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Protective Effect of Carvacrol Against Oxidative Stress and Heart Injury in Cyclophosphamide–Induced Cardiotoxicity in Rat

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ABSTRACT

Possible protective effects of carvacrol (Car) against cyclophosphamide (CP)-induced cardiotoxicity was examined in this study. Experimental groups of the rats were randomly divided into 13 groups,each including seven animals: Group 1 (control) treated with saline; groups 2, 3, and 4 treated with 50, 100, or 150 mg/kg of CP, respectively; group 5 treated with 0.5 mL olive oil; groups 6 and 7 treated with 5.0 and 10 mg/kg of Car, respectively; groups 8, 9, or 10 treated with respective CP plus 5.0 mg/kg of Car; and groups 11, 12, or 13 treated with respective CP plus 10 mg/kg of Car. Serum alanine transaminase (ALT),aspartat transaminase (AST), lactate dehydrogenase (LDH), malondialdehyde (MDA),creatine kinase-MB (CK-MB), total oxidant state (TOS), oxidative stress index (OSI), and levels were high only in the CP groups. There was a dose-dependence on the CP-induced cardiotoxicity. Hemorrhage, inflammatory cell infiltration and the separation of the muscle fibers in the heart tissue supported the biochemical data. With 5.0 and 10 mg/kg Car, there was an important decrease in the CP toxicity and this was related to the oxidative and nitrosative stress in the CP-induced cardiotoxicity. Reduced inflammation and lipid peroxidation in the heart tissue and increase of serum glutathione (GSH) and total antioxidant capacity (TAS) levels were found when carvacrol was applied. Based on these findings, it could be proposed that Car was a strong candidate in preventing the CP-induced cardiotoxicity but further clinical studies should be done in order to verify its application on humans.

Key words: Cyclophosphamide, oxidative stress, cardiotoxicity, carvacrol, antioxidant, rat

INTRODUCTION

Cyclophosphamide (CP), a cytotoxic alkylating agent, is extensively used as an antineoplastic agent for the treatment of various cancers and also as an immunosuppressive agent in organ transplantation, systemic lupus erythematosus; it is also used for some other benign diseases (Selvakumar et al. 2006). By modulating the DNA synthesis, CP prevents cellular proliferation (Dollery 1999). Numerous studies have shown that CP exposureenhances intracellular reactive oxygen species (ROS) production, suggesting that biochemical and physiological side effects may result from its oxidative stress (Manda and Bhatia 2003). Shanholtz (2001) found out that there was a fatal cardioxicity when CP was administrated at high doses. Hence, cardiotoxicity is one of the limiting side effects of this commonly used anticancer agent. In another study, the cardiocoxic effects of CP were found to be dose-related cardiac damage, morphologically defined

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necrosis, bleeding (Ludeman 1999). It has been suggested that in order to benefit from the CP at higher doses, a protective agent was needed that would eliminate the toxic side effects of CP (Ayhanci et al. 2010). Plasma antioxidant concentration has shown a decreaseof patients who had a high dose chemotheraphy (Sabuncuoglu and Ozgunes 2011). The antioxidants that can eliminate toxic side effects of chemotherapy can provide the use of higher and more effective doses of the anticancer drugs (Simone et al. 2007).

Carvacrol (Car) possesses a wide variety of pharmacological properties such as hepatoprotective (Aristatile et al. 2009), anti-inflammatory (Hajhashemi et al. 2002), antioxidant (Yanishlieva et al. 1999), antitumour (Evangelou et al. 1997; Ipek et al. 2005), antimutagenic (Ipek et al. 2005) antimicrobial (Shelef 1983), antibacterial and antiviral activities (Sokmen 2004). Car also inhibits liposome phospholipid peroxidation and has a higher antioxidant (AO) activity than various syntethic antioxidants (Baser 2008). Car can prevent CP's dose limiting toxic effects and may also allow to use high doses of CP, which would lead to a better clinical outcome for the threapy. Hence, in this study, different doses of CP was applied with/without different doses of Car and heart tissue damage histologically, serum levels of creatinekinase-MB (CK-MB), glutathione (GSH), malondialdehyde (MDA), alanine transaminase (ALT), aspartate transaminase (AST), lactate dehyrogenase (LDH), total oxidant level (TOS), total antioxidant capacity (TAS) and oxidative stress index (OSI) biochemically were analysed.

