Section A-Research paper

Formulation Development and Pharmacological Evaluation of Fixed Dose Combination of Saffron, Olive Oil and Coenzyme Q10 Against Doxorubicin Induced Cardio Toxicity In Rat.

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ABSTRACT

The present study aims at formulation development and pharmacological evaluation of fixed dose combination of saffron(*Crocus sativus*)extract, olive oil and coenzyme Q10 (CoQ10) against doxorubicin induced myocardial toxicity in rats.Formulation (emulsion) was prepared using dry gum method (continental method), by using pestle and mortar. The formulation was characterized by performing stability studies which includes flocculation and creaming, cracking, phase inversion and

accelerated studies (temperature and light). In-vivo pharmacological evaluation of *Crocus sativus* extract, olive oil, CoQ10 and its fixed dose combination (FDC) were then performed. The results indicated that developed FDC passed all stability tests and significantly prevented drug induced increase in serum levels of AST, ALT,LDH, Creatinine and lipid profile (TC,LDL,VLDL and TG) and increases the levels of HDL and antioxidant parameters - SOD, GSH and CAT (in heart tissue). It also lowered the doxorubicin induced increase in heart weight due to hypertrophy. These results were also confirmed by histopathology. The results of this study strongly indicate the cardioprotective effect of fixed dose combination of (*Crocus sativus*) extract, olive oil and CoQ10 against doxorubicin induced myocardial toxicity.

Keywords*:* Coenzyme $Q_{10,}$ *Crocus sativus*, doxorubicin, formulation, olive oil

INTRODTION:

Cardiovascular illness (CVD) is an umbrella term for various connected pathologies, normally characterized as coronary illness (CHD), cerebrovascular sickness, fringe blood vessel infection, rheumatic and innate heart sicknesses and venous thromboembolism. All around the world CVD represents 31%of mortality, most of this as CHD and cerebrovascular mishap. (Stewart J, et al 2017) According to data obtained from the World Health Organization, cardiovascular diseases accounted for 31% of all causes of death in 2016, and they are considered as the leading cause of mortality worldwide **(SHIBA Nobuyuki et al 2017).** One of the most risk factors for cardiovascular diseases is metabolic syndrome, a multifactorial entity that includes variables such as obesity, dyslipidemia, hypertension and glucose metabolism dysfunctions (Spahis S et al 2017). Cardiovascular diseases (CVDs), mainly caused by , hyperlipidemia, blood viscosity, obesity, hypertension, atherosclerosis, smoking, aging, physical inactivity, and others, are the leading causes of death worldwide. conversely, the risk of getting CVDs remains a serious threat to social society. Moreever, despite recent advances in the management of CVDs, our understanding of the signaling pathways involved in the pathological and physiological conditions is limited, contributing to the lack of therapeutic targets for CVDs (Shi Q and Yang X., 2016). more than 2000 plants must be listed in the Traditional (Herbal/Alternative) systems of medicine and some of these are given that complete relief to the people anguish from cardiovascular diseases, specially “ischemic heart disease” “hyperlipidaemia” and WHO information indicate that around eighty percent of the overall population still have faith in on botanical drugs and several herbal medicines have advanced to clinical use in current times (Aboul-Fotouh S.2016). Prevention of CVD

by targeting modifiable factors remains a vital public health priority. Diet plays a main role in the aetiology of several chronic diseases including cardiovascular diseases, in that way contributing to a major geographical changeability in morbidity and mortality rates through different countries and populations global(WHO/FAO 2002). A number of dietary factors have been initiate to be connected with CVD risk, such as a low consumption of fruit and vegetables (Begg 2007).The World Health Organization (WHO) gauge that more than 75% of untimely CVD is preventable and hazard factor improvement can assist with decreasing the developing CVD trouble on the two people and medical services suppliers (Ahmed Abdelrahim et al 2021). Whilst age is a realized danger factor for the advancement of CVD, post-mortem examination proof recommends that the most common way of creating CVD in later years isn't inescapable consequently hazard decrease is essential (Kannel WB et al 1987). The INTERHEART study clarified the impact of CVD hazard factors including dyslipidaemia, smoking, hypertension, diabetes, stomach corpulence, while it showed the defensive impacts of utilization of products of the soil, and ordinary actual work. These danger factors were reliable all through all populaces and financial levels examined, assisting with setting up the suitability of uniform ways to deal with CVD essential anticipation around the world (Yusuf S et al 2004). In an effort to improve guidelines to warfare obesity, the American College of Cardiology (ACC) and the American Heart Association (AHA) collaborated with the National Heart, Lung, and Blood Institute to improve clinical practice guidelines for the assessment of cardiovascular risk, way of life modifications to reduce cardiovascular risk, management of blood cholesterol in adults, and management of overweight and obesity in adults (Jensen MD et al 2013). The authors point out that guidelines are meant to be evaluated with respect to the individualized patient; they are not a replacement for clinical judgment. (Jensen MD et al 2013) Therefore, the practitioner must assess the practicality of these guidelines in concurrence with their patient. These guidelines give a primary view of healthy dietary changes and objectives which may reduce cardiovascular risk. The AHA guidelines on the major prevention of cardiovascular disease (CVD) give emphasis to that diets associated with CVD impermanence typically consist of sugar, low-calorie sweeteners, high-carbohydrate diets, low-carbohydrate diets, refined grains, trans fat, saturated fat, sodium, red meat, and processed red meat (such as bacon, salami, ham, hot dogs, and sausage). These guidelines encourage a healthy plant-based or Mediterranean-like diet high in vegetables, fruits, nuts, whole grains, lean vegetable or animal protein (if possible fish), and vegetable fiber. shown to lower the risk of all-cause mortality. (Arnett DK,et al 2019) The authors aptly means that while these guidelines are focused on practice within the

us , they're relevant to the worldwide patient. In addition to these recommendations, several other diets exist with variable long-term cardiovascular outcomes In addition to these recommendations, several other diets exist with variable long-term cardiovascular outcomes. (Chiavaroli L et al 2019) (McDonald TJ et al 2018) (Atallah R et al 2014) 10,69,70 Additionally, newer dietary strategies including the ketogenic diet (Moreno B et al 2016) and intermittent fasting (Patterson RE, Sears DD 2017)have gained popularity. The purpose of this review is to evaluate the clinical evidence of established dietary patterns with regards to reducing cvd risk, as well as to discuss the existing literature regarding newer dietary strategies.

Crocus sativus *L.* var. *cashmirianus,* (Iridaceae) commonly has known as kesar (Hindi), avarakta (Sanskrit) and saffron (English). It comprises of the dried red stigma with a small portion of the yellowish style attached. It contains crocetin which can play a critical role in myocardial injury, since protecting properties of amino acids has just been accounted for fundamentally enhancement of the heart protection (Ahsan et al. 2018). Toxicity studies have demonstrated that the hematological and the biochemical parameters were within a normal range in mice treated with saffron extracts (Nair et al. 2019). Further, a recent work investigated either the acute (up to 3 g, both orally (p.o.) and intraperitoneally (i.p.)) or the sub-chronic effects of crocin (15–180 mg/kg, i.p.) in different biochemical, hematological and pathological parameters in rodents. The results of this study demonstrated that chronic treatment with crocin did not alter the weight of heart, lung, liver, kidney and spleen. Crocin, at the highest dose (180 mg/kg), increased platelets and creatinine levels, and reduced food intake and body weight. A decline in alveolar size in lungs was observed following the highest dose of crocin (180 mg/kg). The authors concluded that crocin, at pharmacological doses, was not shown to markedly damage any of the major organs of the body (Bathaie SZ,and Mousavi SZ., 2010)

Olive Oil is a liquid fat obtained from olives (the fruit of *Olea europaea;* family(*Oleaceae)* Olive oil is approximately 72-percent oleic acid, a monounsaturated fatty acid. (Newmark & Squalene, 1997). Olive oil is unique with respect to the high oleic acid content because the majority of seed oils are composed primarily of polyunsaturated fatty acids, including the essential omega-6 fatty acid, linoleic acid. Compared to polyunsaturated fatty acids, oleic acid is monounsaturated, meaning it has one double bond, making it much less susceptible to oxidation and contributing to the antioxidant action, high stability, and long shelf life of olive oil (Owen & Giacosa, 2000). The Mediterranean diet has been credited with many beneficial effects in the prevention and treatment of cardiovascular diseases

(Walker & Reamy, 2009). including improvement of lipid profiles, reduction in blood pressure, insulin resistance, blood glucose concentrations, inflammatory biomarkers, and total mortality (Martinez *et al.*, 2009)The cardioprotective effects of olive oil, one of the important components of the Mediterranean diet, have been attributed to oleic acid, the major fatty acid in the oil, as well as the polyphenols, hydroxytyrosol and oleuropein (Covas, 2007) (Huang & Sumpio, 2008).

