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Chrysin protects mice liver and kidney from methandienone-induced oxidative stress, inflammation a multi-biomarker approach

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ABSTRACT

Methandienone is a medication that has wide uses by competitors and young people in gyms for fitness. Chrysin (CR) is a characteristic natural flavonoid that has to incorporate being a cancer prevention agent, anti-inflammatory and anti-oxidant agent. Mus Musculus mice were therefore used in the current work to test the effects of methandienone on vital physiological functions as expressed by the liver, renal function tests and oxidation activity. The reverse effects of chrysin were also tested for the treatment of methandienone in order to determine the possibility of lowering levels of side effects. The results showed clear changes in biochemical effects on liver and kidney functions as a result of treatment with methandienone were also evident; The treated mice showed a significant elevation in the concentrations of serum levels of the hepatic function enzymes (ALP, AST, ALT) and kidney functions parameters (urea and creatinine). Combined treatment of mice with (methandienone + 25 mg/kg chrysin) caused a significant reduction in urea level and activities of ALP, ALT and AST ($p<0.05$) as compared to treatment with methandienone alone. Treatment with (methandienone + 50 mg/kg chrysin) also resulted in a significant decrease in the renal level and ALP and AST activities as compared to methandienone-treated mice. The present study concludes that methandienone causes damages to the liver and the kidney, as indicated by elevated levels of their functional enzymes. This effect indicates it increases oxidative stress in these organs, possibly due to elevated production of free radicals.

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INTRODUCTION

Methandienone is one of the commonly used anabolic androgenic steroids (AAS) are made and derived from testosterone hormone, later it was modified to reduce its androgenic properties while maintaining its structural properties to build tissues to have lower androgenic properties than testosterone, adopted for the purpose of improving the physical performance of athletes and young people(Jassim *et al.,* 2015). Methandienone can enhance protein synthesis; also, it has been utilized as an anti-anaemia drug by increasing red blood cells production. Also, it has been utilised in the treatment of iron deficiency, wounds and corneal ulcers(Pope *et al.,* 2014). Unfortunately; the use of AAS has been shown to be associated with a series of significant side effects such as hepatotoxicity, cardiotoxicity, rheumatism, dyslipidemia, and testicular toxicity. The testicular-induced testicular toxicity can be as obvious as testicular infarction, hypothyroidism, infertility (Hartgens and Kuipers, 2004, Fronczak *et al.,* 2012, Garevik *et al.,* 2011), oily skin, high lipid production, acne, body/ facial hair growth and high water retention in the muscles. A state of the thyroid tumour was additionally

reported as liver cell because of high consumption of methandienone(Hernandez-Nieto *et al.,* 1977, Amjed Haseeb Khamees, 2018).

Chrysin (CR) a natural flavonoid found in many plant extracts such as *Radix Scutellaria*, *Passiflora incarnate*, and *Pelargonium crispum*. Also, it can be found in propolis, honey, and mushrooms. Chrysin has various medicinal effects such as being anti-inflammatory, anti-asthmatic and antioxidant, as well as has protective effects against diseases of several organs such as the heart, kidneys, lungs, brain, and liver, chrysin activity relied upon its bioavailability and solvency(Samarghandian *et al.,* 2017, Shawkat *et al.,* 2018). Therefore, it is considered as an anti-cancer, antihypertensive agent and anti-ageing, and has a role in the protection of liver against chemotherapy(Kandemir *et al.,* 2017). The aim of this study was to investigate the antioxidant and anti-inflammatory effects of chrysin against methandienone induced liver and kidney toxicity.

MATERIALS AND METHODS

Drug and Chemicals

Methandienone (tab 10 mg, LA Pharma S.r.l., Thailand) and chrysin powder (Sigma-Aldrich, China) were purchased and dissolved in distilled water to prepare desired doses. Cyclophosphamide was purchased from (Zydus, Germany). All the other chemicals and reagents were utilised at analytical grade level. In our study CR25, mg/kg and CR50, mg/kg were given orally and prepared according to previous studies(Pushpavalli *et al.,* 2010).

