International Journal of Pharmacology

ISSN 1811-7775

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2020.136.144

Research Article
Beneficial Role of Rosemary Aqueous Extracts Against Boldenone Induced Cardiac Toxicity, Injury and Oxidative Stress, in Male Rats

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Abstract
Background and Objective: Boldenone is an androgenic steroid that progresses the development and nourishment change in nourishment creating creatures. In most nations around the world, this anabolic steroid is illegal for human employments and for meat generation because it was created for veterinary utilize. As of late, it is utilized by bodybuilders in both low season and pre-contest, where it is well known for expanding vascularity whereas planning for a weight training challenge. So, the current study was aimed to investigate the possible prophylactic effect of rosemary extracts response of to boldenone induced blood alterations, cardiac toxicity, injury and oxidative stress, in male rats. **Materials and Methods:** A total of 20 adult male rats were divided into 4 groups (1st group was control, 2nd group was treated with rosemary, 3rd group was boldenone group include rats that injected intramuscular boldenone (5 mg kg^{-1} b.wt./week) for 4 weeks, 4th group was post treated group include rats that injected intramuscular boldenone every week for 4 weeks and then treated with rosemary for another 4 weeks). **Results:** Intramuscular injections of boldenone induced a significant increase in serum lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase isoform (CK-MB), myoglobin, malondialdehyde (MDA) and cardiac tissue injury while a significant increase in serum glutathione (GSH), superoxide dismutase (SOD) and catalase activity as compared with their relative values in the control group. These findings explained the common phenomena in athletics and bodybuilders who suffer from problem in cardiovascular system, cardiac toxicity following injection with some steroids drugs as boldenone to build muscles or for enlarge lips or cheeks or breast or buttocks as well as included in many cosmetics. **Conclusion:** Rosemary extracts could scavenge free radicals and produce beneficial effects against boldenone induced damage in heart, oxidative stress and blood alternations.

Key words: Anabolic steroid, boldenone, rosemary, rats, cardiac functions, structure, blood

Citation: Rehab M. Elgharabawy, Maha A. Aldubayan, Mariah A. Alshaifani and Amira S. Ahmed , 2020. Beneficial role of rosemary aqueous extracts against boldenone induced cardiac toxicity, injury and oxidative stress, in male rats. Int. J. Pharmacol., 16: 136-144.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anabolic-androgenic steroids (AASs) are characterized as manufactured subsidiaries of the endogenous sex hormone testosterone, which is fundamental for advancing significant physiological changes within the male body: regenerative tissue advancement in men[1]. A group of elite athletes who use steroids while lifting weights to improve muscle size and enhance exercise regimens to improve athletic performance or physical appearance. This group was the leading athletes who used AAS to enhance their performance[2]. In addition to improving their performance or physical appearance, prohibited drugs utilized to compensate for stress. In some circumstances, doping is wholly intended at aesthetic application to give a sense of wellness[3]. The abuse of steroids become increasingly in both male and females populations. On the other hand, AASs are causing severe side effects on the human body when misused, it occur potential health consequences[1]. Steroid usage has been connected with the incident of severe cardiovascular, including hypertension, decrease in ventricles function and strokes also in active young athletes[4]. Steroid influences the musculoskeletal system. The massive increases in muscle mass related to anabolic steroids lead to an increase in musculoskeletal injuries through defeat the tendons and ligaments of users[5]. Bones stop growing prematurely in child or adolescent that use anabolic steroids because they have unnaturally high levels of sex hormone[5].In most countries around the world, AAS is banned either for meat production or human uses because of unwanted outcomes such as cardiovascular disorders, hepatic dysfunction, renal disease, testicular problems, behavioral disorders[1,4,6]. Some of the veterinary products assigned as Schedule three drugs. These include boldenone, equipoise, ganabol, equigan and ultragan[7].