MATERIAL AND METHODS

Animals

All the animal studies were conducted according to the approval of *ESOGU Experimental Animals Ethic Comitte's.*Provided from *Public Health Center*. The animals were fed in a standard environment with ordinary tap water and pellet food. Albino rats (*Sprague dawley*, 3-4 months old, male, weight 220 ± 20 g healthy) were divided in 13 (n=7) groups together with the control group (control, 50-100-150 mg/kg CP groups, olive oil 5.0 and 10 mg/kg Car groups, CP+5 Car and CP+10 mg/kg Car groups). Before the first injection and killing, animals were weighed. At the end of the experiment, in accordance with ethical guidelines, animals were sacrifized with heart puncture under ketamine/xylazine anesthesia and blood was drawn. Conrol group received 0.5 mL saline intraperitonally (i.p.). For the groups, which received Car together with CP, Car application started three days before the CP aplication and continued till the end of experiment (six days). On the 4th day, animals were weighed, CP doses were calculated and CP+Car were given together. Only for the CP given groups, anaesthesia has been applied three days after the CP application. Thus, on the 4^{th} and 7^{th} days, hearts were taken out from the animals and intracardiac blood was drawn under anaesthesia.

Preparation of Serum Samples and biochemical analysis

Blood samples were centrifuged at 3000 *g* for 10 min. Serum samples were analyzed for alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehyrogenase (LDH), creatinekinase MB (CK-MB) enzyme with total antioxidant level (TAS), total oxidant level (TOS) and glutathione (GSH) levels and plasma malondialdehyde (MDA) levels. ALT, AST and LDH measures were done by using HITACHI – 917 oto analyser (Human Gesellschaftfür Biochemicaund Diagnostica GmbH, Wiesbaden Germany) and with the commercial kits.

Defining PlasmaMalondialdehyde (MDA) Levels

Malondialdehydeamountsin plasma samples were measured by the Tiobarbituric Acid Reactive Substance method developed by Yagi (1984). Lipid peroxidation product (MDA) was measured with spectrophotometer at 520 nm.

Measurement of Serum Glutatyon (GSH) Levels

GSH amount has been measured at 412 nm using Sedlak and Lindsay method. Samples were precipitated with 50% trichloroacetic acid TCA) and were centrifuged at 1000 *xg* for 5 min. From the top phase 0,5 mL was taken, to which 2.0 mL Tris-EDTA buffer (0,2 M, pH 8,9) and 0.1 mL 0,01 M 5,5'-ditiyo-bis-2-nitrobenzoic acid was added. This mixture was kept at room temperature for 5 min and its absorbances was measured at 412 nm (Sedlak and Lindsay 1968) by a spectrophotometer(UV-1700 Shimadzu).

Measurement of Total Oxidant Level (TOS) and Total Antioxidant Capacity (TAS)
Total oxidant level (TOS) levels were measured by using the commercial colorimetric-assay kit (RelAssay, Ref. RL27, Turkey) following the protocols of the manufacturing firm. The absorbance of the samples was measured by using VERSA maxtunable microplate reader (Molecular Divices, California, USA) at 530 nm and results were given as µmolH_2O_2equivalent/L type (Erel 2005).

Measurement of Oxidative Stress Index (OSI)
Oxidative Stress Index (OSI) was calculated by taking TOS/TAS proportion. For this, the unit of TAS value was changed from the mmol Troloxequivalent/L type to µmol Troloxequivalent/L type. OSI was calculated as below:
OSI= *[(TOS, µmol H_2O_2 equivalent/L) / (TAS, µmolTroloxequivalent/L) x 100*] formula (Aycicek et al. 2005).

Histological analysis
Hearts were fixed with 10% formaldehyde solution.Through routine histological preperations, samples were embedded in paraffin and 5.0 µm thick serial sections were made, which were stained with Hematoylin-Eosin and histopathologic features were evaluated.

Statistical Analysis
The data analyzes were performed with SPSS 20.0 and SigmaStat software packages. Independent measurements and continuous data with a normal distribution were analyzed with One Way Anova. Kruskal-Wallis test was applied to score variants with abnormal distribution. Differences among the experimental groups were significant if $p<0.05$.