Laboratory Animals

Mus musculus male mice weighing (25.00 ± 2.00) gm and (8-12) weeks in age were procured from the Laboratory Animal House of University of Technology, Baghdad, Iraq. The mice were acclimatised prior to the experiments and were divided into seven groups; each including 5 mice. All of the experiments were done according to the "rules of the Ethics Committees" which was approved by the University of Technology (Baghdad-Iraq), Animal Ethical Committee.

Experimental Design

A total of 35 male mice were divided into seven groups, each containing five mice. The negative control group received 0.1 mL distilled water; the second group control positive injected 20 mg/kg cyclophosphamide twice a week; third group received 1 mg/kg B.W methandienone for 7 days; fourth, fifth groups received 25 and 50 mg/kg B.W of chrysin respectively for 7 days; sixth and seventh groups received methandienone plus 25 and 50 mg/kg B.W of chrysin, respectively for 7 days. All the groups' administrations were done orally except the second group was intraperitoneally injected of CFA. Mice from all groups were sacrificed after 7 days of treatment, and blood samples were collected from the heart.

Biochemical tests of blood serum

Using anticoagulant -free test tubes, blood samples were centrifuged (4500 rpm; 15 min). After serum collection with a micropipette, it was divided into 2 parts and stored in dry tubes in the freezer until use, urea, creatinine, AST, ALT, and ALP (Tiffany *et al.,* 1972).

Biochemical tests of liver homogenate

Directly after dissection, the liver was excised into a screw-capped test tube where PBS (pH= 7.2-7.4) was added, and the samples were frozen. After homogenisation in the next day, samples were put on plane tubes and cool-centrifuged (1200 rpm) for 20 min. The supernatant was then collected and used for the previously mentioned tests of enzymatic activities (Kandemir *et al.,* 2017).

Determination of Malondialdehyde (MDA) Level

MDA level was determined based on the method of Guidet and Shah that depends on the reaction between Thiobarbituric acid with MDA which is one of the main products of lipid peroxidation. The reaction occurs in acidic pH where a colored product is formed for which the absorbance is measured at 534nm.

Glutathione determination

It was determined by the use of Elman's reagent containing 5,5-Dithio bis-2, nitrobenzoic acid-DTNB that is readily reduced by the sulfhydryl group of glutathione, producing a yellow compound that can be measured under absorbance of 412nm. Glutathione concentration in liver homogenates was calculated following the formula;

The Conc. Of GSH (μ mol/L) = Abs (sample)/E × L
Where E= 13600 L/mole/cm
L= optical path= 1 cm

Determination of Catalase activity

CAT activity was determined using the method of (Huo *et al.,* 2011). Liver homogenate (0.1ml) was added to PBS (1.9ml) in a cuvette, then 1.0ml of prepared hydrogen peroxide was added to the solution. Average hydrogen peroxide was measured by spectrophotometer under 240nm with readings performed after 10 and 30 min. The difference between the two readings is considered as an estimate for CAT activity. Activity was expressed in unit per ml after determination of protein in the tested samples.

Assay of inflammation

Cytokine production in the liver tissue was measured by (enzyme-linked immunosorbent assay-ELISA) by utilizing commercial kits and following the manufacturer's instructions. Interleukin-33 (IL-33) and interleukin-1β (IL-1β) levels were measured using a mice ELISA kit from KOMA-BIOTECH (Korea). Tumour Necrosis Factor-alpha (TNF-α) was measured by using Murine TNF-α ELISA kit Murine from Biotech (France). Interleukin-6 (IL-6) was measured by using Mouse IL- 6 ELISA Complete kit from KOMABIOTECH (Korea). The plates were read at 450 nm by using the ELISA microplate reader Bio-Tek (USA).