Doxorubicin is an anthracycline, is widely used as antineoplastic agent. A number of mechanisms have been proposed for cardiotoxic effects of doxorubicin, but the exact causal mechanism of doxorubicin induced cardiomyopathy remains unclear (Sinha et al. 1987), however till date, at least three major hypotheses have been raised to explain DIC, including activated reactive oxygen species (ROS) 1, 3, 4, 5, inhibition of topoisomerase II-β (TOP2β) 6, 7, and modulation of intracellular calcium release (Ma et al. 2018).

Coenzyme Q_{10} was discovered by Fredrick Crane in 1957 Coenzyme Q_{10} a member of the ubiquinone family, is an essential component of the mitochondrial electron transfer chain, which is required for ATP synthesis and functions as an antioxidant in cell membranes and lipoproteins (Ernster, & Dallner, 1995). CoQ_{10} is also a powerful antioxidant not only within the mitochondria but also in other organelle membranes containing CoQ_{10} (Overvad *et al.,* 1993). Research has confirmed the effectiveness of CoQ10 in improving angina episodes, arrhythmias, and left ventricular function in patients having acute myocardial infarction (Singh Ram et al. 1998). Acute and sub-acute toxicity study reveals that Q10 up to a dose of 2.25 g/kg does not show any mortality or adverse effects in rats (Fu et al. 2009). *CoEnzyme Q10* Coenzyme Q10 (CoQ10), is located within the mitochondrial inner membrane (Greenlee et al. 2012). Research has confirmed the effectiveness of CoQ10 in improving angina episodes, arrhythmias, and left ventricular function in patients having acute myocardial infarction (Singh Ram et al. 1998). Acute and sub-acute toxicity study reveals that Q10 up to a dose of 2.25 g/kg does not show any mortality or adverse effects in rats (Fu et al. 2009).

The aim of the study ‘formulation development and pharmacological evaluation of fixed dose combination of Saffron, Oliveoil and CoenzymeQ_{10} against Doxorubicine induced cardiotoxicity in Rats’.

MATERIAL AND METHODS:

Drugs and chemicals

The entire chemicals which were used are of analytical grade. Crocus sativus (Saffron) and olive oil were purchased from local market, Lucknow, India, Coenzyme Q10(Cipla), Doxorubicin

Hydrochloride Injection (GLS Pharma Ltd), Methanol (ChangshuYangyuan chemical, China), Formaldehyde, Diethyl ether (Fisher scientific Ltd, Mumbai, India), Normal saline (0.9%) (Albert David Ltd, Ghaziabad, India)

Procurement and authentication of plant material

The study materials was procured from local market *crocus sativus* of the family **Iridaceae.** Authenticated by Dr. Mohd Arif and Dr. Mohd Khalid at faculty of pharmacy, integral university, lucknow the accession no. for the specimen is **IU/PHAR/HRB/17/08.**

Preparation of saffron aqueous extract

About 1 g of saffron stigmas Clean and fresh Saffron powder was mixed with distilled water (1:50 w/v) and left on a shaking incubator at 8 °C for 48 hr. The solution was centrifuged (4000 g for 10 min). The yielded supernatant will be retained and sediment will be suspended in the half amount of mentioned distilled water and placed on the shaking incubator for another 24 hr. The centrifugation will be repeated again and resulting supernatant will be concentrated under reduced pressure at a temperature less than 15 C^0 and will be store at $4C^0$ for further use. (Christodoulou E et al., 2019)

Preparation of emulsion:

For small scale preparation of emulsion, pestle and mortar is most widely used equipment. Dry gum method was used for the preparation of emulsion which is also known as continental method and is mainly used for emulsions prepared from dry gum emulgents (such as acacia). The proportion of gum used depends upon the nature of oil. The formula for fixed oils (almond oil) is 2:1:4 (oil:gum:water) for w/o emulsions. Measured quantity of oil was thoroughly triturated with acacia in the mortar. Small amount of aqueous vehicle was added to the contents of the mortar and rapidly triturated till a thick cream was formed. This product was known as primary emulsion and was characterized by a clicking sound on trituration. Remaining quantity of aqueous vehicle was added gradually to the primary emulsion with trituration. The emulsion was transferred to a measure and the volume was adjusted. The product was finally stirred to make a uniform solution (Jain and Sharma, 2008).

Thermodynamic stability of emulsions:

To evaluate the stability, emulsions were subjected to thermodynamic stability testing, which comprises of heating cooling cycle, centrifugation studies and freeze thaw cycle. Physical stability was continuously monitored throughout the experiment. Various aspects like flocculation, phase separation, creaming, cracking, etc. at room temperature were observed (Akhter *et al*., 2008).

Freeze thaw cycle: Selected emulsions were kept in deep freezer (at -20oC) for 24hrs. After 24hrs the emulsions were removed and kept at room temperature. The thermodynamically stable emulsions returned to their original form within 3 minutes. 3 such cycles were repeated.

Centrifugation studies: Emulsions after freeze thaw cycle were subjected to centrifugation studies where they were made to undergo centrifugation for 30 minutes at 5,000 rpm in a centrifuge. The stable formulations did not show any phase separation.

Heating cooling cycle: Emulsions were kept at 37±0.5oC for 24hrs. After that the emulsions were kept at room temperature. The stable emulsion should not show any sign of turbidity, cracking, creaming, phase separation, etc during the entire cycle.

Animals

Adult Sprague Dawley rats (120–250 g) were procured from Central Drug Research Institute (CDRI), Lucknow. They were kept in departmental animal house, Integral University. The animals were housed separately in polypropylene cages for acclimatization at a temperature of (23 ± 2 °C) and 50–60% relative humidity, with a 12 h light/ dark cycle 1 week before and during the commencement of the experiment. Animals were kept on standard pellet diet (Dayal animal feed) and drinking water ad libitum throughout the study period. The study protocols were approved by Institutional Animal Ethical Committee (IAEC), Faculty of Pharmacy, Integral University, Lucknow (Reg no. 1213/PO/ Re/S/08/CPCSEA, 5th June 2008) having approval no. IU/ IAEC/18/17.

Experimental design

Rats were weighed and randomly divided into 7 groups (5 each) prior to dosing the animal was allow for acclimatization to the laboratory conditions for 7 days. Treatment schedule was given as per Table 1. At the end of the study (i.e. day 15th) blood was collected through optical puncture under anaesthesia (thiopental sodium) for biochemical evaluation and rats were euthanized by cervical decapitation and the hearts were dissected out immediately, a portion was used for biochemical estimation and remaining was stored in formalin for histological examination. The serum was separated by centrifugation at 5000 rpm at 4 °C for 15 min and was used for the estimation of marker enzymes.

Table. 1 Treatment schedule

Groups (N=7)	**Treatment**	**Dose, Route and & Duration**
I (NC)	Vehicle	10ml/kg p.o. once a day for 15 days

II (TC)	Vehicle + Doxorubicin	10ml/kg p.o. once a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
III (Std.)	K.A.H.A.W+ Doxorubicin	800mg/kg p.o. onc a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
IV Treated	Saffron extract+Doxorubicin	20mg/kg p.o once a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
V Treated	Olive oil+Doxorubicin	1ml/kg p.o once a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
VI Treated	CoQ_{10}+Doxorubicin	5 mg/kg p.o once a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
VII	FDC+Doxorubicin	FDC orally once a day for 15 days + 2.5mg/kg i.p once on alternate days for 15 days
VIII	FDC	FDC 2.4 ml/kg p.o once a day for 15 days

Each 2.4 ml contain 1ml olive oil + 20mg extract + 5mg CoQ10 + water q.s. Vehicle = 3ml/kg (water + acacia) NC = Normal control, TC = Toxic control, Std. = Standard, p.o. = per oral, i.p = intraperitoneal, CoQ_{10} = Coenzyme Q_{10}, K.A.H.A.W. = Khamira Abresham Hakim Arshad Wala.