RESULTS AND DISCUSSION

Results of CP application at 50, 100 and 150 mg/kg doses are shown in Figures 1, 2, 3, 4. Evidently, theLevels of CK-MB, a specific marker of heart muscle damage, were high in CP applied experimental groups and it showed a direct proportional increase with CP dose increase. The cardiotoxic side effects of CP increased with the increase in dose. However, Car at doses of 5.0 and 10 mg/kg ameliorated the toxicity by decreasing CK-MB level almost to the control level (Fig. 5).

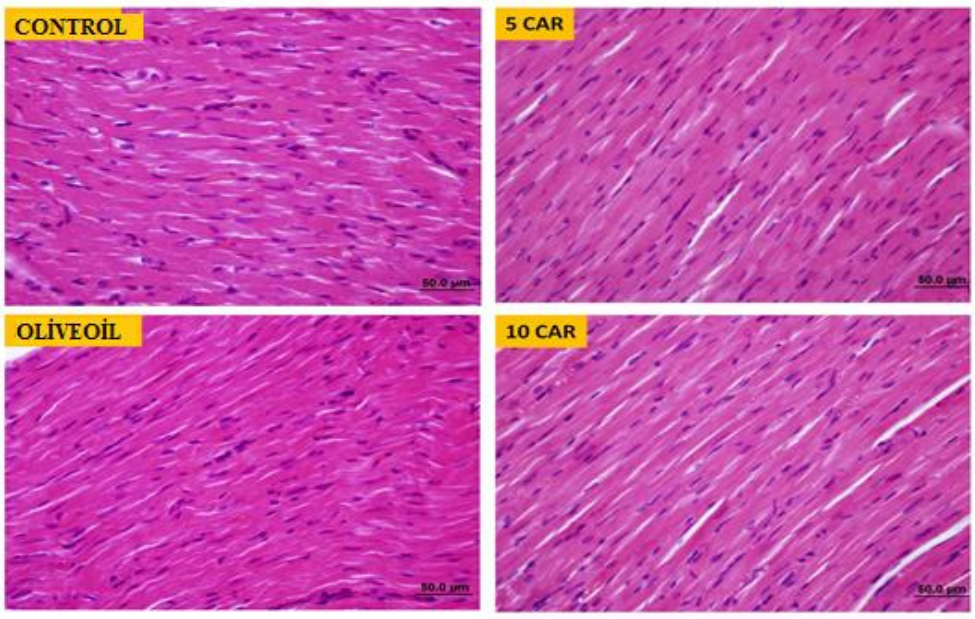

Figure 1 - Each of the four groups of heart tissues are observed to be in normal histological appearance.