Statistical Analysis

The obtained data were presented as mean ± standard error (SE) for all groups. The distinctions were evaluated between the control (-) group and the mean values of each group by determining the value of the t-test. $P < 0.05$ was statistically significant.

RESULTS AND DISCUSSION

Effects of Chrysin on Serum Levels of Urea and Creatinine and Activities of *AST, ALT,* and *ALP*

Results in table 1 show a significant increase in serum levels of urea and creatinine and activities of AST, ALT, and ALP in mice treated with CFA as compared to the negative control group ($p<0.01$). This drug is known for its effects on the genetic material as well as on protein and enzyme synthesis. As related to the group of mice treated with 25 mg/kg chrysin, it exerted significant increases in activities of ALT and ALP ($p<0.01$), whereas those treated with 50 mg/kg chrysin demonstrated significant increases in activities of AST, ALT and ALP ($p<0.01$) as compared to the negative control.

In methandienone-treated mice, a significant increase in urea level ($p<0.05$) and activities of AST, ALT and ALP ($p<0.01$) were observed in comparison to the negative control. In contrast, these parameters showed a significant reduction in mice treated with methandienone + 25 mg/kg CR as compared to mice treated with methandienone alone ($p<0.05$). However, mice treated with methandienone + 50 mg/kg had significantly decreased urea level ($p<0.05$) and ALP activity ($p<0.01$), as well as significantly increased AST activity ($p<0.01$), as compared to mice treated with methandienone alone.

Increased urea level and enzymatic activities in mice treated with methandienone might indicate liver and kidney tissue damage, as it was reported that this increase along with increased creatinine reflects damages that might be attributed, in the kidneys for instance, to dehydration. In a case study on methandienone consumption, acute kidney injury was reported (Daher *et al.,* 2009). In addition, methandienone was shown to exert toxic effects on the liver that is related to increased activity of these enzymes or possibly due to elevated levels of free radicals and oxidative stress (Lee *et al.,* 2006).

Supplementary drugs are usually used to reduce the negative effects of stimulants. Therefore, chrysin was administered to mice in the present work in an attempt to measure its antioxidant potential and ability to prohibit the damaging effects of methandienone. The results showed clear such potential of chrysin with better results when using the dose of 25 mg/kg. These correcting effects of chrysin might be due to its antioxidant activity or due to its ability to cause changes in membrane permeability, ultimately altering the balance between enzyme anabolism and catabolism processes (Rana *et al.,* 1996).

Effects of Chrysin on *MDA* and some Antioxidant Activities

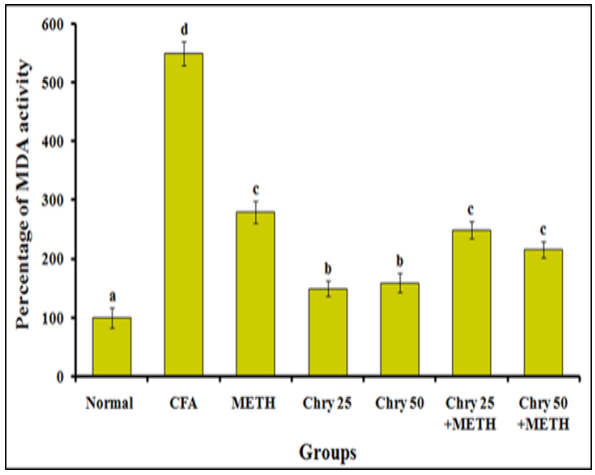

Figure 1: Percentage of MDA effectiveness in liver homogeneity of mice (b = significant difference P <0.05 with control, c = significant difference P <0.01 with control, d = significant difference P <0.001 P <with control, similar characters = no significant difference)

Figure 1 shows the percentage activity of MDA in mice liver homogenate, where a significant increase was observed in mice treated with CFA as compared to the negative control ($p<0.001$). A significant increase in MDA activity was also recorded in mice treated with 25 and 50 mg/kg chrysin as compared to the negative control group ($p<0.05$). In methandienone-treated mice, a significant increase in MDA activity ($p<0.01$) was recorded as compared to the negative control. However, no significant differences were observed between groups treated with 25 and 50 mg/kg chrysin as compared to the group treated with methandienone alone.