Parameters assessed

Physical parameters

Estimation of heart weight:body weight ratio Rats were weighed before euthanasia and were sacrificed under high dose of thiopental sodium and hearts were removed immediately and washed with the ice cold saline. Heart blood was removed from the heart by tapping the heart on the kim wipe (absorbent

pad) or surgical compress. This process was repeated until the heart totally dried. The weight of dry heart was recorded (Firoz et al. 2011). Heart weight: body weight ratio = Heart wt (gm)/Body weight (gm) × 103

Gross examination of rat heart

The dissected hearts were washed with ice-cold saline. Then hearts were visually examined for inflammation, redness, capillary dilatation, scar formation and colour in all part of the heart and grading was performed (Rona et al. 1959). The grading of heart was done by the following Table 2.

Table 2. Grading of gross examination of rat heart

Grade no.	Characteristices
Grade-0	No lesion.
Grade-1	Inflammation and redness, capillary dilatation.
Grade-2	Edema, capillary dilatation, ventricle portion yellowish.
Grade-3	Scar formation, yellowish colour of atrium and ventricle part of heart.
Grade-4	Diffuse hear, absolute scar formation, increased necrosis portion

Biochemical parameters

In blood serum

Aspartate aminotransferase/glutamate oxaloacetate transaminase (AST/SGOT) AST reagent kit was used for the quantitative determination of SGOT of serum (SGOT reagent kit, M.L. No. 799, Pharmapore, India). A standard curve was done and the experiment was carried out following the kit instruction according to the method of Reitman and Frankel (1957).

Alanine aminotransferase/glutamate pyruvate transaminase (ALT/SGPT)

Alanine aminotransferase/glutamate pyruvate transaminase (ALT/SGPT) For the quantitative determination of GPT of serum, Alanine Aminotransferase (ALT) reagent kit was used. The experiment was carried out following the kit instruction (ALT test kit, Ref no. 3977, Span Diagnostics, India) according to the method of Reitman and Frankel (1957).

Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) was evaluated according to the technique of (James1965). Take 1.0 ml of the buffered substrate, 0.1 ml of enzyme preparation was added and the tubes were incubated at 37 °C for 15 min. After adding 0.2 ml of NAD+ solution, the incubation was continued for another 15 min. The reaction was stopped by addition of 1.0 ml of DNPH reagent and then the tubes were incubated for a additional period of 15 min at 37 °C. After the incubation period, 6.0 ml of 0.3 N sodium hydroxide

solution was added and the colour developed was measured at 420 nm in a Shimadzu spectrophotometer. Suitable aliquots of the standards were also analyzed by the same procedure.

Creatinine

The serum creatinine concentration was estimated by alkaline picrate method(Bonsnes and Taussky 1945) using the commercially available kit (Cresent biosystems, Goa, India).

Triglyceride

Triacylglycerol was estimated by the method of (Rice et al. 1970).

Cholesterol

For Total Cholesterol content was estimated by the method of (Parekh and Jung 1970). About 0.1 ml of test sample was made up to 10 ml with ferric acetate uranyl acetate reagent. 0.1 ml of the aliquot of the complete lipid extract was taken and it was evaporated to dryness. The dried extract and standards were made up to 30.0 ml with ferric chloride uranyl acetate reagent. Then 20.0 ml of sulphuric acid ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 min, the colour developed and read at 540 nm using a Shimadzu UV spectrophotometer. Total Cholesterol level was expressed as mg/dl for plasma and tissue cholesterol as mg/g of fresh tissue.

For HDL cholesterol HDL-Cholesterol fraction was separated by the precipitation techniques of (Burstein et al. 1970). and the cholesterol content was determined by method of Parekh and Jung (1970). To 1.0 ml of serum, 0.18 ml of heparin-manganese chloride reagent was added and mixed. The solution was allowed to stand at 4 °C for 30 min and then centrifuged in a refrigerated centrifuge at 1800×*g* for 30 min. The supernatant represented the HDL-C fraction. An aliquot of supernatant was used for cholesterol estimation.

The values were expressed as mg/dl. For LDL cholesterol LDL-C was calculated using the following equation:

LDL-C = Total cholesterol − (HDL-C + VLDL-C). The values were expressed as mg/dl. For VLDL cholesterol VLDL—cholesterol was calculated using the following equation(Friedewald et al. 1972).VLDL-C = Triglycerides/5. The values were expressed as mg/dl.

In heart tissue

Tissue glutathione (GSH)

Total reduced glutathione was determined by the method of (Sedlak and Lindsay 1968). modified according to the method of (Moron et al. 1979). 0.1 ml of the test sample was precipitated with 5% TCA. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant, added 2.1 ml of 0.8

mg of DTNB in 0.2 M phosphate buffer (pH 8.9). The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar manner were also run to determine the glutathione content. The amount of glutathione was expressed as mg/dl for plasma and mg/100 g of tissues.

Catalase

Catalase was analyzed according to the method of (Takahara et al. 1960). To 1.2 ml of 0.01 mM phosphate buffer (pH 7.0), 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of 0.2 mM hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm for every 30 s up to 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as µ moles of hydrogen peroxide decomposed/ minute/mg of protein.

Superoxide dismutase (SOD)

Superoxide dismutase was examined according the method of (Misra and Fridovich (1972). Reaction mixtures contained sodium carbonate (.1 ml, 50 mM), nitroblue tetrazolium (0.49 ml, 28 µm) and freshly prepared hydroxylamine hydrochloride (0.3 ml, 0.2 mM). The reaction mixtures were mixed by inversion followed by the addition of a clear supernatant of tissue homogenates (0.2 ml, 1:10 w/v). The change in absorbance of samples was recorded at 560 nm.

Estimation of total protein

The protein content was estimated according to the method of (Lowry et al. 1951). To 0.2 ml of homogenate/serum, 10.0 ml of water and 4.9 ml of alkaline copper reagent were added and kept at room temperature for 10 min. To this, 0.59 ml of Folin's reagent (1:3) was added and the blue colour developed was read after 22 min at 640 nm. Protein content was expressed as g/dl of plasma.

Histopathological Studies

The rats were be sacrified and heart were dissected out, wash with normal saline, weigh and kept in 10% of formaline solution for histopathological examination (myocardial fibrosis, vascular lesion, myocardial cell size,etc.). The heart sample were sending to the R.S. diagnostic Pvt. Ltd. For histopathological studies.

Statistical Analysis

The analysis was expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by using one way ANOVA followed by Dunnett: compare all Vs control (Graph Pad Instat, USA).

RESULTS

Stability study of FDC

Physical stability

The physical stability of prepared FDC at room temperature was continuously monitored for 3 months and was and was found that no creaming, flocculation, cracking and phase separation was observed in the FDC.

Thermodynamic stability

Freeze thaw cycle

No creaming, flocculation, cracking and phase separation was observed in the FDC during freeze thaw cycle for 3 months.

Centrifugation studies

No creaming, flocculation, cracking and phase separation was observed in the FDC during centrifugation studies for 3 months.

Heating cooling cycle

No creaming, flocculation, cracking and phase separation was observed in the FDC during heating cooling cycle for 3 months.

Parameters assessed

Physical Parameters

Effect of FDC on gross examination of heart. The visually examination of all part of heart gives the information regarding the degree of necrosis in heart tissues.

