Cardioprotective Effect of Cornus Mas Fruit Extract against Carbon Tetrachloride Induced-Cardiotoxicity in Albino Rats

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ABSTRACT

In the present study, cardioprotective effect of *Cornus mas* fruits extract (CMFE) was evaluated in a rat model having acute cardiotoxicity, induced by single dose of carbon tetrachloride (CCl_4) (1ml/kg i.p.). Pre and post treatment of *C. mas* fruit extract (300 and 700mg/kg) in CCl_4-treated rats significantly decreased the increased levels of serum lactate dehydrogenase, serum creatine kinase and myocardial lipid peroxides and significantly increased the myocardial endogenous antioxidants (glutathione peroxidase, superoxide dismutase and catalase) levels. The results of biochemical observations in the serum and heart tissues were supplemented by histological examination of rats' heart sections to confirm the myocardial injury. The present results provide evidence for the first time, that *Cornus mas* fruit extract (CMFE) treatment ameliorated myocardial injury and enhanced the antioxidant defense against CCl_4-induced cardiotoxicity in rats and exhibited cardioprotective properties.

KEYWORDS: *Cornus mas*, carbon tetrachloride, lipid peroxidation, cardiotoxicity.

INTRODUCTION

Carbon tetrachloride (CCl_4), a clear, colorless, volatile, heavy and nonflammable liquid, is a well-known model compound for producing chemical tissue toxicity by generation of free radicals in many tissues (Adaramoye, 2009) such as liver, kidneys, heart, lung, testis, brain and blood (Ahmad et al., 1987, Ozturk et al., 2003). It is biotransformed by hepatic microsomal cytochrome P450 to trichloromethyl-free radical ($CCl_3^\bullet$ or $CCl_3OO^\bullet$) (Rechnagel et al., 1973, Brattin et al., 1985, Rikans et al., 1994, Shenoy et al., 2001), which in turn, initiate lipid peroxidation process (Yuan, 2008, Upur, 2009, Adewole et al., 2010).

The most widely accepted mechanism of CCl_4 induced cardiotoxicity is the formation of free radicals which is a rate limiting process in tissue peroxidative damage (Plaa and Witschi, 1976). This free radical and related reactive species may cause oxidative stress, which produces major interrelated rearrangements of cellular metabolism, increase in intracellular free calcium, damage to membrane ion transport and permeability, and destruction of the cells by lipid peroxidation (Giordano., 2005). The accumulating of lipid peroxides introduces hydrophophilic moieties hydrophobic phase and thus alter membrane permeability and cell function. This leads to loss of myocardial structural integrity and depressed cardiac function resulting in cardiotoxicity and congestive cardiac failure (Plaa and Witschi, 1976).

However, several endogenous protective mechanisms have been evolved to limit ROS and the damage caused by them (Sies, 1993), but this protection may not be complete, or when the formation of ROS is excessive, additional protective mechanisms of dietary antioxidants may be of a great importance (Lieber, 1996, Cervinkova and Drahota, 1998). Maintaining the balance between reactive oxygen species and natural antioxidants is therefore crucial, and could serve as a major mechanism in preventing damage by oxidative stress induced by toxic agents (Donder et al., 1999, Türkdoğan et al., 2001). Cooperative defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes (Sreelatha et al., 2009). Antioxidant and radical scavengers have been used to study the mechanism of CCl_4 toxicity as well as to protect tissue cells from CCl_4 induced damage by breaking the chain of lipid peroxidation (Weber et al., 2003). Numerous studies have shown that among horticultural crops, fruits are sources of diverse antioxidant properties, which can protect body against CCl_4 induced oxidative stress (Yilmaz et al., 2009).

Cornelian cherry (*cornus mas*), a medicinal plant has been mentioned for the treatment of inflammation in an ancient system of medicine (Yilmaz et al., 2009). Chemical characterization of *Cornus mas* fruit has shown that it is

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a rich source of phenolic and antioxidant compounds. In addition, it contains vitamins such az E, B_2, B_1, C, anthocyanins, flavonoids, and plenty of oxalic acid is found in (Zargari, 1996, Sreelatha et al., 2009). *C. mas* also contains antioxidant substances such as butyl hydroquinone, butylated hydroxyanisole and butylated hydroxytoluene and has the potential to fight cancer (Yilmaz et al., 2009).