Table 1: Effects of Chrysin and Methandienone on Serum Levels of Urea and Creatinine and Activities of AST, ALT, and ALP

Groups	Urea (m.Mol/L)	Creatinine (U.Mol/L)	AST (U/L)	ALT (U/L)	ALP (U/L)
Negative Control	40.11±0.65**	0.30±0.02*	114.00±7.26**	26.20±0.63**	146.28±8.15**
Positive Control (CFA)	82.85±0.95	0.47±0.04	484.50±26.94	91.75±6.71	514.50±25.09
CR 25 mg/kg	42.85±1.56	0.31±0.03	262.38±8.36**	61.22±3.04**	200.00±1.00**
CR 50 mg/kg	34.20±5.11	0.30±0.05	384.50±2.51**	71.25±0.75**	253.50±11.85**
Methandienone 1 mg/kg	64.14±5.40*	0.35±0.03	273.00±6.00**	70.50±9.31**	348.00±12.30**
(Methandienone 1+CR25) mg/kg	43.65±3.84*	0.27±0.04	246.50±0.98*	45.00±3.04*	177.00±43.93*
(Methandienone 1+CR50) mg/kg	46.20±2.08*	0.28±0.11	411.43±24.34**	64.50±0.50	196.37±24.86**

p-value: ** <0.01, *<0.05

Determination of Glutathione Level in Liver Homogenate

Figure 2 presents glutathione percentage activity in liver homogenates of mice, where a highly significant increase was recorded in mice treated with CFA as compared to the control group ($p<0.001$). However, a significant decrease in activity was shown in methandienone ($p<0.01$), 25 mg/kg ($p<0.01$), and 50 mg/kg chrysil ($p<0.05$) treated mice as compared to the negative control. However, a significant increase in glutathione activity was recorded in mice treated with methandienone + 25 mg/kg CR ($p<0.05$) and methandienone + 50 mg/kg CR ($p<0.01$) as compared to those treated with methandienone alone.

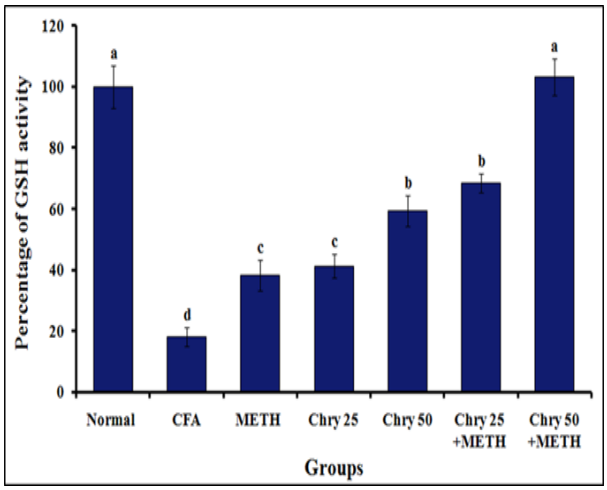

Figure 2: Percentage of GSH effectiveness in liver homogeneity of mice

The results indicate a significant effect of methandienone on reducing glutathione activity, whereas the activity was increased when methandienone was followed by treatment with chrysin. The optimal effects were observed with the dose of 50 mg/kg CR, where glutathione reached almost to normal values recorded in the negative control group.

Determination of Catalase Activity

Figure 3 presents results of catalase activity, where a significant difference was recorded between the CFA and negative control groups ($p<0.05$), whereas the other groups did not show any significant differences compared to the negative control. No significant differences were also observed between mice treated with methandienone+CR as compared to those treated with methandienone alone.