Group I (NC i.e. vehicle treated) and VIII (Perse i.e. FDC treated) showed no sign of lesions or necrosis (Grade-0). Group II (TC i.e. Doxorubicin treated) showed diffuse heart, absolute scare formation and increased necrosis portion showing grade-4 cardiac damage. Group III (STD i.e. K.A.H.A.W. + Doxorubicin treated) and VII (FDC + Doxorubicin treated) showed marked reduction in edema, capillary dilation, and scar formation showing grade-1 cardiac damage. Group IV (Extract + Doxorubicin treated), V (olive oil + Doxorubicin treated) and VI (CoQ10 + Doxorubicin treated) showed slight decrease in inflammation, redness, capillary dilatation and scar formation showing grade-2 type of cardiac damage(Table 2. Fig.1)

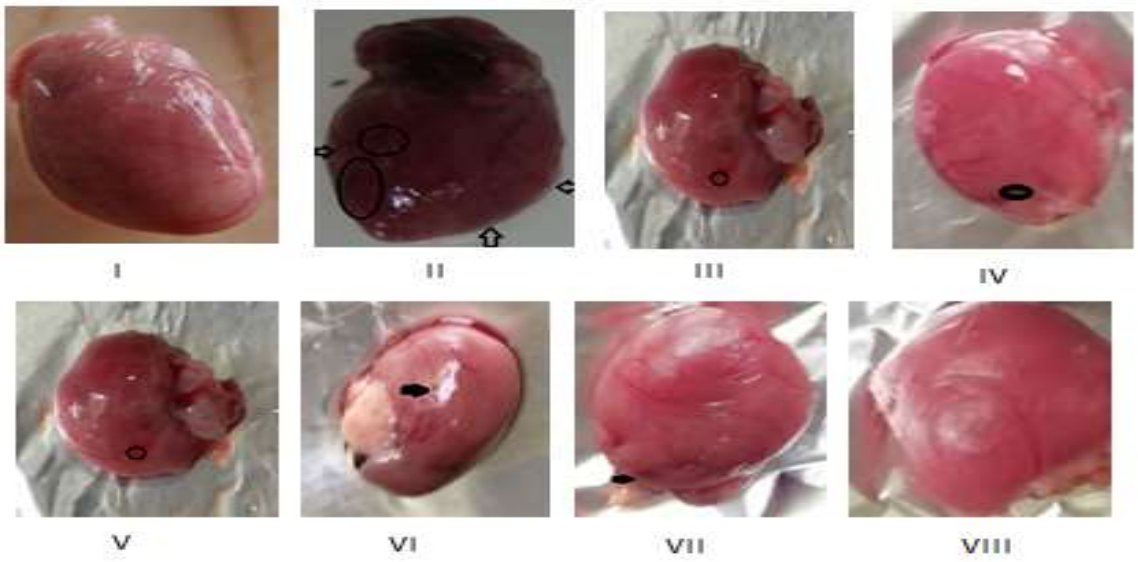

Figure 1. Photographs of hearts of different experimental group (I) Vehicle treated heart (II) Doxorubicin treated heart (III) K.A.H.A.W.+ Doxorubicin treated heart (IV) saffron extract+ Doxorubicin treated heart (V) olive oil+ Doxorubicin treated heart (VI) CoQ10+ Doxorubicin treated heart (VII) FDC+ Doxorubicin treated heart (VIII) FDC treated heart.

Table 3. Effect of FDC on grading of heart

Groups	Treatment	Grading Result	Parameters
I	Vehicle	Grade 0	No lesion
II	Vehicle+Doxorubicin	Grade 4	Diffuse heart, absolute scare formation, increased necrosis portion
III	K.A.H.A.W.+Doxorubicin	Grade 1	Inflammation and redness, capillary dilatation.
IV	Saffron +Doxorubicin	Grade 2	Edema, capillary dilatation, ventricle portion yellowish.
V	Olive oil +Doxorubicin	Grade 2	Edema, capillary dilatation, ventricle portion yellowish.
VI	CoQ_{10} +Doxorubicin	Grade 2	Edema, capillary dilatation, ventricle portion yellowish.

VII	FDC +Doxorubicin	Grade 1	Inflammation and redness, capillary dilatation.
VIII	FDC	Grade 0	No lesion

Effect of FDC on heart weight/body weight ratio

The heart weight/body weight ratio was analysed in various group (Table 4/Fig.2). Group II (TC) showed significant ($p<0.01$) increase in heart weight/body weight ratio due to hypertrophy, whereas no change was observed in Group VIII (Perse), i.e. ($p>0.05$) when compared with group I (NC). Group III (STD) and VII (FDC) showed significant ($p<0.01$) reduction in heart/body weight ratio when compared with group II (TC). Group IV (Extract), V (olive oil) and VI (CoQ_{10}) showed less significant ($p<0.05$) reduction in heart/body weight ratio when compared with group II (TC).

Table 4. Effect of FDC on heart /body weight ratio

Groups	**Treatment**	**Heart weight : Body weight ratio**
I	Vehicle	3.13 ± 0.14
II	Vehicle +Doxorubicin	$3.63 \pm 0.28^{**}$
III	K.A.H.A.W. +Doxorubicin	$3.24 \pm 0.08^{\#\#}$
IV	Saffron +Doxorubicin	$3.49 \pm 0.24^{\#}$
V	Olive oil +Doxorubicin	$3.98 \pm 0.24^{\#}$
VI	CoQ_{10} +Doxorubicin	$2.92 \pm 0.2^{\#}$
VII	FDC +Doxorubicin	$3.32 \pm 0.13^{\#\#}$
VIII	FDC	3.27 ± 0.18^{ns}

All values are expressed as Mean±SEM for five animals. $^{**}P<0.01$= significant & ns $P>0.05$ = non significant when compared with normal control (Gr-I), # $P<0.05$ and ## $P<0.01$= significant when compared with Toxic group (Gr-II).

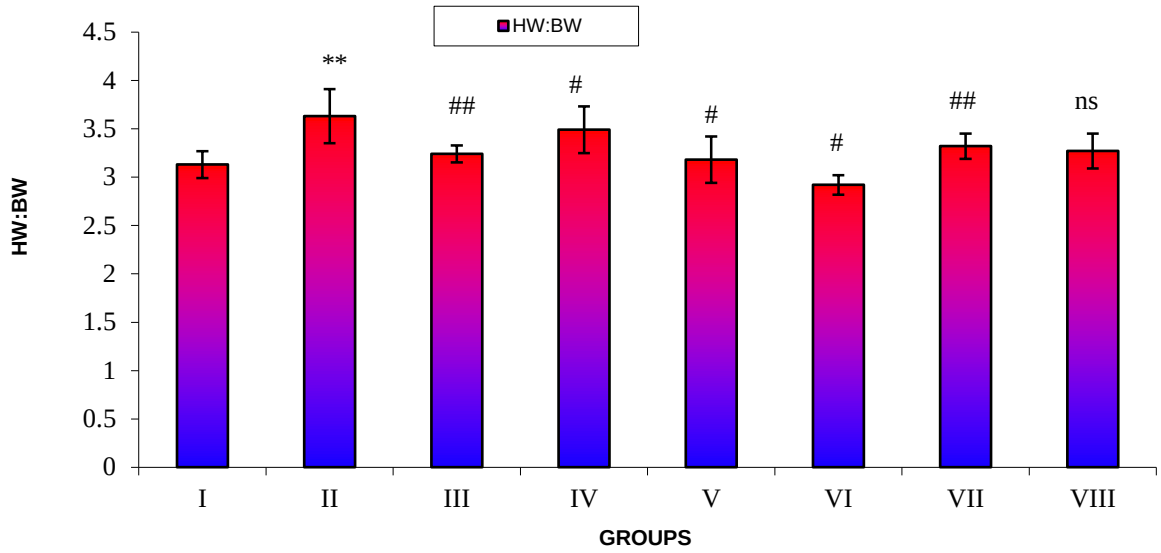

Figure.2 Effect of FDC on heart weight /body weight ratio

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

Biochemical parameters

In blood serum

Effect of FDC on cardiac enzyme concentration

The effect of FDC on serum marker enzymes AST, ALT and LDH are outlined in (Table 5/ fig 3,10) Group II (TC) showed significant (p<0.01) increase in concentration of serum marker enzymes, whereas no change was observed in Group VIII (Perse), i.e. (p>0.05) when compared with group I (NC). Group III (STD) and VII (FDC) showed significant (p<0.01) reduction in cardiac marker enzymes when compared with group II (TC). Group IV (Extract), V (olive oil) and VI (CoQ10) showed less significant (p<0.05) reduction in concentration of AST, ALT and LDH when compared with group II (TC). But FDC shows the similar effect as K.A.H.A.W.

Table 5. Effect of FDC on serum AST, ALT and LDH levels.