Boldenone is injectable AASs that can directly be utilized by athletes to enlarge and build their muscles. Women use it as well to enlarge their lips, cheeks, buttocks and breast and, it also exists in many cosmetics. Besides, humans may indirectly be affected by meat consumption from animals that treated with boldenone consumption[8]. In veterinary field, boldenone is one of the items that devoted to expanding the development rate of food-producing creatures by the advancement of protein amalgamation which picked up from the dehydrogenation of testosterone, that appears solid anabolic and tolerably androgenic properties[4,7]. Its part for expanding the muscular mass is because of the promotion of positive nitrogen stability through exciting protein generation in addition to decreasing protein degradation. Furthermore, it provides maintenance in body water, blood electrolytes ions[9]. Boldabol, ganabol, equigan and ultragan were produced mostly to veterinary application, basically for horse therapy. In the majority nations, AAS may be taboo for both, human and meat production[7]. In the USA, BOL is not approved for human usage and only accessible for veterinary centers. Because of the high undecylenate ester related to the parent steroid, BOLs' half-life is long (up to one and a half years). Therefore, minimum quantities of this remedy can easily be discovered even after months from terminated use[10]. In 2015, a published study adopted that the main risk factors for cardiovascular disorders such as peripheral arterial disease are infrequency glomerular filtration rate and albuminuria[11]. Several plant extracts have been the basis of treatment on human history and this medicine is still widely practiced till today. Medicinal plants acknowledged having significant antioxidant activity which may be related to the treatment of several fatal disorders such as blood and heart toxicity[11,12].

Rosemary is a sweet-smelling evergreen herb that has leaves alike to pine needles used as a flavoring in cooking. Rosemary is endemic in Mediterranean and Asia and can resist dehydration, surviving for long periods. The shape of rosemary plant is upright tall between (1.5-2) cm and trailing. The leaves are high and wide between (2-5) mm, green-up, white down and have thick small soft hair. Flowers have many colors like white, pink, purple, or deep blue[13]. Rosmarinic acid can be absorbed in the gastrointestinal (GI) tract[14]. *Rosmarinus officinalis* (rosemary) is a medical plant that used to cure a variety of disorders. Its contain phytochemicals compounds such as rosmarinic acid, caffeic acid, betulinic acid, camphor, ursolic acid and antioxidants carnosic acid[14]. Laboratory studies have shown rosemary to be rich in varied bioactivities such as antioxidant agents[13]. According to 2017 statistics, cardiovascular disorders were the major causes of mortality and disability globally. The burden of these disorders on affected individuals and communities is enormous[15]. Therefore, this study was focused to evaluate rosemary outcomes on growth promoter boldenone induced changes in rats blood and cardiac function and structure.

MATERIALS AND METHODS

This study was conduct at college of pharmacy, Qassim University, KSA during the period from September to November, 2019.

Chemical and drug: Rosemary aqueous extracts and boldenone undecylenate vial (50 mg mL^{-1} vehicle) contain oily solution was obtained from Laboratorios Tornel Company.

Animals: This study respected the ethical guidelines that are outlined by the Committee of National Research Center of Saudi Arabia. The protocol utilized in this study was approved by the Animal Ethics Committee of the Faculty of Pharmacy at the Al-Qassim University, Al-Qassim, Saudi Arabia. Albino Sprague Dawley rats was used in this study. The rats were all males, they were 20 and they each weighing between 200-250 g. During the four weeks of experiment, water and food were provided.

Experimental design: Randomly animals were separated to 4 groups (5 rats in each):

- Group 1: Control group included rats that experience an intramuscular injection with olive oil
- Group 2: Rosemary group included rats that experience rosemary extract (intragastrically 220 mg kg^{-1}) this lasted for four weeks[14]
- Group 3: Boldenone group included rats that experience boldenone undecylenate (5 mg kg^{-1} intramuscular injection weekly) this lasted for 4 weeks[16]
- Group 4: Post treated group (boldenone+rosemary) included rats that experience for 4 weeks boldenone intramuscular injection. Then they were treated with rosemary extract for four weeks

Blood sampling: After the experiment weeks had done, rats were euthanized using Sodium pentobarbital via intravenous injection. The inferior vena cava of each rats were used as the site for blood collection. These samples were collected in non-heparinized blood tubes then left for 30 min at standard temperature to form clots. Samples were centrifuged at 3000 rpm for 15 min. Then, the serum were stored in -18°C until analysis for estimation of cardiac enzymes including, creatine kinase isoform (CK-MB), lactate dehydrogenase (LDH) and myoglobin. Also, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase were used for oxidative stress parameters estimation.

Measurement of the cardiac biomarkers: The activity of serum LDH was assayed by using El Atrash *et al.*[17] method. Meanwhile, CK-MB activities in serum levels was assayed using Bishop *et al.*[18] method. Activities of myoglobin concentration in serum levels was assayed using cummins *et al.*[19] method.