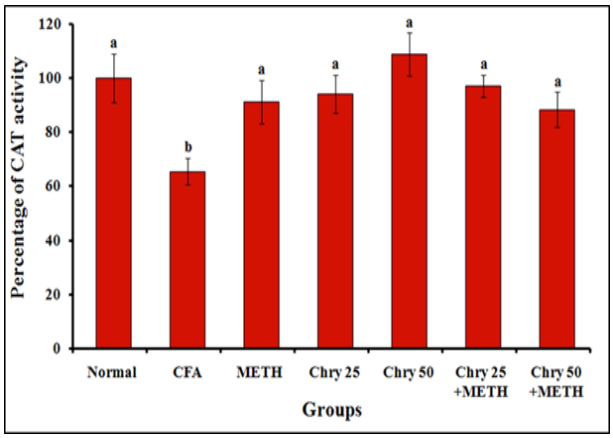

Figure 3: Percentage of CAT effectiveness in liver homogeneity of mice

The liver is the main organ responsible for removing toxic materials from the body, and it is the first organ exposed to the effects of drugs after their consumption. High doses of medicines usually cause various hepatic disorders through the production of ROS that is capable of damaging the cells through different mechanisms such as affecting cellular biomolecules like lipids, proteins and DNA (Ziech *et al.,* 2010).

Previous studies demonstrated that physical training could cause an imbalance between ROS and antioxidants, referred to as oxidative stress. Such studies resulted in wide marketing of dietary antioxidant supplements that were used by athletes as

a tool to overcome the oxidative stress (Urso and Clarkson, 2003). Most of the biomedical research on the toxic effects of AAS physical stimulants' abuse 'reported mortality cases with pathogenic abnormalities in the heart, blood vessels, reproductive system, and liver, along with variations in blood lipid levels (Barceloux and Palmer, 2013). However, the exact mechanism behind the restated hepatic toxicity is not yet totally understood, although it is thought to be related to oxidative stress. Antioxidants exert the ability to provide protection against toxicity stimulated by AAS, with positive correlations for both anabolic resistance and androgenic potency to the level of hepatic toxicity (Bond *et al.,* 2016).

From the present results on effects of methandienone treatment on indicators of activities related to oxidative stress and antioxidant activity, it is possible to state that this treatment resulted in elevated oxidative stress reactions and inhibition or reduction of the general level of cellular antioxidant activities in liver cells.

The role of chrysin that has antioxidant effects becomes prominent through its capacity to reduce oxidative stress and lipid peroxidation. Chrysin is one of the important flavonoids with several biological activities and medicinal effects (Mani and Natesan, 2018). It has the ability to prevent the damage of oxidative materials through different mechanisms, including removal of oxygen radicals (Woodman and Chan, 2004). Consuming chrysin also reduces lipid peroxidation and improves antioxidants that regulate the balance between pro-oxidant and antioxidant compounds during carcinogenesis (Kasala *et al.,* 2015).

In the present study, treatment with chrysin alone causes a significant increase in the percent activity of MDA, along with significantly reduced in that for GSH, with no difference in that for CAT. Combined treatment with chrysin and methandienone did not cause a significant change in the effect of the drug that increased MDA. However, this treatment caused an elevated glutathione level as a positive effect of chrysin, with best results recorded under 50 mg/kg CR that returned levels to those close to normal. Combined treatment did not show a significant change in the effects of the drug alone on CAT activity. In general, the positive effect of chrysin was sufficient to restore the normal level of glutathione, with the need for higher stimulation to this enzyme to overcome the damaging effect of increased mDA level. This effect might be possible to reach by using higher doses of chrysin.

Determination of cytokine activities in liver homogenate: Levels of four cytokines (TNF-α, 1βIL, 1L-33, IL-6) were measured in liver homogenate of mice treated with different treatments of CFA, methandienone, and chrysin as compared to negative control.