Groups	AST (U/L)	ALT (U/L)	LDH (U/L)
I	83.28 ± 6.43	74.31 ±6.79	128.36 ±6.44
II	124.25 ± 10.4**	92.45 ± 10.87**	179.54 ± 10.49**
III	67.45 ± 5.48##	63.32 ± 5.65##	136.56 ± 5.32##
IV	76.24 ± 9.68#	75.54 ± 16.94#	144.35 ± 9.45#
V	83.55 ± 7.76#	77.32 ± 8.13#	148.66 ± 7.53#
VI	80.81 ± 6.04#	70.89 ± 7.84#	145.92 ± 6.61#
VII	61.16 ± 6.56##	58.23 ± 8.19##	139.26 ± 6.14##
VIII	74.45 ± 5.64 ns	69.31 ± 4.71 ns	130.34 ± 4.84 ns

AST- Aspartate Transaminase; ALT- Alanine Transaminase; LDH – Lactate Dehydrogenase

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

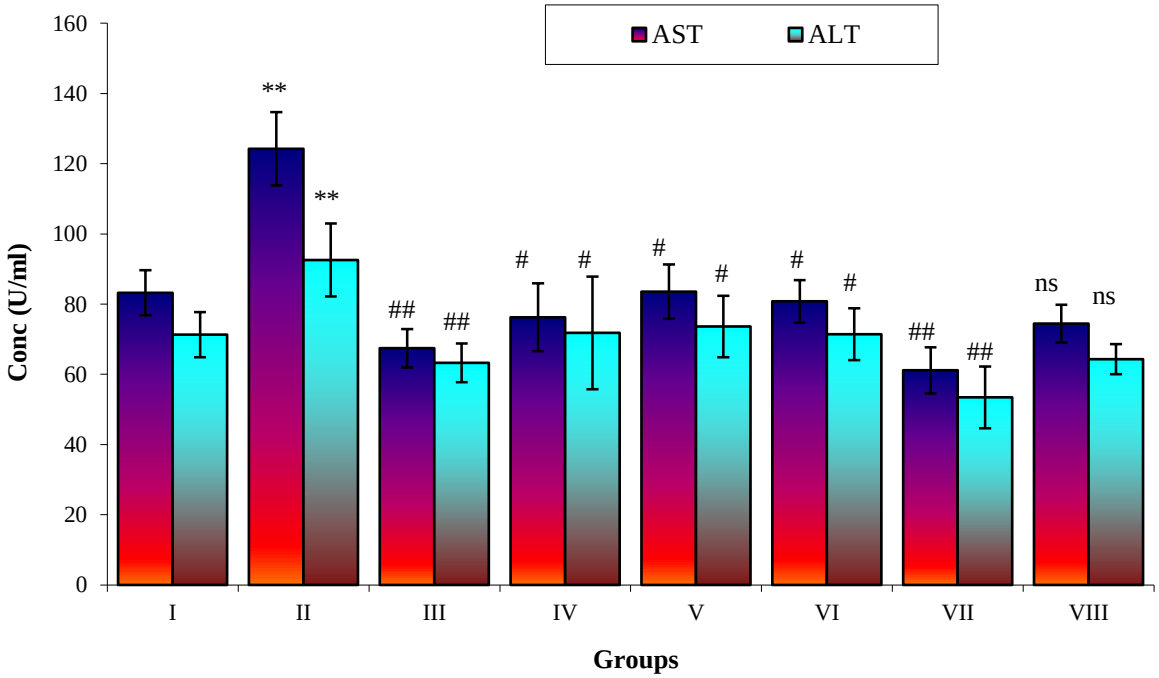

Figure. 3 Effect of FDC on serum AST, ALT. AST- Aspartate Transaminase; ALT- Alanine Transaminase;

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

Effect of FDC on serum Creatinine level

The effect of FDC on serum creatinine is shown in table 6/fig.4. Level of creatinine was significantly (p<0.01) increased in group II (TC), whereas no change was observed in Group VIII (Perse), i.e. (p>0.05), when compared to the group I (NC). The concentration of creatinine was significantly (p<0.01) decreased in group III (STD) and VII (FDC) as compared with group II (TC). The concentration of creatinine was less significantly (p<0.05) decreased in Group IV (Extract), V (Almond oil) and VI (CoQ10) IV when compared with group II (TC).

Table 6. Effect of FDC on serum Creatinine level

Groups	Creatinine (mg/dl)
I	0.69 ±0.12
II	$2.44 \pm 0.22^{**}$
III	$0.69 \pm 0.08^{\#\#}$
IV	$0.78 \pm 0.07^{\#}$
V	$0.83 \pm 0.37^{\#}$
VI	$0.88 \pm 0.02^{\#}$
VII	$0.66 \pm 0.04^{\#\#}$
VIII	0.72 ± 0.28^{ns}

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

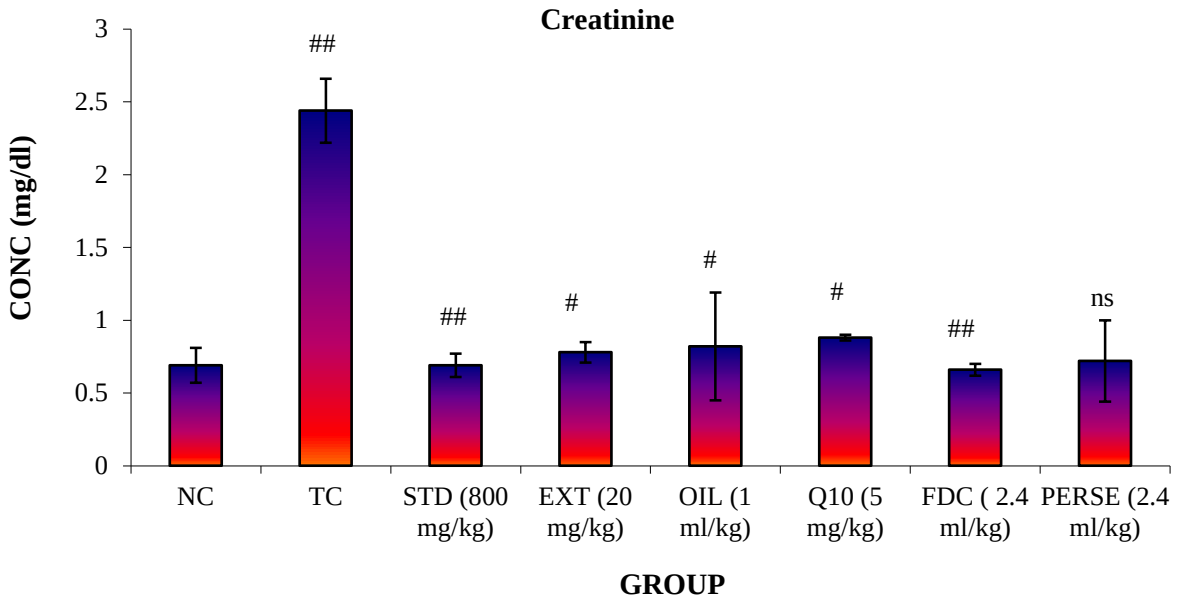

Figure.4 Effect of FDC on serum Creatinie level.

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

Effect of FDC on serum Triglycerides (TG) level

The effect of FDC on serum TG is shown in table 7/fig.5. Circulating level of triglyceride was significantly (p<0.01) increased in group II (TC), whereas no change was observed in Group VIII (Perse), i.e. (p>0.05), when compared to the group I (NC). The concentration of triglyceride was significantly (p<0.01) decreased in group III (STD) and VII (FDC) when compared with group II (TC). The concentration of triglyceride was less significantly (p<0.05) decreased in Group IV (Extract), V (olive oil) and VI (CoQ10) IV when compared with group II (TC).

Table 7. Effect of FDC on serum Triglyceride (TG) level.