Cornus mas, a medicinal plant has been mentioned for the treatment of circulation disorders and blood dilution in an ancient system of medicin in Iran, but this plant has not been subjected to systematic investigation to assess its cardioprotective effect. So the aim of the present study was to investigate cardioprotective effect of *Cornus mas* fruit extract against CCl_4-induced cardiotoxicity in male rats.

MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA) and Ethylen diamine tetra acetic acide (EDTA) were obtained from Sigma–Aldrich Chemical Co. Ltd. (USA). thiobarbituric acid (TBA) and Carbon tetrachloride (CCl_4) were obtained from Merck Co. (Germany). Assay kits for the estimation of biochemical factor such as lactate dehydrogenase (LDH) and creatine kinase (CK) were purchased from Pars Azma (Iran) and all other chemicals used were of analytical grade were obtained from either Sigma–Aldrich or Merck (Germany).

Plant material and extraction

Cornus mas fruits obtained from suburbs of kaleibar (East Azarbaijan, Iran) at the end of spring 2012. The plant fruit parts were air-dried, protected from direct sunlight, and then powdered. The powder was kept in a closed container at 8 °C. The air-dried fruit of *Cornus mas* were made into a coarse powder. Five hundred grams of the powder was extracted with mixture of methanol: water (7:3) at 25±2 °C. The solvent was completely removed by rotary vacuum evaporator at 50 °C. *Cornus mas* fruits extract (CMFE) was freeze dried and stored in a vacuum desicator untile use.

Animals

Male albino rats of Wistar strain (250-300 g) were used for the study. The animals housed in polypropylene cages in a temperature-controlled room (22±2 ◦C) with relative humidity (44-55%) under 12/12 h light and dark cycles for one week before and during the experiments. Animals were provided with a standard rodent pellet diet and clean drinking water ad libitum. The study has got the clearance from the Institutional Animal Ethical Committee (IAEC) the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental design

Animals were divided into 6 groups of six animals each

- Group I served as a normal control and received drinking water for 16 days orally and on the 16th day olive oil (1ml/kg i.p.).
- Group II served as a toxic control and received drinking water for 16 days orally and on the 16th day CCl_4 (1ml/kg i.p.) in 1:1 dilution with olive oil.
- Group III and IV served as pre-treatment groups (prophylactic). They received CMFE at the dose of 300 and 700 mg/kg, orally for 16 days respectively, and on the 16th day received CCl_4 (1ml/kg i.p.) in 1:1 dilution with olive oil, 2 h after administration of the last dose of extract.
- Group V and VI served as post-treatment groups (curative). They received drinking water orally for 16 days and on the 16th day they received CCl_4 (1ml/kg i.p.) in a 1:1 dilution with olive oil, followed by plant extract at a dose of 300 mg/kg (Group V) and 700 mg/kg (Group VI) orally at 2, 6, 12, 24 and 48 h after CCl_4 intoxication.

Assessment of cardiac functions

All animals were sacrificed by ether anaesthesia then 50 h after CCl_4 administration. Blood samples were collected from left ventricular of heart. Serum was separated by centrifugation at 3000 rpm for 15 min and used for biochemical estimations and was used freshly for the assessment of heart function tests. The lactate dehydrogenase and creatine kinase levels in all the sample sera were estimated by standard diagnostic test kits (Pars Azma, Iran).

Preparation of heart homogenate

Heart tissues were homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA:pH 7.4) and centrifuged at 12000 rpm for 20 min. The supernatant was used for the measurement of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Total protein contents were determined by the method of (Lowry et al., 1951), using bovine serum albumin as a standard.

Measurement of cardiac lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction method (Berton et al., 1998). In brief, samples were mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25-N hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 30 min and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatant was measured at 535 nm. MDA, a measure of lipid peroxidation, was calculated using an extinction coefficient of 1.56×10^5/M cm. The results were expressed as nmol/mg protein.

Determination of antioxidant enzymes

Catalase activity was measured according to the method of Aebi, 1984 (Aebi, 1984). One unit of catalase was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 1 min. The reaction was initiated by the addition of 1.0 ml of freshly prepared 20 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 2 min. The enzyme activity was expressed as U/mg protein.