Enzymatic and non-enzymatic antioxidant assays: In serum, MDA was detected as a marker for lipid peroxidation by using Saggu *et al.*[20] method. GSH was determined, according to Aldubayan *et al.*[21] and the activities of SOD in serum levels were assayed by using Saggu *et al.*[20] and Aldubayan *et al.*[21] method. While the catalase activities in serum were assayed in consonance with the method of Moustafa *et al.*[22].

Histological examination: After execution, dismemberment was held for hearts from all studied groups. Tests were extricated and overflowed in 10% unbiased buffered formalin. At that point, dried, clarified by utilizing Xylene and embedded in liquid Paraffin.

By rotary microtome portions of 7 microns' depth will be cut then fixed on sterile slides. Portions were dyed by Ehrlich's Haematoxylin then counterstained by Eosin according to Massoud *et al.*[23] method.

Immunohistochemical evaluation of p53 immunore activities: The avidin biotin complex (ABC) approach was used in accordance with Tousson *et al.*[8] to evaluate apoptotic p53 immunoreactivity (p53-ir) in the sections[23].

Statistical analysis: SPSS version 23 was the statistical software employed for examination of the findings. The data generated was presented as Mean±standard deviation. This was calculated by one way ANOVA, which allowed the establishment of whether significant differences existed between treatment groups. Regarding the biochemical information gathered, results at $p<0.05$ were counted statistically significant.

RESULTS

Effect of boldenone and rosemary on biomarkers for cardiac injury: Figure 1 revealed a significant increase in serum lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB (CK-MB) and myoglobin in treated rats with boldenone (G3) when compared with control (G1) and rosemary (G2) groups. On the other hand, a significant decrease in serum LDH, CK, CK-MB and myoglobin in post treated rats with boldenone and rosemary (G4) when compared with treated rats with boldenone (G3).

Effect of boldenone and rosemary on oxidative stress: Figure 2 revealed a significant increase in MDA levels, in contrast a significant decrease in catalase (CAT), superoxide dismutase activity (SOD) and reduced glutathione (GSH) levels in the serum of treated rats with boldenone (G3) as compared to control (G1) and rosemary (G2) groups. On the other hand, a significant decrease in MDA levels and, a significant increase in CAT, SOD and GSH levels in the serum of post treated rats with boldenone and rosemary (G4) when compared with treated rats with boldenone (G3) (Fig. 2).

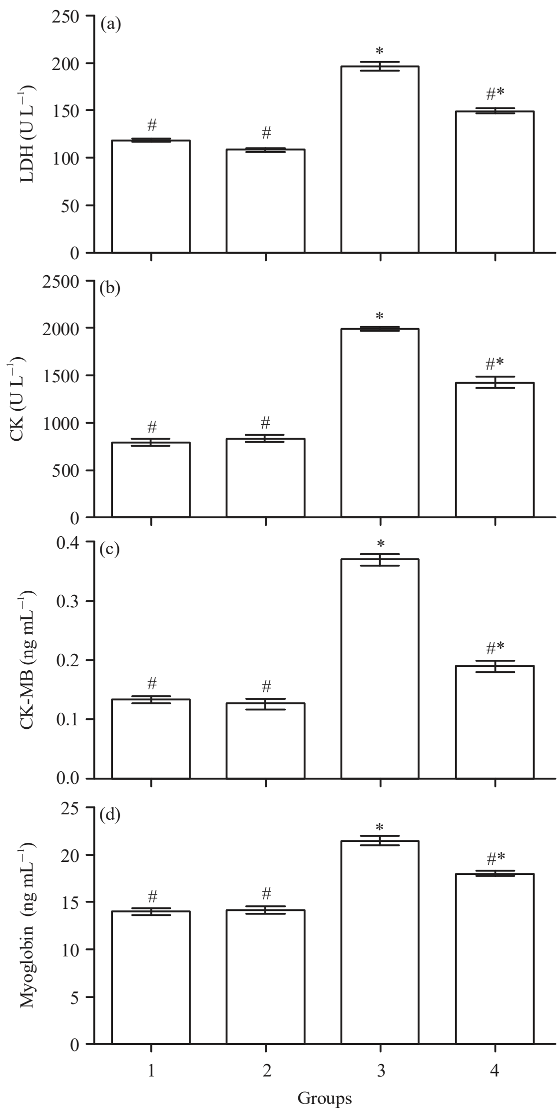

Fig. 1(a-d): Changes in (a) Serum lactate dehydrogenase (LDH), (b) Creatine kinase (CK), (c) Creatine kinase MB (CK-MB) and (d) Myoglobin in different groups