Groups	TG (mg/dl)
I	33.08 ±4.12
II	72.57 ± 8.09**
III	43.31 ± 3.91##

IV	$48.02 \pm 7.47^{\#}$
V	$39.56 \pm 4.47^{\#}$
VI	$49.56 \pm 5.94^{\#}$
VII	$35.14 \pm 5.37^{\#\#}$
VIII	34.51 ± 5.49^{ns}

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

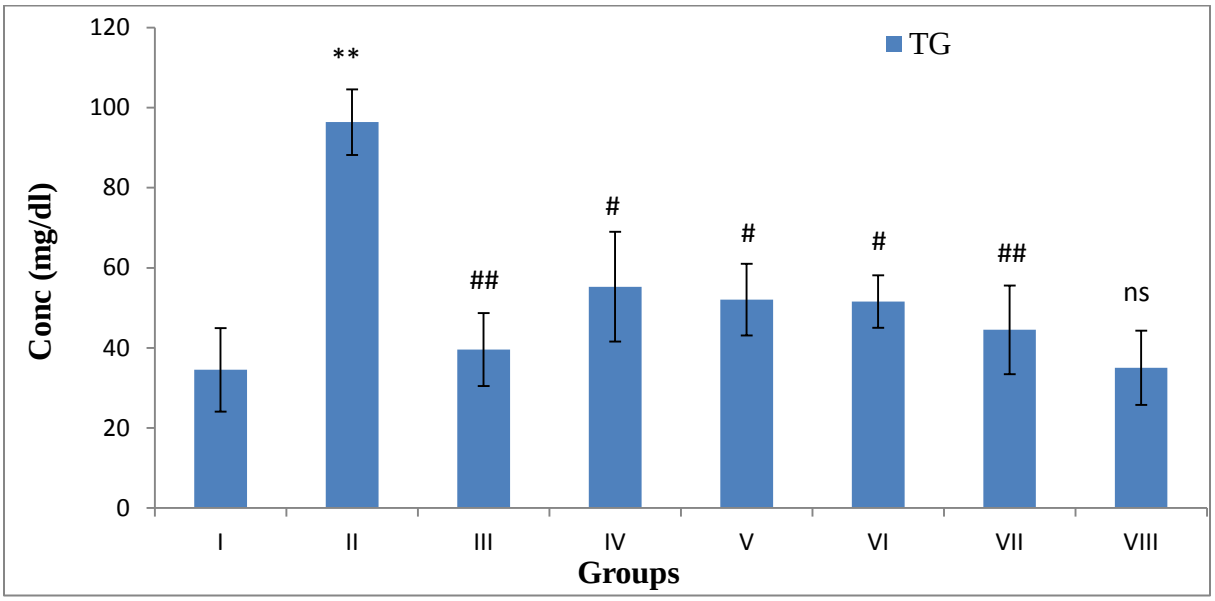

Figure 5. Effect of FDC on serum Triglyceride (TG) level.

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

Effect of FDC on serum TC, HDL, LDL and VLDL levels

The effect of FDC on serum levels of TC, HDL, LDL and VLDL are outlined in table No. 8/ fig. 6. Group II (TC) showed significant ($p<0.01$) increased in concentration of serum TC, LDL and VLDL, whereas no change was observed in Group VIII (Perse), i.e. ($p>0.05$) when compared with group I (NC). Group III (STD) and VII (FDC) showed significant ($p<0.01$) reduction in serum TC, LDL and VLDL when compared with group II (TC). Group IV (Extract), V (olive oil) and VI (CoQ10) showed less significant ($p<0.05$) reduction in concentration of TC, LDL and VLDL when compared with group II (TC).

In case of HDL, its level significantly ($p<0.01$) decreased in Group II (TC), whereas no change was observed in Group VIII (Perse), i.e. ($p>0.05$) when compared with group I (NC). Group III (STD) and VII (FDC) showed significant ($p<0.01$) increase in serum HDL level when compared with group II (TC). Group IV (Extract), V (olive oil) and VI (CoQ10) showed less significant ($p<0.05$) increase in concentration of HDL when compared with group II (TC).

Table 8. Effect of FDC on serum TC, HDL, LDL and VLDL levels

Groups	TC(mg/dl)	HDL(mg/dl)	LDL(mg/dl)	VLDL(mg/dl)
I	118.31 ± 5.29	50.39 ± 4.35	60.39±2.12	7.5 ± 0.67
II	$162.3 \pm 10.77^{**}$	$26.02 \pm 3.07^{**}$	$91.01 \pm 8.3^{**}$	$13.27 \pm 1.48^{**}$
III	$124.9 \pm 10.32^{\#\#}$	$59.94 \pm 4.85^{\#\#}$	$58.39 \pm 6.35^{\#\#}$	$8.31 \pm 0.77^{\#\#}$
IV	$128.35 \pm 5.22^{\#}$	$46.3 \pm 6.74^{\#}$	$62.25 \pm 6.8^{\#}$	$9.15 \pm 1.74^{\#}$
V	$129.58 \pm 8.59^{\#}$	$44.7 \pm 3.79^{\#}$	$72.02 \pm 6.34^{\#}$	$8.61 \pm 1.19^{\#}$
VI	$129.98 \pm 6.59^{\#}$	$47.07 \pm 1.41^{\#}$	$69.5 \pm 10.19^{\#}$	$9.93 \pm 1.1^{\#}$
VII	$120.17 \pm 7.13^{\#\#}$	$59.51 \pm 2.95^{\#\#}$	$53.94 \pm 10.05^{\#\#}$	$7.28 \pm 1.12^{\#\#}$
VIII	115.6 ± 5.23^{ns}	57.57 ± 6.69^{ns}	63.75 ± 5.6^{ns}	6.95 ± 0.97^{ns}

TC- Total Cholesterol; HDL- High Density Lipoprotein; LDL- Low Density Lipoprotein; VLDL- Very Low Density Lipoprotein.

All values are expressed as Mean±SEM for five animals. $^{**}P<0.01$= significant & ns $P>0.05$ = non significant when compared with normal control (Gr-I), # $P<0.05$ and ## $P<0.01$= significant when compared with Toxic group (Gr-II).

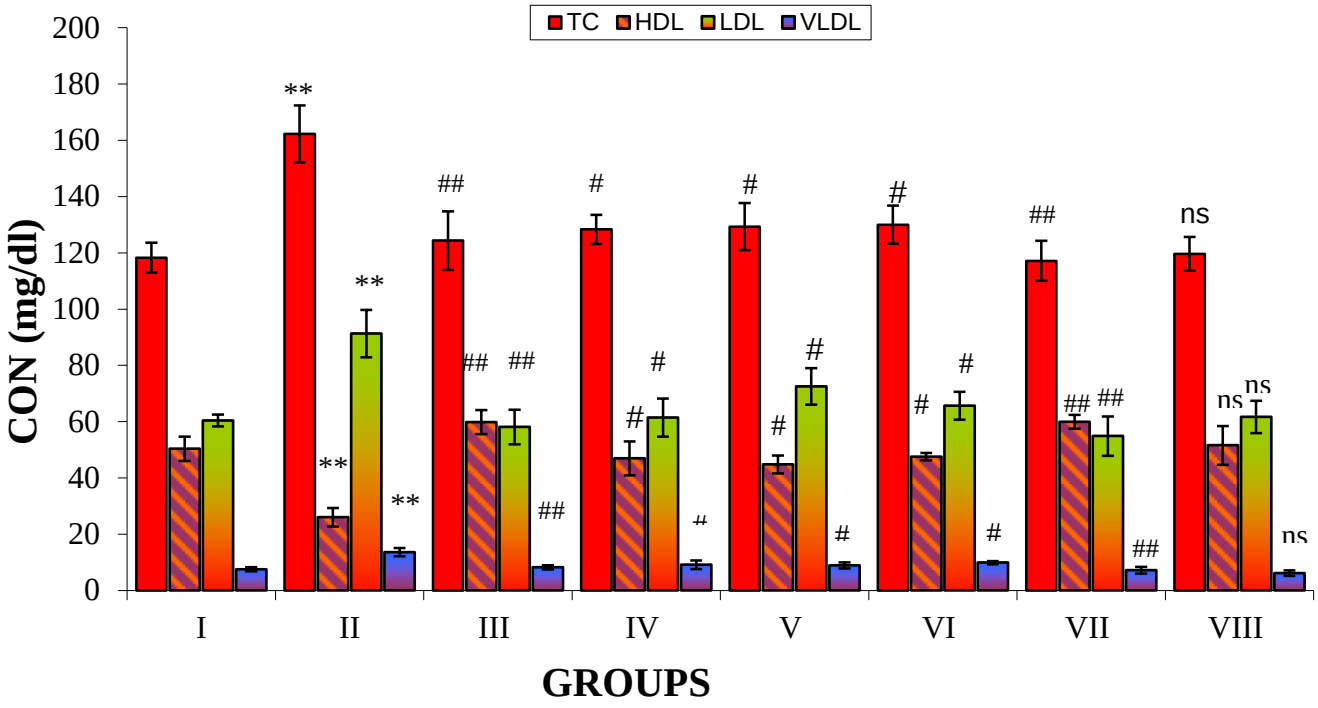

Figure 6 Effect of FDC on serum TC, HDL, LDL and VLDL levels.

TC- Total Cholesterol; HDL- High Density Lipoprotein; LDL- Low Density Lipoprotein; VLDL- Very Low Density Lipoprotein.