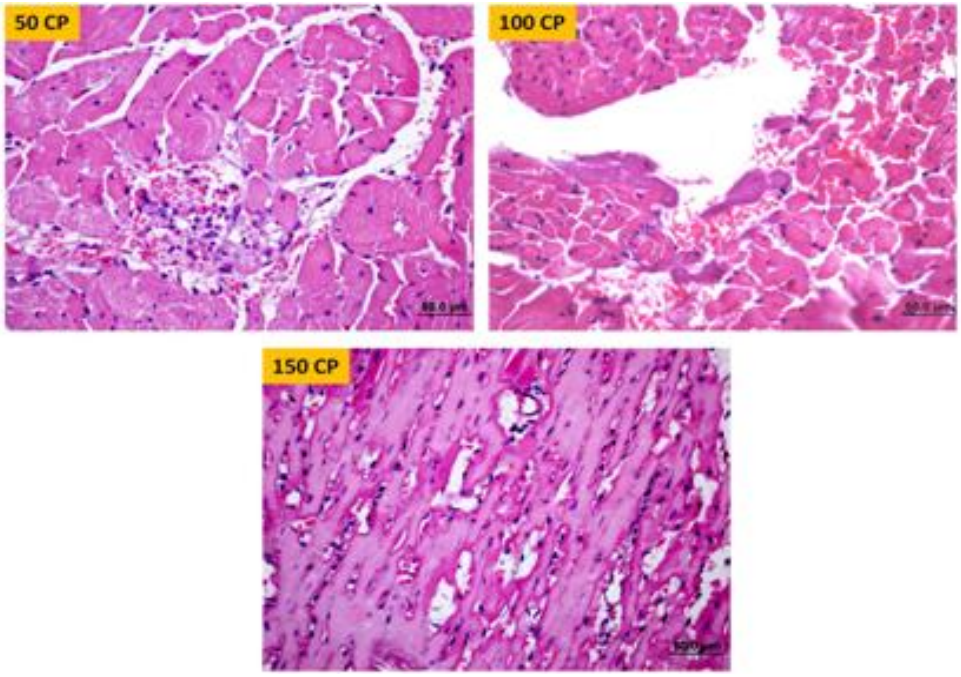

Figure 2 - Purulent cell foci in heart section belonging to 50 CP group, acidophilic stained muscle cells and heterochromatic cell nucleuses, erythrocytes indicate bleeding between muscle fibers in 100 CP group, separation of muscle fibers from each other and fading of the stain indicating degeneration in 150 CP group. In 100 CP group, separation of muscle fibers from each other and stain loss indicate degeneration in 150 CP group.

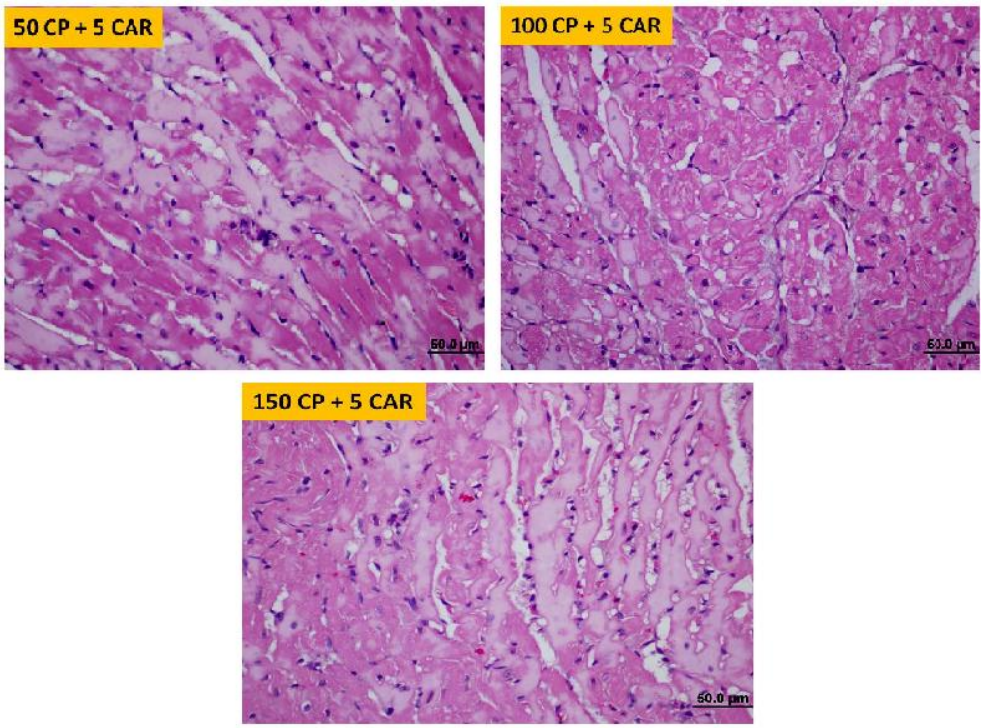

Figure 3 - It is observed that that there are divisions among muscle cells in sections of 50, 100 and 150 CP and 5 mg/kg dose Car applied groups, cells lose their normal staining properties, vacuoles occur within cells and sufficient improvement with low doses of Car is not available.

