 0 | 5 | 0 | 1 | 3 | 0 | |
| **Liver assessment** | No damage | 5 | 0 | 0 | 0 | 0 | 0 | <0,001 |
| | Damage is light | 1 | 0 | 4 | 2 | 1 | 3 | |
| | Damage moderate | 0 | 2 | 2 | 3 | 3 | 3 | |
| | Damage is high | 0 | 4 | 0 | 1 | 2 | 0 | |

Table 4. **Semi-quantitative histopathological scoring of the kidney and liver tissues**

Figure 4: **H&E staining of the kidney and liver tissues of the groups.**

When the immunohistochemical semi-quantitative scores of the kidney and liver tissues were evaluated, with the exception of eNOS and NFKB activities in the liver tissue ($p>0.05$), a statistically sginificant difference was determined in all the other parameters between the control group (Group I) and all the other groups ($p<0.05$) (Tables 5, 6).

Table 5
Semi-quantitative immunohistochemical scoring of the kidney tissues

| | | Group I (n=6) | Group II (n=6) | Group III (n=6) | Group IV (n=6) | Group V (n=6) | Group VI (n=6) | *P* |
|---|---|---|---|---|---|---|---|---|
| **Renal iNOS** | No damage | 3 | 0 | 4 | 0 | 0 | 2 | <0,007 |
| | Damage is light | 3 | 3 | 2 | 5 | 6 | 4 | |
| | Damage moderate | 0 | 3 | 0 | 1 | 0 | 0 | |
| | Damage is high | 0 | 0 | 0 | 0 | 0 | 0 | |
| **Renal eNOS** | No damage | 3 | 0 | 6 | 0 | 0 | 1 | <0,001 |
| | Damage is light | 3 | 3 | 0 | 4 | 5 | 5 | |
| | Damage moderate | 0 | 3 | 0 | 2 | 1 | 0 | |
| | Damage is high | 0 | 0 | 0 | 0 | 0 | 0 | |
| **Renal VEGF** | No damage | 0 | 0 | 0 | 0 | 0 | 0 | <0,021 |
| | Damage is light | 5 | 1 | 6 | 4 | 4 | 6 | |
| | Damage moderate | 1 | 5 | 0 | 1 | 1 | 0 | |
| | Damage is high | 0 | 0 | 0 | 1 | 1 | 0 | |
| **Renal NFKB** | No damage | 0 | 0 | 0 | 0 | 0 | 0 | <0,002 |
| | Damage is light | 5 | 0 | 5 | 1 | 0 | 4 | |
| | Damage moderate | 1 | 2 | 1 | 3 | 5 | 2 | |
| | Damage is high | 0 | 4 | 0 | 2 | 1 | 0 | |

iNOS: Inducible nitric oxide synthase, eNOS: endothelial nitric oxide synthase, VEGF: Vascular Endothelial Growth Factor,

NF-κB: Nuclear factor-κB

Table 6
Semi-quantitative immunohistochemical scoring of the liver tissues

| | | Group I (n=6) | Group II (n=6) | Group III (n=6) | Group IV (n=6) | Group V (n=6) | Group VI (n=6) | *P* |
|---|---|---|---|---|---|---|---|---|
| **Liver iNOS** | No damage | 3 | 0 | 0 | 0 | 0 | 3 | <0,001 |
| | Damage is light | 3 | 2 | 6 | 3 | 3 | 3 | |
| | Damage moderate | 0 | 4 | 0 | 3 | 0 | 0 | |
| | Damage is high | 0 | 0 | 0 | 0 | 3 | 0 | |
| **Liver eNOS** | No damage | 0 | 0 | 0 | 0 | 0 | 1 | 0,137 |
| | Damage is light | 5 | 3 | 6 | 2 | 3 | 5 | |
| | Damage moderate | 1 | 3 | 0 | 4 | 2 | 0 | |
| | Damage is high | 0 | 0 | 0 | 0 | 0 | 0 | |
| **Liver VEGF** | No damage | 6 | 0 | 0 | 0 | 0 | 0 | <0,001 |
| | Damage is light | 0 | 6 | 6 | 5 | 0 | 6 | |
| | Damage moderate | 0 | 0 | 0 | 1 | 5 | 0 | |
| | Damage is high | 0 | 0 | 0 | 0 | 1 | 0 | |
| Liver **NFKB** | No damage | 1 | 0 | 1 | 0 | 0 | 0 | 0,590 |
| | Damage is light | 5 | 6 | 5 | 5 | 5 | 5 | |
| | Damage moderate | 0 | 0 | 0 | 1 | 0 | 1 | |
| | Damage is high | 0 | 0 | 0 | 0 | 1 | 0 | |

iNOS: Inducible nitric oxide synthase, eNOS: endothelial nitric oxide synthase, VEGF: Vascular Endothelial Growth Factor,