The activity of SOD was measured according to the method of McCord, 1994 (McCord, 1994). For the determination of SOD activity, xanthine and xanthine oxidase were used to generate superoxide radicals reacting with 2-(4-iodophenyl)-3-(4-nitrophenol)- 5-phenyl tetrazolium chloride to form a red formazan dye. SOD activity was then measured at 505 nm. Data were expressed as U/mg of protein.

GPx activity was determined by the method described by Paglia and Valentine (Paglia and Valentine, 1967). The reaction mixture consisted of 400 μL, 0.25 M potassium phosphate buffer (pH 7.0), 200 μL supernatant, 100 μL GSH (10 mM), 100 μL NADPH (2.5 mM) and 100 μL glutathione reductase (6 U/mL). Reaction was started by adding 100 μL hydrogen peroxide (12 mM) and absorbance measured at 366 nm at 1 min intervals for 5 min using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. Data were expressed as U/mg of protein.

Histopathology

For microscopic evaluation heart were fixed in a fixative (absolute alcohol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4μm, subsequently stained with hematoxylin/eosin and observed under a light microscope mainly for inflammatory, cellular damage and necrotic changes with respect to control healthy rats' heart.

Statistical analysis

All results are expressed as mean ± SE. One way analysis of variance (ANOVA) followed by multiple comparison with the Tukey post hoc test was used to compared different parameters between the groups. A *p* value <0.05 was considered significant.

RESULTS

Effect of CMFE on serum profile in rat

Activities of LDH and CK in serum of normal and experimental rats are presented in Table 1. Administration of rats with a single dose of CCl_4 (1 mg/kg i.p.) caused a significant increase in both plasma CPK and LDH enzyme activities compared to their respective control values. Pre and post treatment with CMFE at doses of 300 and 700 mg/kg significantly (P<0.05) decreased the activities of these enzymes in CCl_4 treated rats as compared to rats treated only with CCl_4.

Effect of CMFE on lipid peroxidation

Activity of lipid peroxidation products (MDA) content of heart in control and experimental animals is presented in Figure 1. There was significant (P<0.05) increase in myocardial MDA levels in the CCl_4 treated compared to control. A significant decrease (P<0.05) in the level of MDA was observed in CMFE treated rats (groups III-VI) compared to rats treated only with CCl_4.

Effect of CMFE on heart antioxidant enzymes activity

Effect of CMFE administration on antioxidant enzymes in heart tissue of control and experimental animals are shown in Table 2. CCl_4 treated rats showed a significant (P<0.05) decrease in the activities of SOD, CAT and GPx compared to control rats. CMFE treated rats (groups III-VI) showed a significant (P<0.05) increase in the activities of these myocardial enzymes compared to rats treated only with CCl_4.

Effect of CMFE on Histopathology of heart

Figure 2A shows the light micrograph of control group heart with normal architecture. The CCl_4 induced rat heart (Fig. 2B), show significant pathological changes, inflammation and edema, to compared with normal control rats.

DISCUSSION

Oxidative stress induced by an increase in free radicals and/or decrease in antioxidant defenses is well documented in several models of cardiotoxicity (M. Rudnicki 2007, Marañón et al., 2008, T. Jayakumara 2008, Botsoglou et al., 2009). CCl_4, a typical toxic agent, exerts its toxic effects by the generation of free radicals. By the activation of liver cytochromes P450, CCl_4 generates methyl trichloride radicals ($CCl_3{}^{\bullet}$), which immediately react with cell membrane (Dashti et al., 1989). Although liver is considered to be the primary target of CCl_4 toxicity, it is not the only target organ of CCl_4 and it causes free radical generation in other tissues also such as kidneys, heart, lung, testis, brain and blood (Ahmad et al., 1987, Ohta et al., 1997, Ozturk et al., 2003, Preethi and Kuttan, 2009). These radicals form covalent bonds with unsaturated fatty acids or take a hydrogen atom from the unsaturated fatty acids in membrane, leading to the production of chloroform and lipid radicals (Kanter et al., 2003). They initiate a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA). Peroxidation of lipids can dramatically change the properties of biological membranes, resulting in severe cell damage and consequently play a significant role in pathogenesis of diseases (Williams and Burk, 1990, Zhang et al., 1996).