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

Effect of FDC on SOD, GSH and CAT levels in heart tissues

The effect of FDC on SOD, GSH and CAT are outlined in table 9/ fig.7,8,9). The level of SOD, GSH and CAT significantly (p<0.01) decreased in Group II (TC), whereas no change was observed in Group VIII (Perse), i.e. (p>0.05) when compared with group I (NC). Group III (STD) and VII (FDC) showed significant (p<0.01) increased the level of SOD, GSH and CAT when compared with group II (TC). Group IV (Extract), V (olive oil) and VI (CoQ10) showed less significant (p<0.05) increase in concentration of SOD, GSH and CAT when compared with group II (TC).

Table 9. Effect of FDC on SOD, GSH and CAT levels in heart tissue.

Groups	SOD (Units/mg protein)	GSH (µg/mg of protein)	CAT (ηmoles of H_2O_2 consumed/minute/mg protein)
I	2.14 ± 0.11	19.23 ± 0.46	0.2473 ± 0.049
II	2.53 ± 0.12**	4.60 ± 0.31**	0.121 ± 0.0083**
III	2.00 ± 0.19$^{\#\#}$	15.59 ± 0.25$^{\#\#}$	1.111 ± 0.0285$^{\#\#}$
IV	1.60 ± 80.22$^{\#}$	10.82 ± 0.39$^{\#}$	0.558 ± 0.018$^{\#}$
V	1.55 ± 0.19$^{\#}$	9.94 ± 0.57$^{\#}$	0.396 ± 0.031$^{\#}$
VI	1.60 ± 0.23$^{\#}$	11.39 ± 1.04$^{\#}$	0.513 ± 0.054$^{\#}$
VII	1.73 ± 0.28$^{\#\#}$	14.73 ± 0.44$^{\#\#}$	1.141 ± 0.033$^{\#\#}$
VIII	1.82 ± 0.38^{ns}	18.68 ± 0.75^{ns}	0.241 ± 0.049^{ns}

SOD- Superoxide Dismutase; GSH- Glutathione; CAT- Catalase

All values are expressed as Mean ± SEM for five animals. $^{**}P<0.01$= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01= significant when compared with Toxic group (Gr-II).

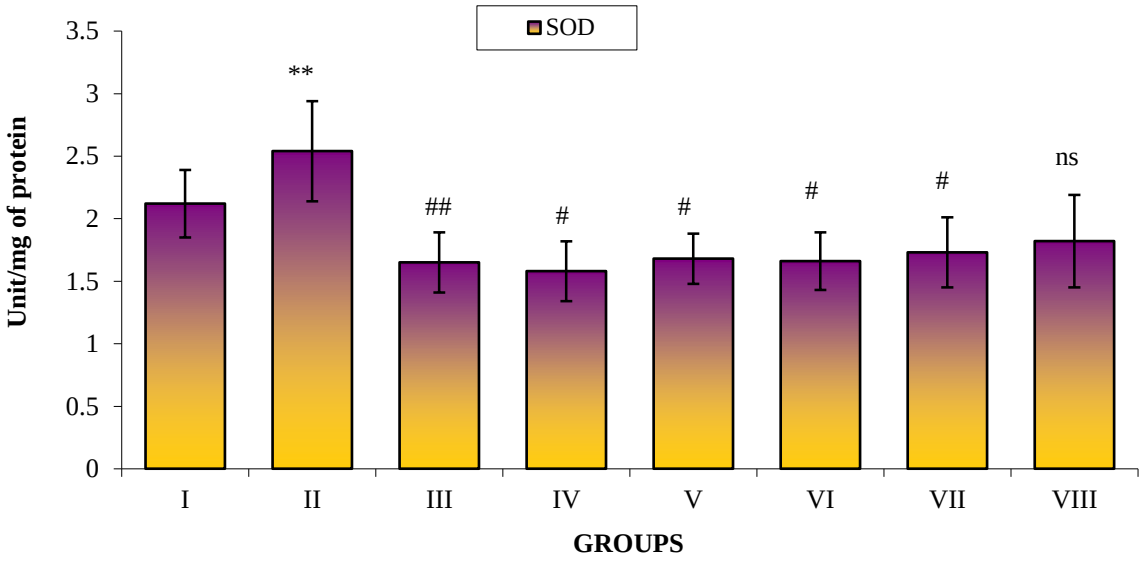

Figure.7: Effect of FDC on SOD levels in heart tissue.

SOD- Superoxide Dismutase

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

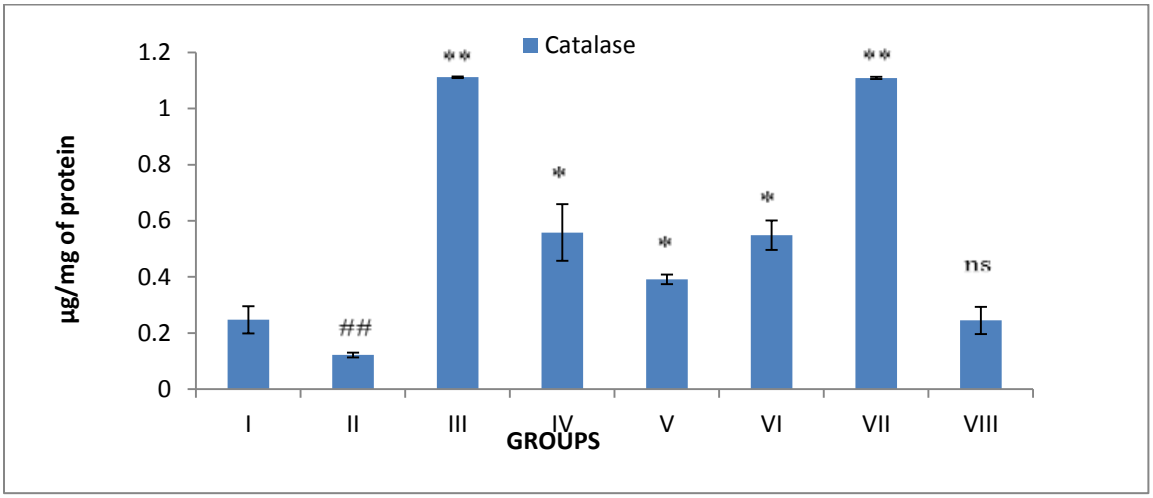

Figure .8 Effect of FDC on CAT levels in heart tissue.

CAT- Catalase

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

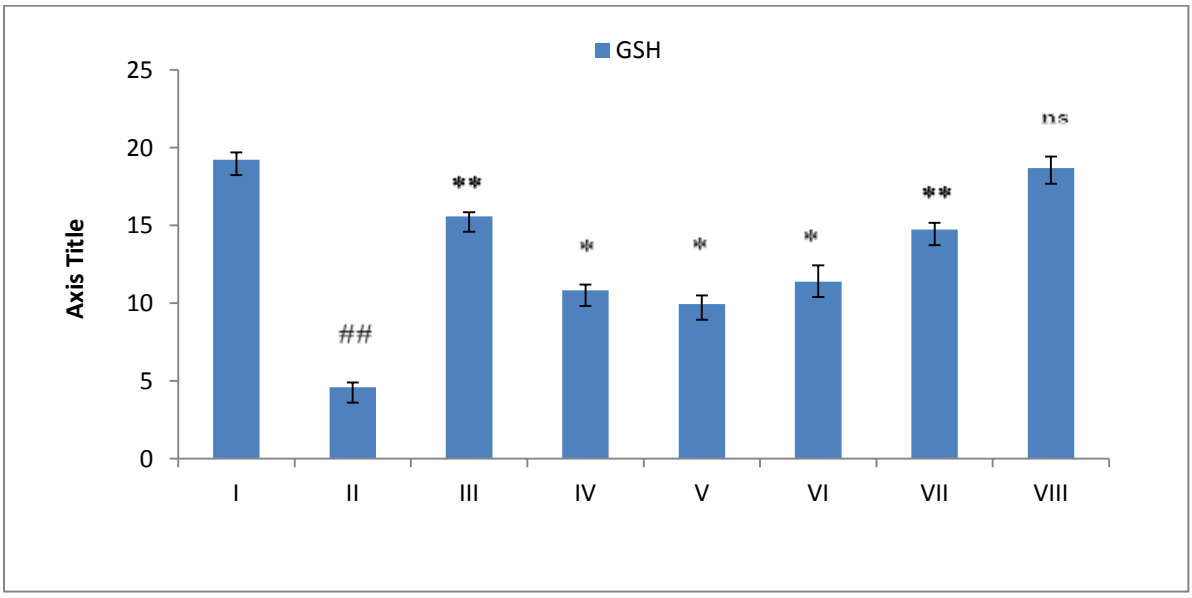

Figure .9 Effect of FDC on GSH levels in heart tissue.