NF-κB: Nuclear factor-κB

When compared with the control group (Group I), a statistically significant difference was only determined in the potassium dichromate group (Group II) in the kidney tissue iNOS (NOS2), eNOS (NOS3), VEGF, and NFKB activities and the liver tissue iNOS (NOS2) and VEGF activities ($p<0.05$). In the comparison of all the treatment groups (Groups III-V) with the potassium dichromate group (Group II), the damage in these tissues was seen to have been significantly ameliorated only in the group administered 10mg/kg CGA before potassium dichromate ($p<0.05$) (Tables 5, 6). (Figure 5,6).

Table 5. **Semi-quantitative immunohistochemical scoring of the kidney tissues**

Figure 5: **Immunohistochemical staining of the kidney tissues shows the changes**

Table 6. **Semi-quantitative immunohistochemical scoring of the liver tissues**

Figure 6: **Immunohistochemical staining of the liver tissues shows the changes**

# Discussion

The aim of this study was to investigate the hepato and nephro protective role of chlorogenic acid against oxidative damage and disruption to the antioxidant defence system caused by potassium dichromate in rats in the light of biochemical, histopathological, and immunohistochemical evaluations.

Oxidative stress is one of the important factors playing a role in the toxic effects of many environmental pollutants such as heavy metals. Recent experimental studies have drawn attention to the use of various antioxidants to be able to overcome oxidative events due to potassium dichromate, which is thought to play a role in tissue damage formed by oxidative stress (Awoyomi et al 2021, El-Demerdash et al 2021b, Bashandy Samir et al 2021, Fedala et al 2021). Although chlorogenic acid is known to havee potent antioxidant and anti-inflammatory properties, its protective effect against oxidative damage created by potassium dichromate has not yet been investigated. This study is the first to have investigated the above-mentioned effects of CGA on acute potassium dichromate toxicity.

Several metals with redox potential, such as chrome, damage the lipid components of the cell membrane by leading to an increase in the production of reactive oxygen species (ROS) such as hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet -}$), or $H_2O_2$ (hydrogen peroxide), mediated by the redox cycle and Fenton reactions, and consequently cause an increase in the malondialdehyde (MDA) level, which is one of the end products of lipid peroxidisation (Stohs et al 1995, Shi 1999a). Several previous studies have reported that exposure to potassium dichromate causes a decrease in reduced glutathione (GSH) levels to cope with oxidative stress and the increase in serum and liver and kidney tissue MDA levels (El-Demerdash et al 2021a, El-Demerdash et al 2021b, Al Jameil et al 2017, Mary Momo et al 2019). In contrast, other studies have shown that the MDA level does not change or decreases in oxidant exposure (Garcia et al 2020, Lima et al 2019, Ubani-Rex et al 2017). The results of the current study showed that the serum, and liver and kidney tissue MDA levels did not change in the group administered potassium chromate when compared with the control group, and although the serum GSH level decreased, which was consistent with the findings of other studies, it did not change in the liver tissue, and was seen to have significantly increased in the kidney tissue. A previous study that was methodologically similar to the current study also found no change in the plasma and kidney MDA and GSH levels (García-Niño et al 2015).

These conflicting results can be attributed to differences in the administration route, duration and dose of potassium dichromate. It has also been reported in acute studies that the GSH level can be increased in some organs to be able to detoxify $H_20_2$, which has been over-produced against a high dose of foreign

substance administered to the body (Onate et al 2017, Somade et al 2016). Therefore, the elevated GSH level in the kidney tissue of the potassium dichromate group compared to the control group in the current study could be associated with over-production of $H_20_2$.