The present study revealed that injection of CCl_4 to rats induced oxidative heart tissue damage which is proved by an increase in the lactate dehydrogenase (LDH) and creatine kinase CK (enzymes responsible for ATP regeneration) and malondialdehyde (MDA) level (index of membrane lipid peroxidation) and a decrease in the antioxidant enzymes, SOD, CAT and GPx, level (antioxidant defence system) in hearts of CCl_4-treated rats when compared with normal rats, indicating that the heart is one of the target organs affected by CCl_4 toxicity . These results are in agreement with previous studies demonstrated that CCl_4 can cause oxidative damage and generation of reactive oxygen species (ROS) in different tissues including heart (Ohta et al., 1997, T. Jayakumara 2008, Botsoglou et al., 2009).

Our experimental results have shown that CCl_4 induced a significant increased in the glycolytic enzymes, LDH and CK. The present increase in the activity of diagnostic marker enzyme, LDH and CK (enzymes responsible for ATP regeneration) in cardiac tissue of CCl_4 intoxicated rats versus normal animals, is considered index of cardiac tissue damage. This may be attributed to a generalized increase in membrane permeability and is particularly useful in the diagnosis of muscular dystrophy (Kaczor et al., 2007). Also, it has been reported that the increased levels of LDH result from superoxide anions and hydroxyl radicals in the presence of transition metal ions which cause oxidative damage to the cell membrane (Yadav, 1997). As well as, similar result was obtained in brain of rats under the effect of CCl_4 toxicity (Nishitani et al., 2005). Our results were in agreement with the previous reports of (Van Vleet et al., 1980, Tesoriere et al., 1994, Kang et al., 1996, Wu and Kang, 1998, Monnet and Christopher Orton, 1999, Nagi and Mansour, 2000, Al-Majed et al., 2002, Liu et al., 2002, Yagmurca et al., 2003) in cardiotoxicity by toxic agents. Prophylactic and curative administration of the used antioxidant *Cornus mas* fruits extract (CMFE) in combination to CCl_4 intoxicated rats beneficially up-regulated the increased level of these metabolizing enzymes in their cardiac tissue in relation to CCl_4 treated animals. The best result was obtained with the combination of CMFE as it restored these bioenergetic markers to their normal level. From this result it may be suggested that CMFE may have antioxidant potential action in protection and stabilizing cellular membranes by mainipulating the oxidative tissue damage, thus reducing the release of enzymes.

The increasing in such enzyme may attributed to the ability of CCl_4 to induce lipid peroxidation which has the main cause of many deleterious effects on the cell membrane and coupled with inactivation of membrane bound enzymes (Jadon et al., 2007). It has been reported that lipid peroxidation is one of the major causes of CCl_4-induced toxicity, mediated by the production of free radical derivatives of CCl_4 (Khan et al., 2009). The cardiotoxicity and oxidative damage induced by CCl_4 administration are also manifested by a significant increase in the cardiac content of MDA. MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid (Vaca, 1988). The elevated cardiac content of MDA, found in this study, strongly proves the oxidative damage caused by CCl_4 (Mohamed, 2010). Treatment with CMFE in CCl_4-induced rats recovered the increased MDA level with significant lowering of cardiac MDA content. The significant decrease in the cardiac MDA concentration confirms that treatment with CMFE could effectively protect against the cardiac lipid peroxidation induced by CCl_4.

To prevent the damage caused by oxygen-free radicals, tissues have developed an antioxidant defense system that includes nonenzymatic antioxidants (e.g., glutathione, vitamins C and E) and enzymatic activities such as that of superoxide dismutase, catalase, and glutathione peroxidase (Rechnagel et al., 1973, Castillo et al., 1992, Melin et al., 2000). Superoxide dismutase is found in the cytosol as a zinc/copper-containing enzyme and in mitochondria as a