Value represents Mean±SD of 5 rats, *Significant difference from the control group at p<0.05, #Significant difference from boldenone group at p<0.05

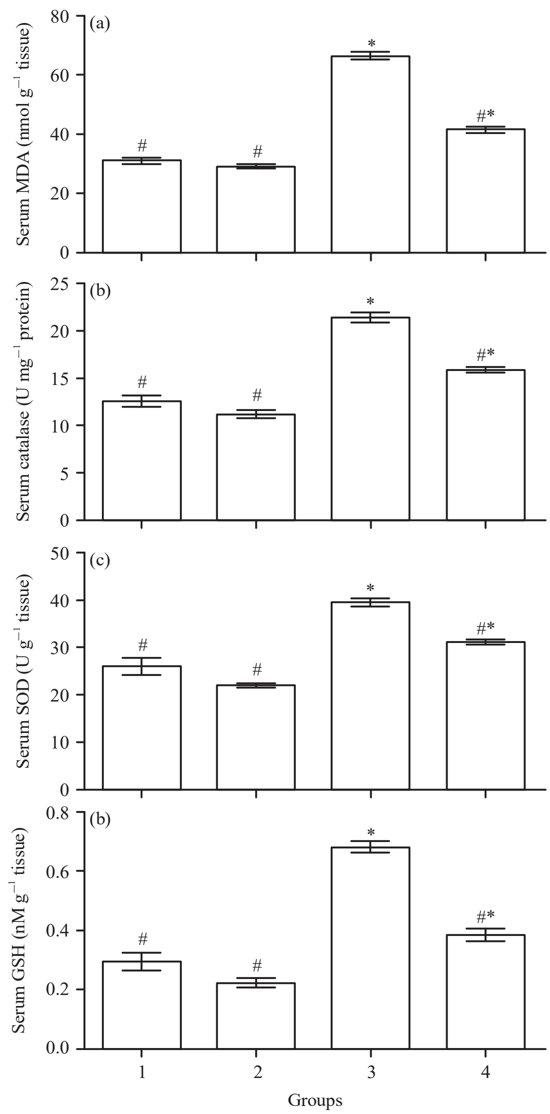

Fig. 2(a-d): Changes in (a) Serum malondialdehyde (MDA), (b) Catalase (CAT), (c) Superoxide dismutase (SOD) and (d) Reduced glutathione (GSH) activities in different groups

Value represents Mean±SD of 5 rats, *Significant difference from the control group at p<0.05, #Significant difference from boldenone group at p<0.05

Effect of boldenone and rosemary on cardiac tissues: Light microscopy of the rat heart sections in control and rosemary groups revealed a normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils (Fig. 3a, b). Heart sections in treated rat with boldenone revealed marked degree of myocardial injury with marked myocardial hypertrophy, nuclear pyknosis, cytoplasmic vacuole and marked hydrophobic changes of myofibrillar structure with striations and moderate leukocyte infiltration (Fig. 3c). Heart sections in post treated rats with boldenone and rosemary revealed mild degree of myocardial injury with only mild myocardial hypertrophy (Fig. 3d).

Effect of boldenone and rosemary on p53 immunoreactivity: Figure 4 indicates the expression of p53 in the various experimental groups. A cardiac section in control and in the treated rats with rosemary exhibits a faint to mild positive reaction for p53 expression (Fig. 4a, b), while moderate positive reactions for p53 expression were detected