Organisms also work together with the enzymatic antioxidant defence system such as SOD, CAT and GPx to ameliorate oxidative stress or repair macromolecules damaged due to exposure to xenobiotics or during normal metabolism. SOD protects against superoxide radical ($O_2^-$) which damages the cell membrane and its biological structure. SOD converts $O_2^{-'}$ to hydrogen peroxide ($H_2O_2$) and then, CAT and GPx are the enzymes responsible for the water detoxification of the created $H_2O_2$ (Turkmen et al 2019). CAT is found in all the major tissues and organs of humans and animals. GPx may also be significantly affected by the removal of lipid hydroperoxide. $O_2^-$ and hydroxyl radicals ($OH^{\bullet}$) are known to be agents leading to significant damage in organs and tissues (Kilic et al 2014).

In the current study, there was observed to be no change in SOD, CAT (except liver tissue CAT), and GPx activities in the potassium dichromate group compared to the control group. Consistent with these results, Garcia-Nino et al (2015) also reported no change in these antioxidant enzymes in brain, heart, lung, kidney, spleen, pancreas, stomach and intestine tissues in rats administered 15mg/kg potassium dichromate intraperitoneally. However, in the current study, the serum and liver tissue CAT enzyme activity was determined to be increased in the group that received 20 mg/kg CGA. The increase in CAT activity may reduce free radicals formed by potassium dichromate and CGA may prevent hepatotoxicity due to potassium dichromate. The current study results are consistent with the findings of Susa et al (1997) and Cengiz et al (2016), which demonstrated that antioxidant applications increased CAT enzyme activity.

Reactive oxygen species (ROS) are important signalling molecules that play a role in many inflammatory diseases. ROS produced by polymorphonuclear neutrophils (PMN), which are a component emerging in the immunological defence of the inflammatory region, cause endothelial dysfunction and tissue damage (Mittal et al 2014).

Cytokines are low-molecular weight, soluble proteins, which provide cell-cell interaction and communication. These proteins function in the regulation of immune system responses, the inflammatory response and tissue healing. Pro-inflammatory cytokines, such as nuclear factor-κB (NFκB), interleukin-1β (IL-1β), and TNF-α are transcription factors necessary for gene regulation and activation (Kuçukler et al 2021). There are studies in literature showing that potassium dichromate plays an active role in the transcription of some proinflammatory cytokines, primarily TNF-α and IL-1β, by increasing NFκB (Shi et al 1999b). In a previous study in which acute kidney damage was created with potassium dichromate, it was reported that intraperitoneal administration of 4mg/kg potassium dichromate for 35 days increased kidney NFκB and TNF-α mRNA activities and increased proinflammatory cytokine levels such as TNF-α, IL-1β, and IL-6 in liver tissue compared to the control group, and the administration of melatonin suppressed the inflammatory mediators induced by potassium dichromate (Han et al 2019). In the current study, a single intraperitoneal dose of 15mg/kg potassium dichromate only increased the serum IL-6 level compared to the control group, did not affect kidney tissue proinflammatory cytokine

levels, and was found to reduce the liver tissue TNF-α and IL-1β levels. In addition, the administration of CGA at different doses to the potassium dichromate groups was not found to lead to a statistically significant decrease in proinflammatory cytokine levels compared to the potassium dichromate only group. In contrast to these results, in a mouse model of kidney damage created with lead, Zang et al (2019) showed that increasing serum TNF-α, IL-1β, and IL-6 levels were prevented by CGA in a dose-related manner. According to another study by El-Khadragy et al (2021) in which testis damage in mice was created by administering arsenic for 4 weeks, the application of CGA was shown to reduce the proinflammatory cytokine levels in the testis tissue related to the arsenic. From the different results obtained in the current study, it was concluded that the results could be related to the dose and duration of use of the toxic agents.

Nitric oxide and isoforms are extremely common signalling molecules which have an important role in the physiology and pathophysiology of the liver and kidneys (Iwakiri et al 2015, Lee 2008). In mammals, NO is synthesized by 3 different isoforms, of the enzyme group known as nitric oxide synthase, stated as 1 inducible form (iNOS or NOS2) and 2 structural forms (nNOS or NOS1 and eNOS or NOS3) (Li et al 2020). Edothelial NOS (eNOS) produces NO in small amounts in response to stimuli such as blood pressure and vascular endothelial growth factor (VEGF), and the eNOS-mediated derived NO protects the liver from homeostasis and prevents pathological conditions in the liver. In contrast, under pathological conditions, inducible NOS (iNOS) produces large amounts of NO originating from reactive nitrogen species (Iwakiri et al 2015). In a study in which a nephrotoxicity model was formed of vancomycin origin, treatment with CGA was found to reduce oxidative/nitrosative stress, inflammation, and apoptosis (Qu et al 2020). The protective effect of CGA has also been shown in experimental hepatotoxic models created with various xenobiotic agents such as drugs and heavy metals (Dkhil et al 2020, Wei et al 2018, Cheng et al 2017, Ali et al 2017). In the current study, the application of potassium dichromate was found to lead to an increase in kidney tissue iNOS, eNOS, and VEGF activities, and liver tissue iNOS and VEGF activities, and these inflammatory activities in the tissues were determined to be reduced with CGA, especially at the dose of 10mg/kg. These findings can be interpreted as the hepato and nephroprotective effect of CGA being related to its anti-inflammatory properties.