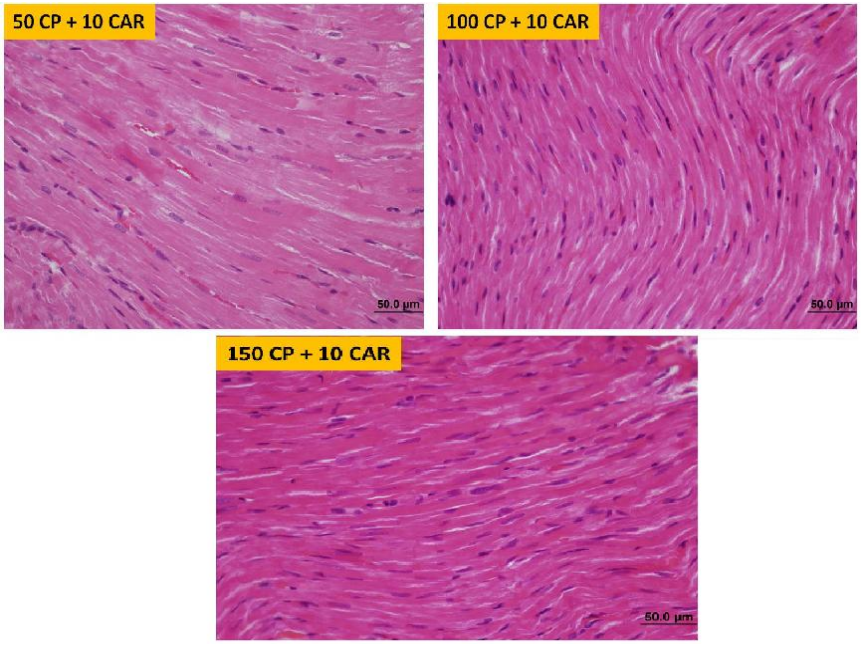

Figure 4 - It is observed that muscle cells have normal staining properties and structure and improvement is provided with high doses of Car in sections of 50, 100 and 150 CP and 10 mg/kg dose Car applied groups.

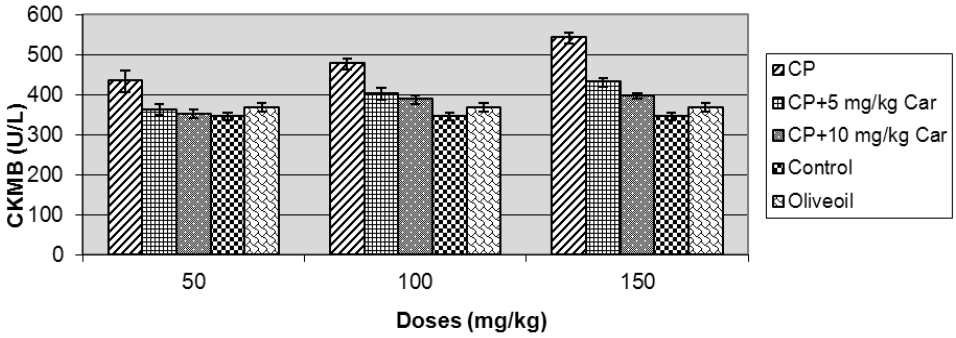

Figure 5 - CK-MB levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

Main marker of the oxidative stress, lipid peroxidation (MDA), increased in CP applied groups (150 mg/kg CP group as the most significant increasing one). Dose of 10 mg/kg Car significantly decreased MDA levels close to the control levels only in 100 mg/kg CP group **(**Fig. 6).

GSH, which protects the cardiomyocytes against reactive oxygen species and plays critical role against the cell damage of oxidative stress, showed a significant decline in CP applied experimental groups. Car at both the doses increased the GSH levels close to the control GSH levels (Fig. 7). The levels of ALT, AST and LDH were generally increased in the CP groups in relation to the dose. Car at 5.0 and 10 mg/kg doses lowered the levels of serum ALT, AST and LDH in the CP groups to nearly the control level (Fig. 8, 9, 10).

In 50, 100, 150 mg/kg CP groups, when TOS increased, TAS decreased, which indicated CP-induced oxidative stress and cardiotoxicity. Accordingly, OSI level was alos at high level. Car at 5.0 and 10 mg/kg prevented the CP toxicity, which explained the decrease in TOS level and increase in TAS level (Fig. 11, 12, 13).