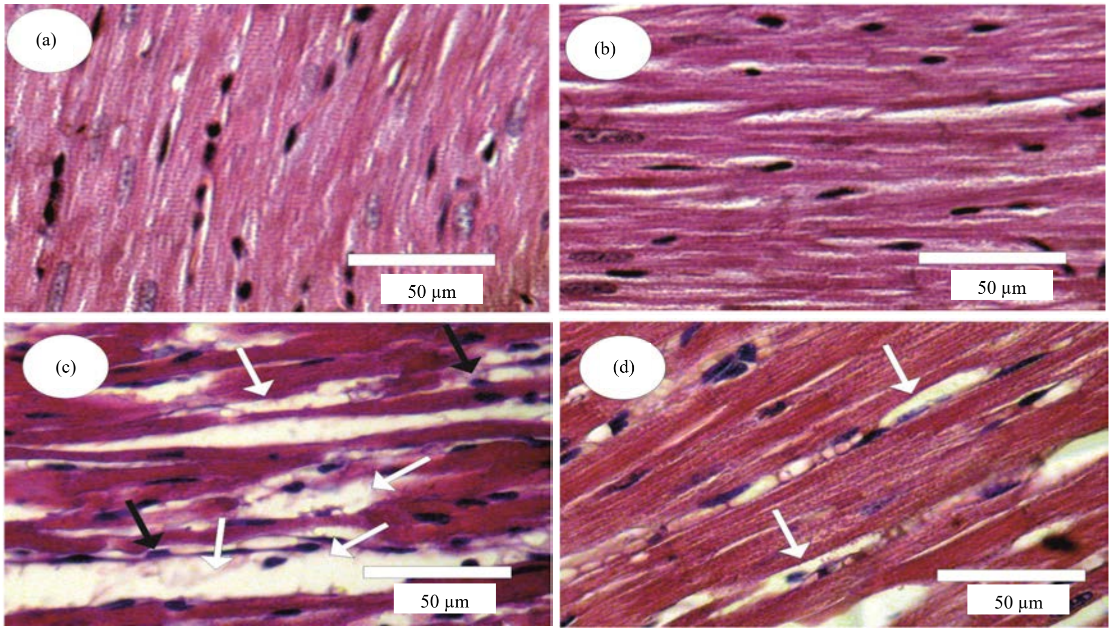

Fig. 3(a-d): Illustrations of section of rat heart presented as photomicrographs. Sections are hematoxlyin and eosin stained for morphology analysis, (a-b) Control group and rosemary group, respectively, they both depict normal myofibrillar structure with striations, (c) Tissue section of the heart of rats treated with boldenone demonstrating myocardial hypertrophy (white arrows) moderate leukocyte infiltration (black arrows) and (d) Heart sections from rats treated with both boldenone and rosemary, these demonstrated good degree of improvements with mild myocardial hypertrophy

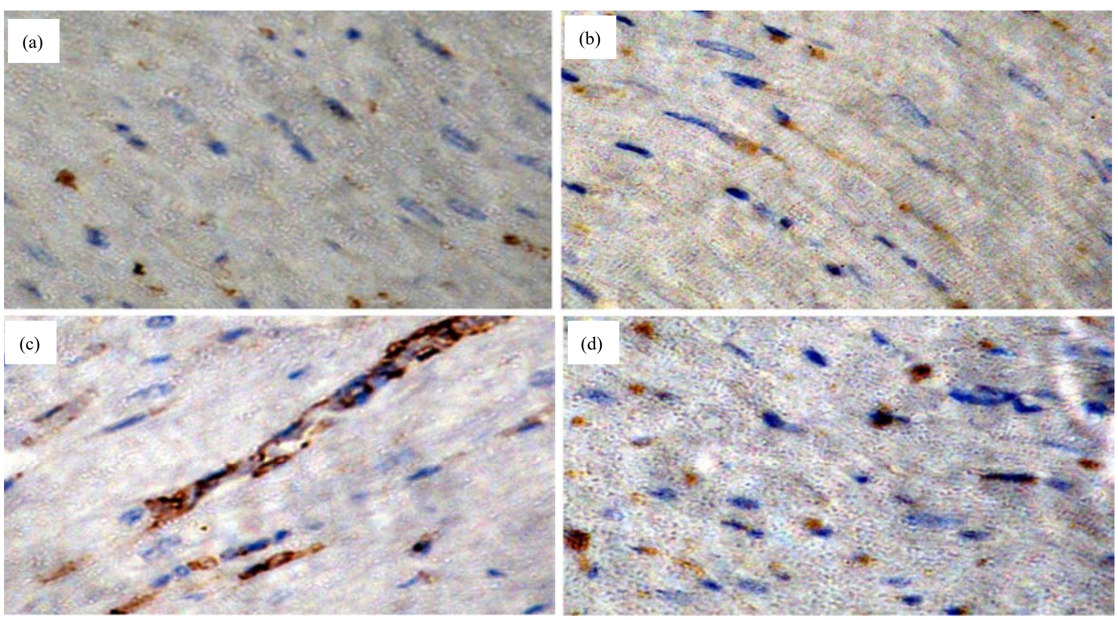

Fig. 4(a-d): Photomicrographs of heart sections stained with p53-ir in the different groups, (a-b) Heart sections in control and rosemary groups showed faint positive affinity for p53, (c) Heart sections of boldenone group showed moderate positive affinity for p53 and (d) Heart sections in treated boldenone with rosemary revealed mild positive affinity for p53 in heart sections in treated rats with boldenone (Fig. 4c). Heart section in post treated rats with boldenone and rosemary revealed mild positive reaction for p53 expression (Fig. 4d).