manganese-containing enzyme. It dismutates the superoxide anion by combining it with protons to form hydrogen peroxide and oxygen (Michiels et al., 1994, M.M. Simibe and Feo., 2001). Catalase is located within the peroxisomes and cytosol of the cell and decomposes hydrogen peroxide to water and oxygen (McCray et al., 1976). Glutathione peroxidase is a selenium containing metalloenzyme, partially located within the cellular membrane, that can remove hydrogen peroxide by converting reduced glutathione into oxidized glutathione. Glutathione peroxidase can also terminate the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane (Singh and Pathak, 1990, Jung and Henke, 1996). Our results indicated the level of antioxidant enzymes such as SOD, CAT and GPx decreased in CCl_4-treated group was recovered by treatment of CMFE. The protective effects of CMFE in maintaining the above enzymes level towards control have increased the capacity of endogenous antioxidant defense and increased the steady state of them and/or its rate of synthesis that confers enhanced protection against oxidative stress.

Furthermore, histopathological observations revealed that *C. mas* fruits extract (CMFE) perevented the degeneration of myofibriller tissue and leucocytic infiltration in myocardial infarction.

Conclusion

In conclusion, the present results demonstrated that the cardioprotective beneficial effect of the current used antioxidant *Cornus mas* fruit extract (CMFE) may be related to their ability to attenuate the extent of peroxidation of membrane lipid, recover the enzymatic antioxidant defence system and up-modulate the bioenergetic state of cardiac tissue and may be used as prophylactic and curative agents against cardio-toxic agents.

Table 1
Effect of CMFE on serum profile in rat.

Treatment	LDH (U/L)	CK (U/L)
Control+ Olive oil	250.33±8.46	151.5±3.33
1ml/kg CCl_4	968.0±14.45[*]	1048.16±7.55[*]
300mg/kg CMFE+CCl_4	692.66±8.92[+]	216.16±4.98[+]
700mg/kg CMFE+CCl_4	632.0±8.32[+]	203.66±5.85[+]
CCl_4+Post 300mg/kg CMFE	469.33±7.51[+]	194.0±3.90[+]
CCl_4+Post 700mg/kg CMFE	410.33±7.48[+]	174.83±1.99[+]

Results are expressed as mean±S.E. ($n = 6$).
* Indicate significance at $P < 0.05$ probability from control group.
+ Indicate significance at $P < 0.05$ probability from CCl_4 group.

Table 2
Effect of CMFE on heart antioxidant enzymes activity

Treatment	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Control+ Olive oil	34.57±0.65	5.45±0.18	0.6±0.01
1ml/kg CCl_4	20.15±0.60[*]	3.43±0.09[*]	0.28±0.18[*]
300mg/kg CMFE+CCl_4	27.25±0.35[+]	4.59±0.16[+]	0.48±0.10[+]
700mg/kg CMFE+CCl_4	29.02±.0.32[+]	4.88±0.14[+]	0.46±0.00[+]
CCl_4+Post 300mg/kg CMFE	30.03±0.33[+]	4.52±0.19[+]	0.51±0.01[+]
CCl_4+Post 700mg/kg CMFE	31.43±0.39[+]	5.15±0.12[+]	0.57±0.01[+]

Results are expressed as mean±S.E. ($n = 6$).
* Indicate significance at $P < 0.05$ probability from control group.
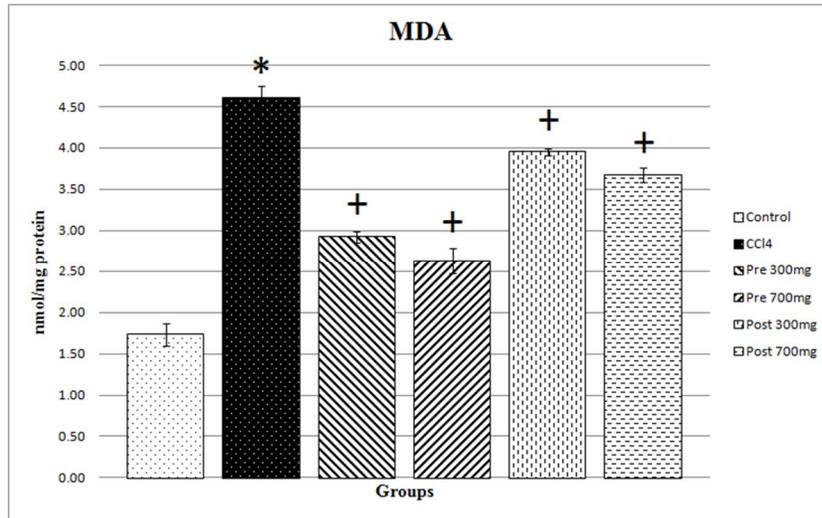+ Indicate significance at $P < 0.05$ probability from CCl_4 group.