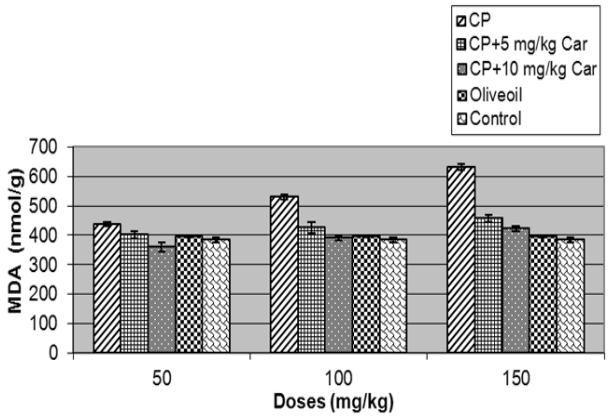

Figure 6 - MDA levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

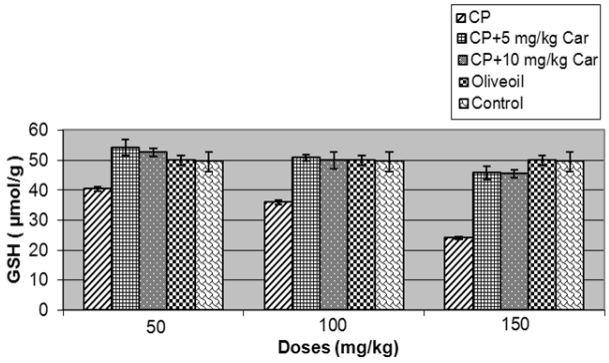

Figure 7 - GSH levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

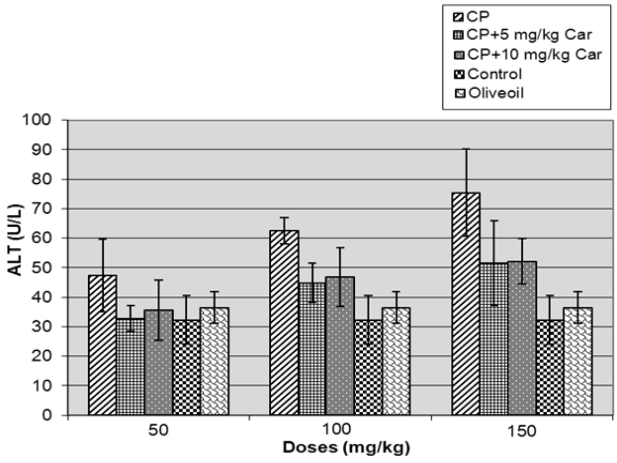

Figure 8 - ALT levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

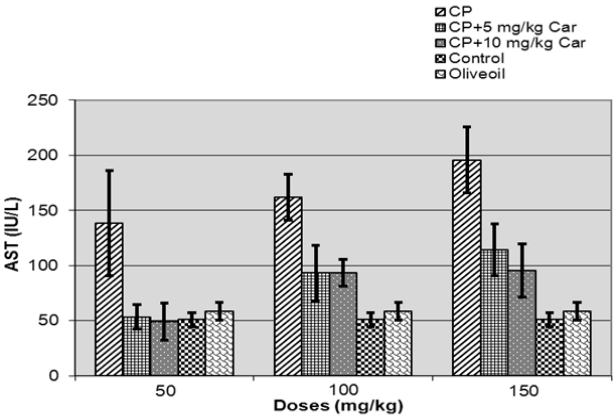

Figure 9 - AST levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

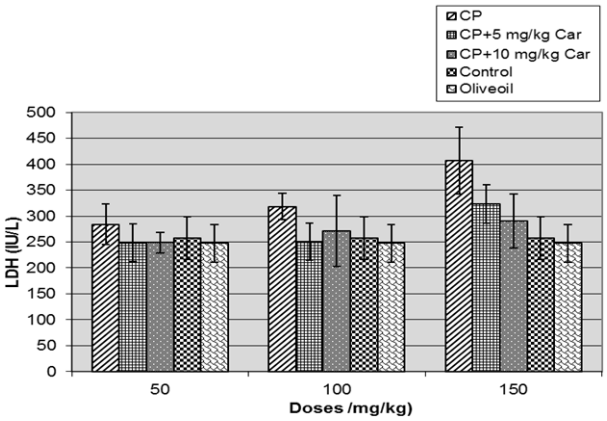

Figure 10 - LDH levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

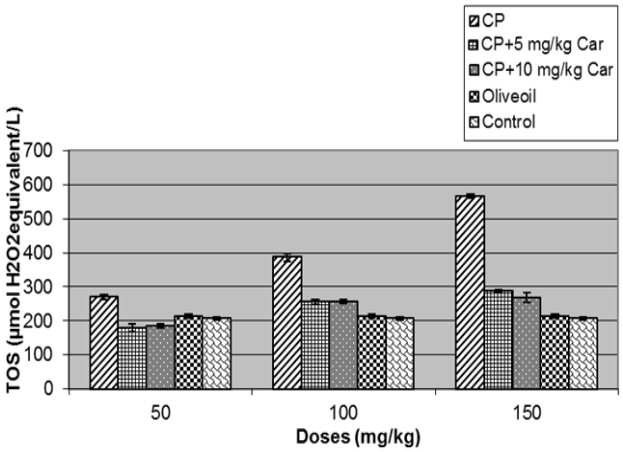

Figure 11 - TOS levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

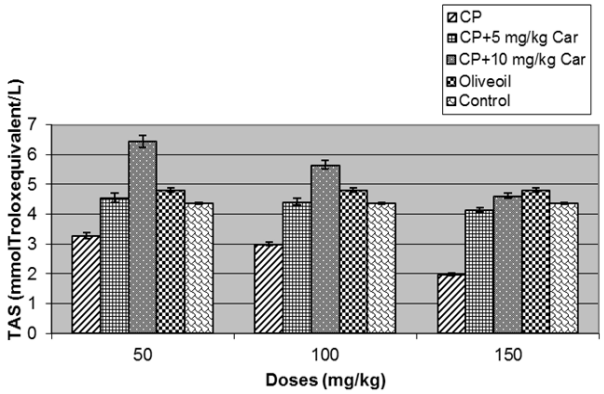

Figure 12 - TAS levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups**.**

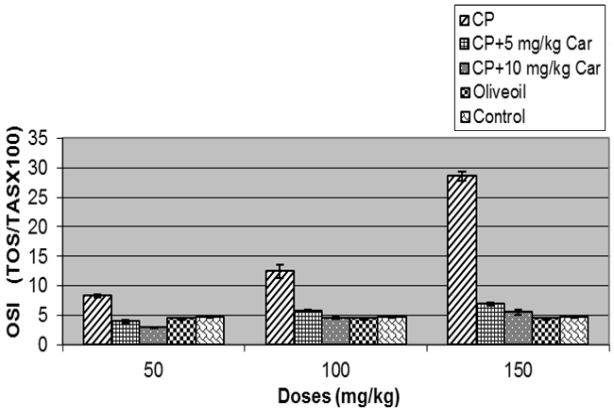

Figure 13 - OSI levels of 50-100-150 mg/kg CP, 50+5, 100+5 and 150+5 mg/kg CP+Car and 50+10, 100+10 and 150+10 mg/kg CP+Car applied experimental groups.

It is reported that a common side effect of CP related anticancer treatments are cardiovascular toxicity and acute fatal cardiomyopathy observed with high dose of CP (200 mg/kg) (Sayed-Ahmed et al. 2014). It has also been reported that 100 mg/kg CP given to the rats caused oxidative stress, increased MDA and NO level, decreased TAS level (Wei et al. 2012). Motawi et al. (2010) found that at higher doses of CP (200 mg/kg), there was heart damage as a result of the high oxidative stress, NO and MDA and decreased GSH and TAS levels. In some clinic and experimental studies (Mythili et al. 2004; Fatani et al. 2010), it has been observed that high doses