DISCUSSION

The diagnosis of cardiac enzymes is known to be necessary. Serum CK activity is a more sensitive indicator in the early stage of myocardial ischemia, while peak rises in LDH are roughly proportional to the extent of the myocardial tissue[24]. The aim of this study was to examine the possible effect of rosemary aqueous extract against growth promoter boldenone induced changes in rats' blood and cardiac function and structure. In addition to, evaluate the beneficial effect of rosemary.

The present study revealed a significant increase in serum LDH, CK, CK-MB and myoglobin in treated rats with boldenone (G3) when compared with control (G1) and rosemary (G2) groups, causing muscle damage. According to brancaccio, CK-MB levels applied to identify myocardial disorders[25]. Current study results matched with research written in 2006 stated that the injecting of testosterone and nandrolone hormones to young rats increases CK-MB levels[26]. Moreover, present study results matched with another research stated that rats, which injected with nandrolone had an increase in CK-MB levels[27]. In 2010, a published research remarked an accretion in CK-MB levels in male rats after testosterone dose; however, our results matched with the study of Tasgin[28,29] who reported no increase in CK-MB levels in female rats. Results discovered a high concentration of LDH in the liver, heart, erythrocytes, skeletal muscles and kidneys. Such elevations have been widely applied as diagnostic indices for kidney, liver, heart and red blood cell dysfunction[30]. Additionally, in the studies of Kanowski and Clague, they reported high levels of serum LDH activity in a variety of cancers[31].

Most pharmacological effects of rosemary are the consequence of the high antioxidant activity of its main chemical constituents, which include carnosol, carnosic acid, ursolic acid, rosmarinic acid and caffeic[14]. The potent antioxidant properties of rosemary have been mainly attributed to its significant diterpenes, carnosol and Carnosic acid, as well as to the essential oil components[32].

Oxidative stress is an indicator of the damage that results from a change in the balance between oxidants and antioxidants in favor of oxidants[33]. In the current study, serum MDA levels significantly increased in the boldenone group, unlike serum GSH, catalase and SOD levels which were significantly decreased in treated rats with boldenone when compared with control and treated rats with rosemary. Reactive oxygen species (ROS) and MDA are linked with the changes in the microscopic examination of cardiac muscle injury. Our results matched with research written in 2003 stated that the anabolic-androgenic steroids produced oxidative stress[34]. Our results matched with research written in 2012 demonstrated that boldenone induces oxidative stress in hepatic and renal tissues[35]. Our results agree with Tousson *et al.*[8], Alm-Eldeen and Tousson[16], El-Moghazy *et al.*[35] and Tousson *et al.*[36], who reported that the injection with boldenone induced oxidative stress in liver and kidney respectively in male rats. According to El-Sherif and Issa[37], the biochemical interaction of boldenone undecylenate steroid hormones operates by stimulation of receptors molecule in muscle cells, which stimulate specific genes to generate proteins. Meyer mentioned that estrogen and androgens mediate their activity via intracellular receptors directly in muscular tissue as well as indirectly via stimulation of growth hormone from the anterior pituitary and other growth factors from liver plus several distant organs[38].

Additionally, in 2015, a study summarized that increased oxidative stress and cardiac oxidation have also been connected with diastolic dysfunction[39]. Our results pointed that rosemary supplementation decreased oxidative stress and there was a significant decrease in MDA levels, in addition to, a significant increase in CAT, SOD and GSH levels in the serum of post-treated rats with boldenone and rosemary (G4) when compared with treated rats with boldenone (G3). In general, our outcomes matched an earlier study reported that rosemary and its compounds provide an antioxidant effect[40]. Also, our results agreed with El-Demerdash *et al.*[41], who find that oxidative stress modulation by *Rosmarinus officinalis* in creosote induced hepatotoxicity. These results suggested that the effect of rosemary extracts might be due to their antioxidant activities restraining the oxidative stress, which is widely associated with boldenone toxicity.