Tumour Necrosis Factor-alpha

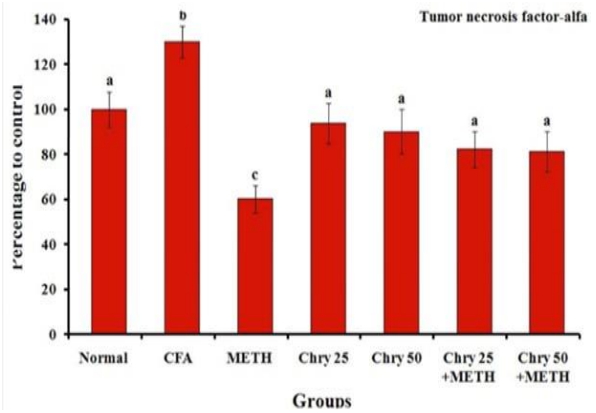

Figure 4: Percentage of TNF-α efficacy in rat homogeneity under the influence of different treatments

The results illustrated in figure 4 show a significant increase in TNF-α level in mice treated with CFA as compared to the negative control ($p<0.05$), while treatment with methandienone caused highly significant reduction as compared to the negative control ($p<0.01$). No significant differences were observed between mice treated with 25 and 50 mg/kg CR and those of the negative control. A significant increase was recorded in mice treated with methandienone+ 25 mg/kg CR and methandienone+ 50 mg/kg CR as compared to those treated with methandienone alone ($p<0.05$). The latter two groups did not show significant differences with the negative control group.

Interleukin-1Beta

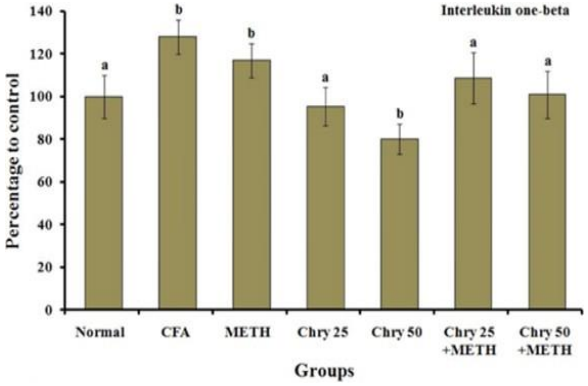

Figure 5: Percentage of IL-1β efficacy in rat homogeneity under the influence of different treatments

The results of IL-1β shown in figure 5 show significant elevation of its level in mice treated with CFA or methandienone as compared to the negative control ($p<0.05$). No significant difference was observed in mice treated with 25 mg/kg CR, whereas treatment with 50 mg/kg CR caused significant decrease as compared to the negative control. Treatment with methandienone+ 25 mg/kg CR and

methandienone+ 50 mg/kg CR showed a significant increase as compared to that of methandienone alone ($p<0.05$).

Interleukin-33

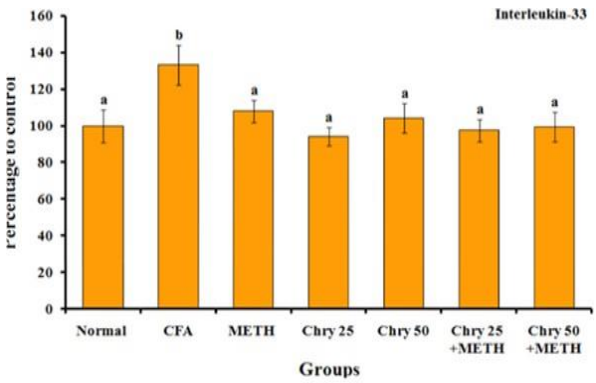

Figure 6: Percentage of IL-33 efficacy in rat homogeneity under the influence of different treatments

The results of IL-33 shown in figure 6 demonstrate a significant increase in mice treated with CFA as compared to the negative control ($p<0.05$). No significant effects were shown as a result of methandienone or chrysin treatments as compared to the negative control. Similarly, no differences were recorded in mice treated with methanedienone+ 25 mg/kg CR and methandienone+ 50 mg/kg CR in comparison to those treated with methandienone alone.