of CP increased the serum levels of CK-MB, LDH, ALT and AST. Zarei and Shivanandappa (2013) reported that the serum levels of ALT, AST, LDH, SOR and lipid peroxidation levels increased and GSH, AO eynzyme activities decreased with the CP treatment in the rats. Yousif (2010) found that in the heart tissue of CP given groups, myocardial dysfunction was seen along with the oxidative stress. Sayed-Ahmedet al. (2014) reported that CP treatment led to increased serum LDH and CK-MB levels and acute heart failure. In a study by Motawi et al. (2010), histological findings showed that there were hemorrhagical lesions on myocardium and siruption of myocardial fibers. Thus, histological findings of the present study were consistent with other studies.

In this study, the tissue damage was also a result of membrane damage caused by the CP metabolites. These pathological changes were consistent with the changes in enzyme activity. Probably, CP induced cardiotoxicity was a result of a mitochondrial dysfunction and resulted in a decrease in ATP due to oxidative and nitrosative stress. These findings showed that Car applied together with CP decreased the tissue damage and abnormal pathological findings such as necrosis, and also protected the heart tissue against oxidative damage. Car also acted as an atioxidant and as a membrane stabilizer to protect the cellular, thus tissue integrity.

CONCLUSION

From the results, it could be concluded that that Car was a good candidate to eliminate the cardiotoxic side effects of CP but in-depth studies would be needed to evaluate its clinical application on humans.

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