The present results showed that intramuscular injection of rats with boldenone has a markedly adverse effect on the cardiac muscles as a marked degree of myocardial injury with marked myocardial hypertrophy, nuclear pyknosis, cytoplasmic vacuole and marked hydrophobic changes of myofibrillar structure with striations and moderate leukocyte infiltration. These findings suggested that the misapplication of boldenone undecylenate associated with constant muscle functional and structural damage that leads to developed conditions. Our results are in agreement with Tousson *et al.*[42] who reported that the boldenone induced muscle hypertrophy and fibrosis in cardiac muscles in male rats. Also, our results are in agreement with McCarthy *et al.*[43], who reported that the anabolic steroid-induced muscle hypertrophy in skeletal and cardiac muscles in humans. Our results are in agreement with several studies which provided evidence that anabolic steroid harms human health[6,35,36]. Our results agreed with Yesalis *et al.*[44] who reported that despite the growth-promoting effects, the unfavorable outcomes of anabolic steroids revealed in cardiovascular, hepatic, muscular and endocrine systems. Cardiac fibrosis designates as an unusual thickening of the cardiac valves as an outcome of an improper accretion of cardiac fibroblasts. However, the term usually applies to the increased precipitation of the extracellular matrix in the cardiac muscle. In the current study, our results revealed that post-treatments of rats with boldenone and rosemary showed a mild degree of myocardial injury with only mild myocardial hypertrophy. Our results are in agreement with Forman *et al.*[45], who reported that; the smallest dose of rosemary supplementation led to heart diastolic function and hypertrophy and it can generate more considerable improvement in post-MI heart function. Apoptosis, or programmed cell death, is a crucial cellular activity in the behavior of mammalian cells in a wide range of pathophysiological conditions[35]. The p53 tumor suppressor protein is a transcription factor that regulates the transcription rate of several genes involved in the regulation of cells cycle, DNA repair and apoptosis[36]. Our immunohistochemical observations of the cardiac tissues showed a significant increase of the apoptotic protein p53 after boldenone injection and the treatments with rosemary able to inhibit this elevation in p53 expressions. Our results are in agreement with El-Moghazy *et al.*[35], who find that; boldenone injection in rats induced apoptosis in liver tissues. Also these results agree with Aldubayan *et al.*[21], who detected that; equigan induced increase in p53 expression in rat testes. Finally, the present study may help to understand the extent of interaction between the studied extracts administration in toxicants causing physiological alterations, its prevention in animal model and allowed for preventive applying in human status.

Also, our results are in agreement with Karthik *et al.*[46] and Liu *et al.*[47], who reported that; rosmarinic acid reduced myocardial damage blood pressure in hypertensive rats fed a high fructose diet and protected the heart against cardiac dysfunction and fibrosis. Moreover, Moreno *et al.*[48] reported that the extracts of rosemary with high search efficiency against a variety of ROS and nitrogen species, it was believed that the ability to examine free radicals is one of the primary mechanisms through which phenolic phytochemicals work as antioxidants. Finally, the present study may help to understand the extent of interaction between the administrations of the studied extracts intoxicants causing physiological alterations, its prevention in the animal model and allowed for preventive applying on human cases.

CONCLUSION AND FUTURE RECOMMENDATIONS

It is concluded that administration of boldenone induced the cardiac toxicity and disrupt the redox balance. Treatment with rosemary extracts ameliorated the damage induced in the heart and regulate the redox system. This new insight into the anti-oxidative activity of rosemary extracts could serve as a basis for developmental improvement of chemopreventive or therapeutic strategies for cardiac toxicity induced by boldenone especially in bodybuilders who suffer from problem in cardiovascular system after exposure to boldenone.

Further studies are needed to establish the role of rosemary in the protection of cardiac injury induced by boldenone. More detailed studies involving administration of different antioxidants like green tea, vitamin C and other antioxidant agents as protective against the oxidative stress induced by boldenone.

Only a few studies about the protective effect of rosemary against the toxic effect of boldenone on cardiac were published. So we need to focus on rosemary and its beneficial effect against other organ toxicities induced by boldenone.

SIGNIFICANCE STATEMENT

This study discover the role of rosemary extracts that can be beneficial for ameliorating the damage induced in the heart and regulate the redox system. This study will help the researcher to uncover the critical areas of treatment of cardiac toxicity that many researchers were not able to explore. Thus a new theory on natural products such as rosemary extracts can ameliorate cardiac toxicity induced by boldenone may be arrived at.

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