Interleukin-6

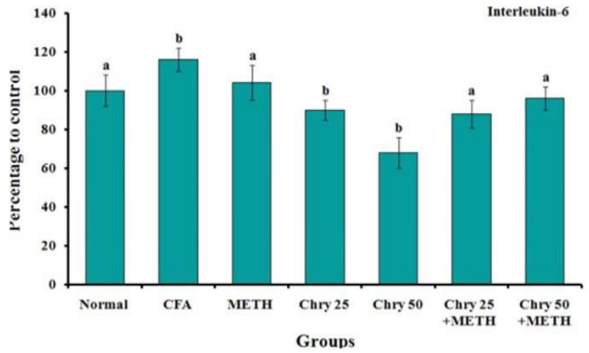

Figure 7: Percentage of IL-6 efficacy in rat homogeneity under the influence of different treatments

The results of IL-6 shown in figure 7 demonstrate a significant increase in mice treated with CFA as compared to the negative control ($p<0.05$). No significant effects were shown as a result of methandienone as compared to negative control. Mice treated with 25 mg/kg CR and 50 mg/kg CR caused significant differences as compared to the negative control ($p<0.05$). No differences were recorded in mice treated with methanedienone+ 25 mg/kg CR and methandienone+ 50 mg/kg CR in comparison to those treated with methandienone alone.

The present results demonstrate that CFA stimulated an inflammatory reaction through the increased levels of TNF-α, 1βIL, 1L-33, and IL-6. This might be due to its toxic effect as a mutagenic and carcinogenic drug. The cytokines TNF-alpha, IL1-beta, IL-33 and IL-6 can inhibit the release of iron from the reticuloendothelial system, resulting in anaemia. Which is related to iron deficiency anaemia (Means *et al.,* 1994). In mice treated with methandienone, there was no significant increase in levels of IL-33 and IL-6, but in IL-1β, indicating an immune response. It was previously shown that doses of nandrolone decanoate and oxymetholone stimulated the production of IL-1β and TNF-α in human peripheral lymphocytes in vitro (Hughes *et al.,* 1995).

In the present results, the levels of this cytokine were significantly reduced, indicating that the toxic effect of methandienone dose used did not reach to a level that was sufficient to cause an increase in TNF-α levels that is enough to result in cell necrosis and death. This might be due to a short period of treatment which was only one week.

Sonya et al. indicated no effects of AASs on the immune system, whereas doses of AASs with intact steroid nucleus showed immune-suppressive effects by decreasing the number and function of cells. In contrast, those with altered intact steroid nucleus had immunostimulation since it stimulates the proliferation of lymphocytes and other immune cells (Brenu *et al.,* 2011).

The current results show that chrysin has anti-inflammatory effects by decreasing the expression of pro-inflammatory cytokines. Furthermore, it has the ability to reduce the immune response of macrophages and monocytes (Bae *et al.,* 2011).

CONCLUSION

The present study concludes that methandienone causes damages to the liver and the kidney, as indicated by elevated levels of their functional enzymes. This effect indicates it increases oxidative stress in these organs, possibly due to elevated production of free radicals. Therefore, in this manner, taking methandienone must pursue medicinal supervision for assurance of the time of treatment and dose. Also, our results confirmed the antioxidative and anti-inflammatory effects of chrysin on methandienone induced liver and kidney damage in mice. Therefore treatment with chrysin for a brief period could decrease these symptoms of treatment with methandienone.

Conflicts of interests

All authors have none to declare

Author contributions: All author contributed equally

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