The liver and kidneys are the most important organs for the metabolism and excretion, respectively, of xenobiotics. According to the histopathological examination and quantitative evaluations made in the current study, potassium dichromate caused significant histopathological changes in the liver and kidney tissues. Compared to the control group, hyalinosis and sclerosis were observed in the renal glomeruli of the rats in the group given potassium dichromate only, inflammation in the tubulointerstitial areas of the cortex, fibrosis, tubular atrophy, dilatation, and hyperemia were also observed, and in the liver, hepatocellular degeneration, necrosis and fibrous areas. The application of CGA, especially at the dose of 10mg/kg was seen to have prevented histopathological damage in the liver and kidneys.

In a study by El-Demerdash et al (2021a, b), hepato and nephrotoxicity was created in rats with intraperitoneal administration of 2mg/kg potassium dichromate for 3 weeks. Damage was reported in the kidneys of cytoplasmic degeneration in the tubules, pyknotic nucleus, necrosis and hemorrhage, together

with dilatation and congestion in blood vessels, and liver damage including hepatocyte degeneration, necrosis, inflammation and vacuolisation. In the same study, it was concluded that the histopathological changes induced by potassium dichromate were corrected by Rosmarinus officinal L., which is a plant rich in phenolic components. There are other recent studies in parallel with the results of the current study showing that CGA prevented histopathological changes due to heavy metal toxication (Dkhil et al 2020, Shi et al 2021,Cheng et al 2019).

There were some limitations to this study, primarily that it was an experimental study with a low number of rats in each group. This problem of a low number of animals in each group was due to ethical concerns related to following the “principle of reduction”. To be able to clarify the small biochemical, histological and immunohistochemical differences between the groups, it would be necessary to use much greater numbers of animals. A second limitation was the use of a subjective scoring system to evaluate the histological changes of CGA and potassium dichromate. To provide a clear result, it would be more correct to use an imaging-analysis program that provides objective and automatic interpretation of histological changes. However, this type of program was not available for this study.

# Conclusion

From the results of this study it was concluded that oxidative stress and inflammation play a key role in the toxicity caused by potassium dichromate and the harmful effects of potassium dichromate are corrected in the presence of chlorogenic acid through the suppression of inflammation. That chlorogenic acid was effective, especially at the dose of 10mg/kg, in protecting against the damage created by reactive oxygen and nitrogen species in the liver and kidney tissue was demonstrated with histopathological and immunohistochemical methods. With the benefit of anti-inflammatory and antioxidant effects, chlorogenic acid could be used for protective purposes to ameliorate or prevent organ damage caused by exposure to potassium dichromate, and thereby contribute to human health.

# Declarations

### Ethical Approval and Consent to participate:

The care of the rats and all the experimental procedures were applied in the framework of the ethical rules and regulations of  the Experimental Animals Local Ethics Committee of Afyon Kocatepe University. The study was approved by the Experimental Animals Local Ethics Committee of Afyon Kocatepe University (protocol no: 2016/54).

### Consent for publication

The authors declare that this article is original, has never been published, and has not been submitted to any other journal.

### Availability of supporting data

Not applicable.

### Consent for publication:

Consent was obtained from all participants.

### Competing interests

The authors declare no competing or financial interests.


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### Authors' contributions

The authors SO,RT,HHD,MA, and FF declared that they have no competing interests. The author SO, RT,HHD,MA drafted the work. SO,RT,FT reviewed and edited the work. SO,RT and MA collected data and revised the figures.MA,RT,HHD substantively revised this manuscript and supervised the work. SO,RT,MA designed and conceptualized this work. All authors read and approved the final manuscript.