Fig. 1. Effect of CMFE on myocardial contents of MDA in rat:
* Indicate significance at $P < 0.05$ probability from control group.
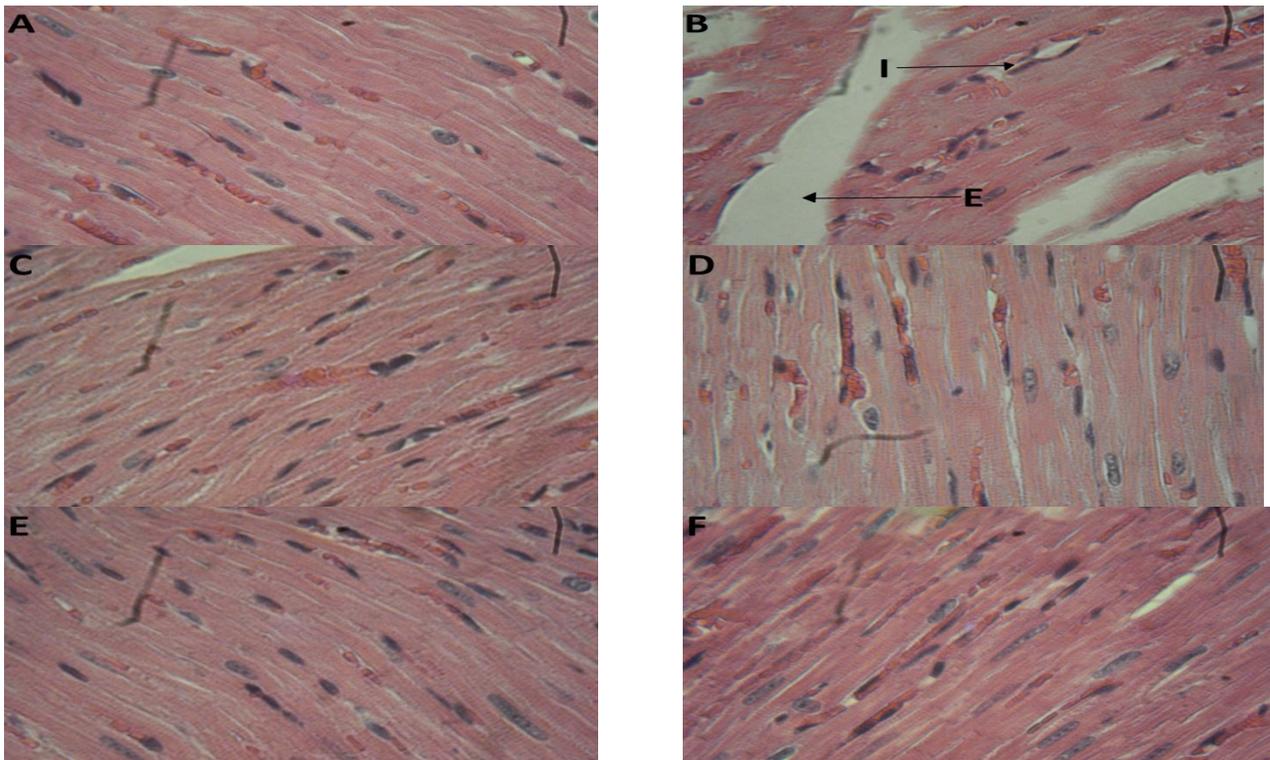+ Indicate significance at $P < 0.05$ probability from CCl$_4$ group.

Fig. 2. Histological examination of heart in experimental animals. (A)- Vehicle control group (Group I) rat showing normal architecture of heart with regular morphology of myocardial cell membrane (40X); (B)- Pathologenic control group (Group II) rat showing inflammatory infiltrate (I) with edema (E) in heart section (40X); (C-F)- CMFE treatment group (Group III - VI) rat showing absence of inflammation and sign of muscle necrosis in heart section (40X).

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