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# Figures

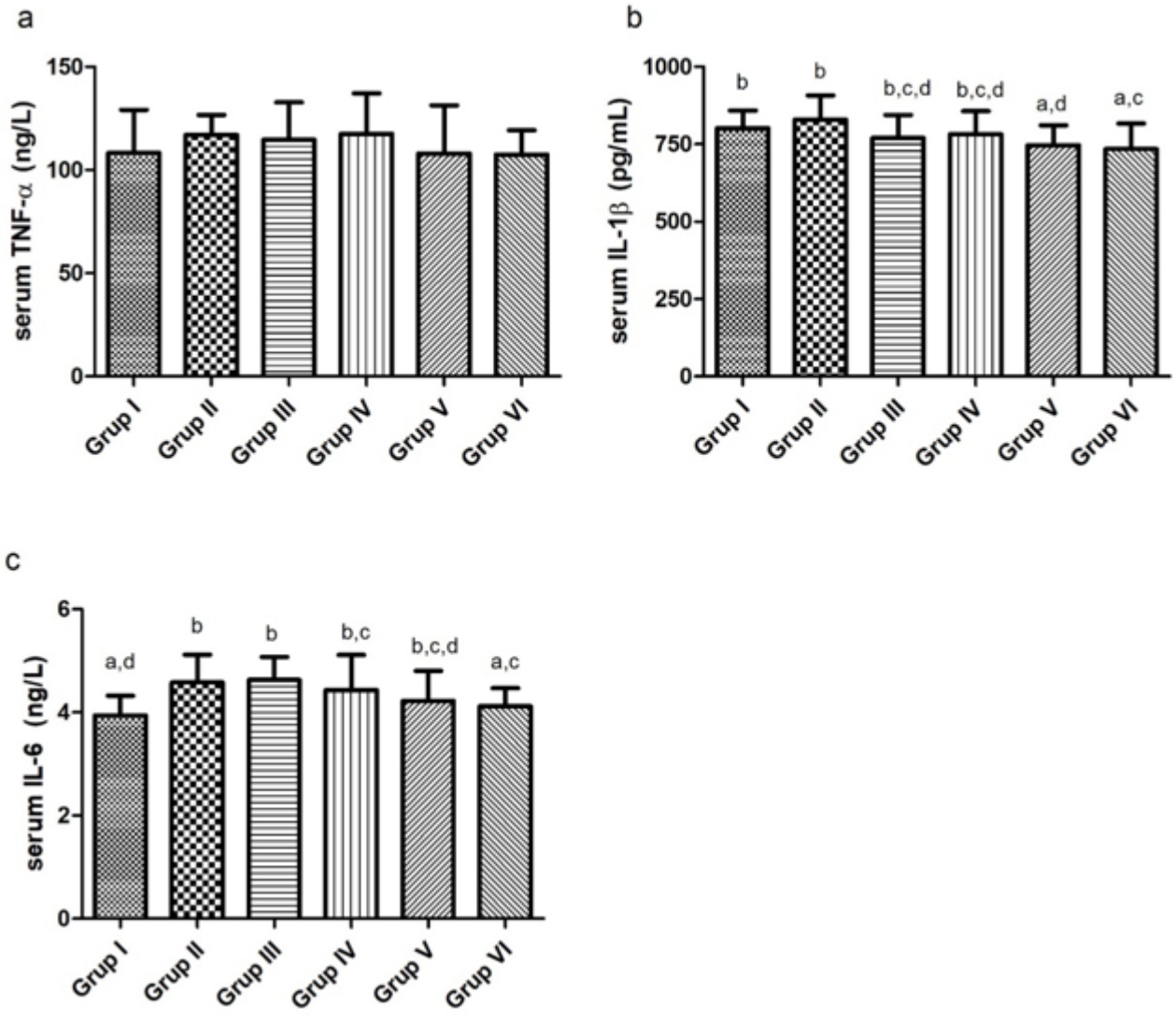
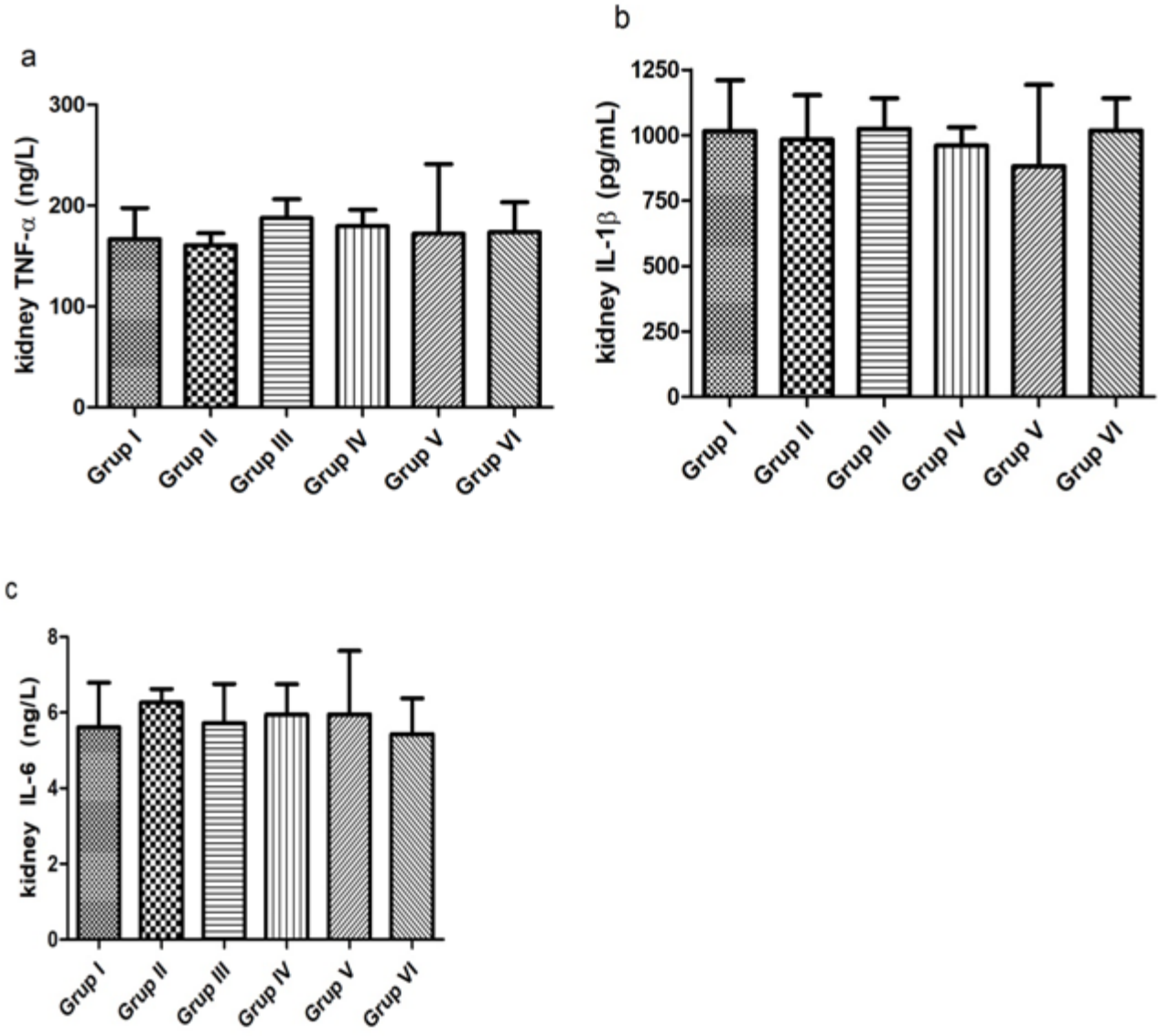


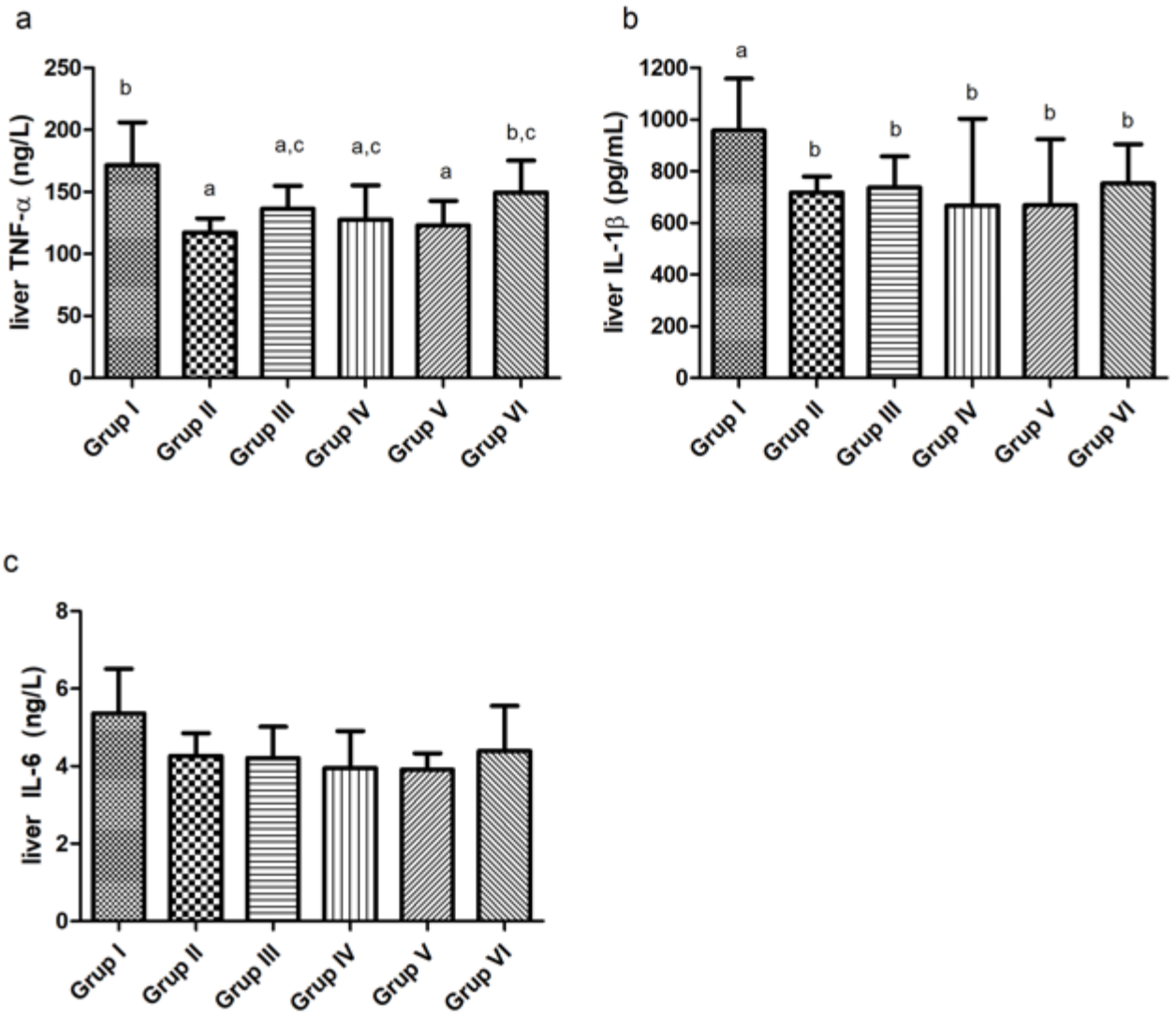

**Figure 1**

**The serum proinflammatory cytokine levels of all the groups**




## Figure 2

## The kidney proinflammatory cytokine levels of all the groups




## Figure 3

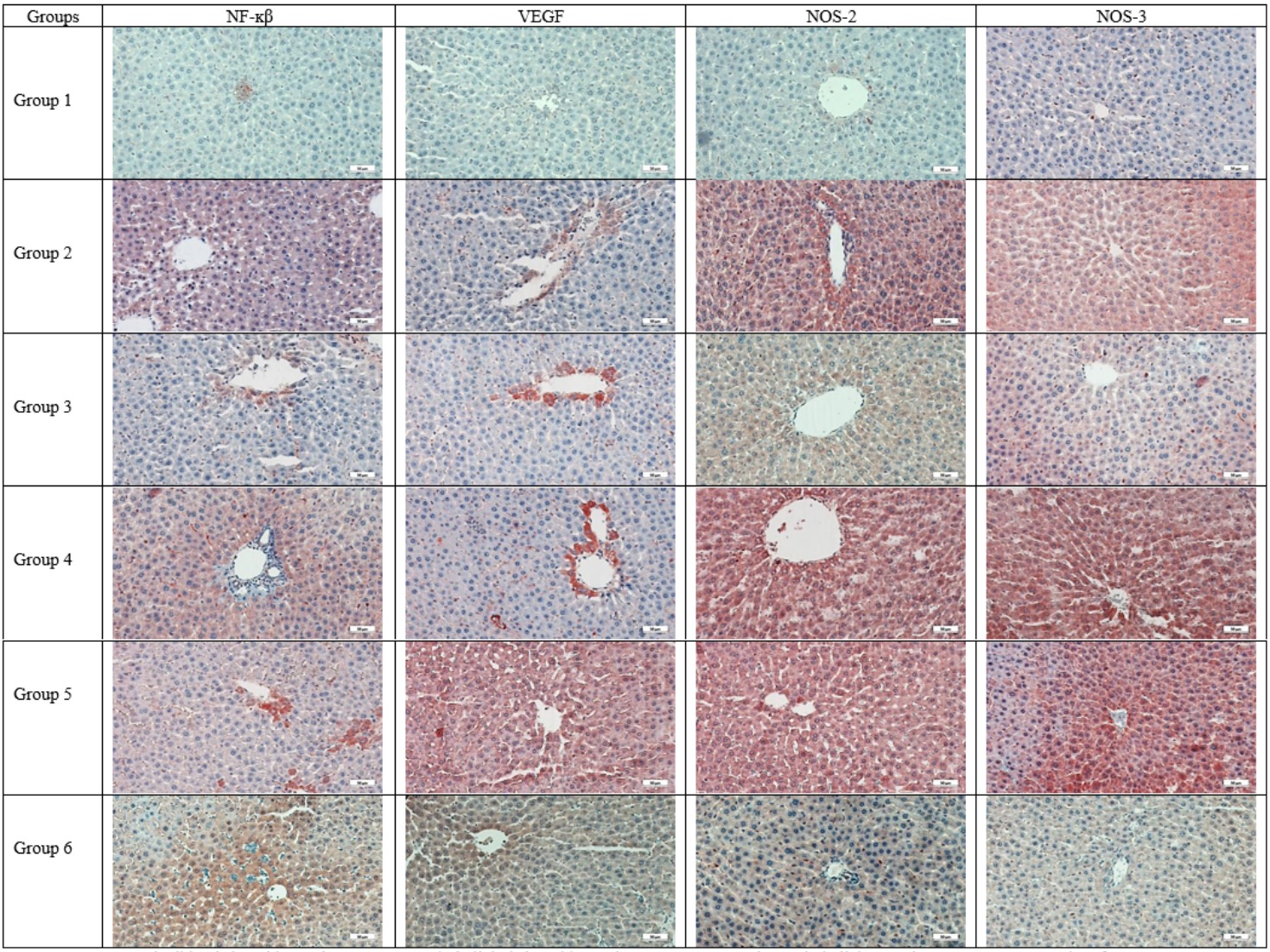
## The liver tissue proinflammatory cytokine levels of all the groups

## Figure 4

H&E staining of the kidney and liver tissues of the groups. The areas of damage in the tissues taken from the renal cortex and the liver tissues are marked with symbols to show the damage that developed due to the application of potassium dichromate and CGA. In Group I, no damage is seen in the control samples. In Group II, areas of severe damage are shown and in Group IV, areas of moderate damage. Scale bar=50μm.

## Figure 5

Immunohistochemical staining of the kidney tissues shows the changes in NF-κβ, VEGF, NOS-2, and NOS-3 expressions in glomerular and interstitial areas due to the application of potassium dichromate and CGA. Compared to the Group I control samples, there was seen to be strong expressions of NF-κβ, VEGF, NOS-2, and NOS-3 in Group II and moderate expression in Group IV. Scale bar=50μm.




2NOS: Inducible nitric oxide synthase, 3NOS: endothelial nitric oxide synthase, VEGF: Vascular Endothelial Growth Factor, NF-κB: Nuclear factor-κB

## Figure 6

Immunohistochemical staining of the liver tissues shows the changes in NF-κβ, VEGF, NOS-2, and NOS-3 expressions in the hepatocellular cell bonds due to the application of potassium dichromate and CGA. Compared to the Group I control samples, there was seen to be strong expressions of NF-κβ, VEGF, NOS-2, and NOS-3 in Group II and moderate expression in Group IV. Scale bar=50μm.