GSH- Glutathione

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05 and ## P<0.01= significant when compared with Toxic group (Gr-II).

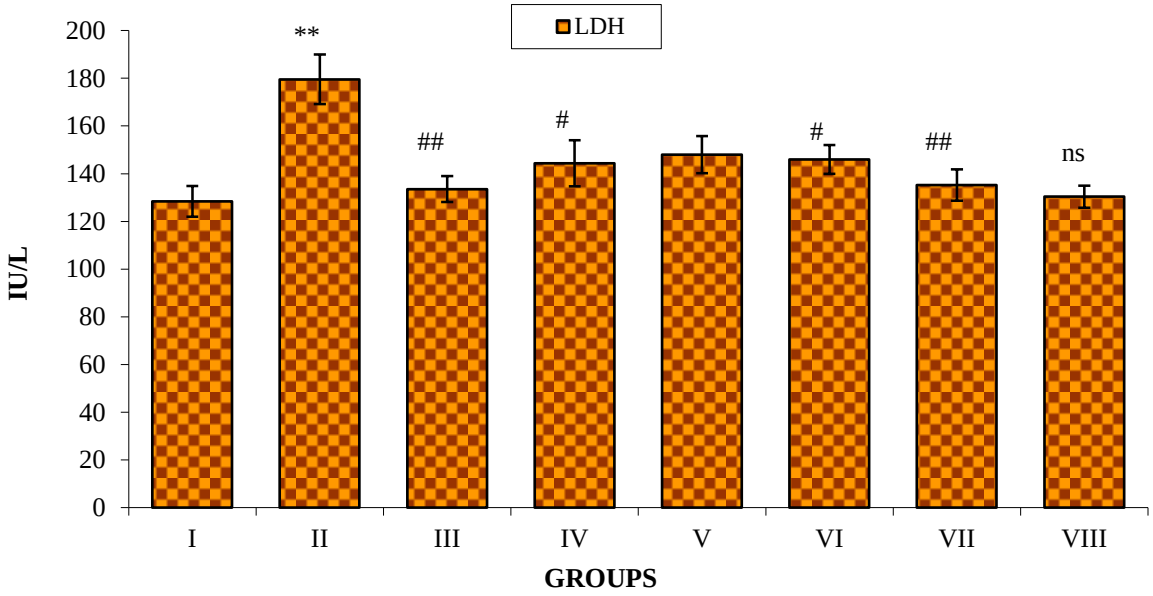

Figure .10 Effect of FDC on LDH levels in heart tissue.

All values are expressed as Mean±SEM for five animals. ***P*<0.01= significant & ns P>0.05 = non significant when compared with normal control (Gr-I), # P<0.05, ## P<0.01= significant when compared with toxic control (Gr-II).

Histopathology

Photomicrograph of rat heart of normal control group (Gr-I) and Perse group (Gr-VIII) shows, the endocardium, myocardium, and epicardium as well as papillary muscles and vasculature were all normal and healthy in both the groups. There was no muscular hypertrophy or evidences of myositis (necrosis and/or round cell infiltrates). Photomicrograph of rat heart of Doxorubicin treated group (Gr-II) shows focal myonecrosis with myophagocytosis and lymphocytic infiltration in sub-endocardium vacuolar changes and prominent oedema along with chronic inflammatory cells. Photomicrograph of rat heart of the K.A.H.A.W. treated group (Gr-III) and FDC treated group (Gr-VII) shows very lesser degree of myonecrosis, myophagocytosis and lymphocytic infiltration, oedema and very little infiltration of inflammatory cells are clearly visible. Photomicrograph of rat heart of the extract treated group (Gr-IV), almond oil treated group (Gr-V) and CoQ_{10} treated group (Gr-VI), compactly arranged

muscle fibres with minimum interstitial tissue, long spindle shaped vesicular nuclei, muscle striation well marked, few blood vessels with fibrous tissue element was seen. Group VII (FDC) showed loosely arranged muscle fibres with fragmentation and increased interstitial tissue, long spindle shaped vesicular nuclei, muscle striation less marked, granular cytoplasm, blood vessels small with fibrous tissue element was seen

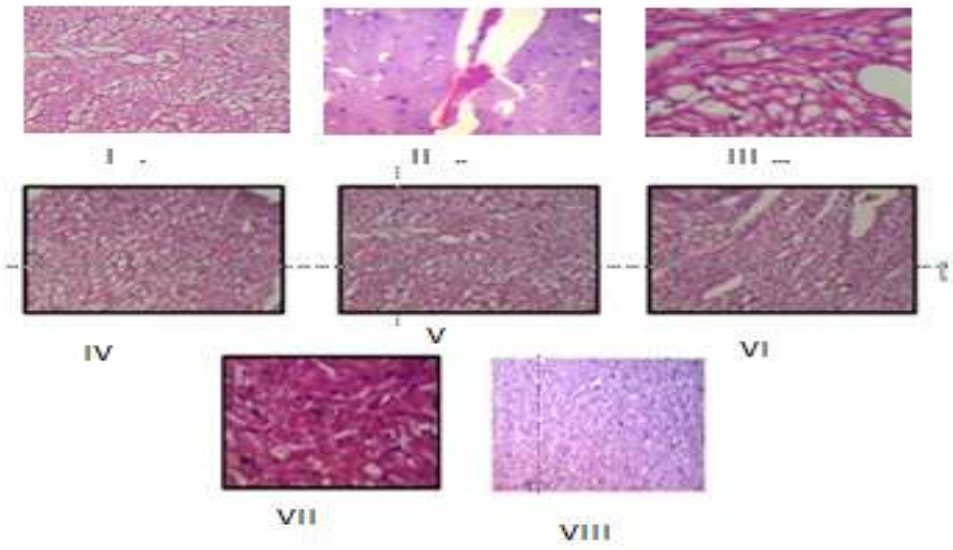

Figure. 11 Effect of FDC on histopathology of rat heart

(I)Vehicle treated heart (II) Doxorubicin treated heart (III) K.A.H.A.W.+ Doxorubicin treated heart (IV) saffron extract+ Doxorubicin treated heart (V) olive oil+ Doxorubicin treated heart (VI) CoQ10+ Doxorubicin treated heart (VII) FDC+ Doxorubicin treated heart (VIII) FDC treated heart.

Discussion

The biochemical markers that are used widely in detection of myocardial necrosis are AST, ALT and LDH (Vennila and Pugalendi 2010). Estimation of high serum enzymes is useful guide for necrosis of myocardium. The present study shows that the FDC of saffron (*Crocus sativus*) olive oil and CoQ_{10} has efficiently protected the myocardium against Doxorubicin induced myocardial toxicity.

The biochemical markers that are used widely in detection of myocardial necrosis are AST, ALT and LDH (Shubhada N, 2001). Estimation of elevated serum enzymes is useful guide for necrosis of myocardium. This might be due to the damage in the heart muscle, rendering the leakage of enzymes in

to the serum. Apart from that, reduction in the levels of antioxidant parameters also indicates the degree of myocardial necrosis (Mohan, 2001).

Doxorubicin causes significant damage of myocardium, hypertrophy and a significant increase in the levels of serum marker enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) (Ayaz et al., 2005). Several mechanisms were suggested for Doxorubicin induced cardio toxicity, such as oxidative stress, produced by increased levels of free radicals (e.g. reactive oxygen species (ROS), intracellular iron and decreased levels of antioxidants e.g. glutathione (GSH). This oxidative stress causes increased intracellular calcium and acceleration of lipid peroxidation that damages the cell membrane and other cellular components (Abhushouk et al., 2017)
In the present study Doxorubicin treated group showed grade-4 cardiac damage (i.e. diffuse heart, absolute scare formation and increased necrosis portion), increased heart/body weight ratio (myocardial hypertrophy), increased the levels of marker enzymes (AST, ALT & LDH), creatinine, TC, LDL, VLDL & TG and decreased the levels of HDL & tissue antioxidants (SOD, GSH and CAT), whereas FDC pre-treated showed grade-1 cardiac damage (i.e. marked reduction in edema, capillary dilation, and scar formation), reduction in heart/body weight ratio, decreased the levels of marker enzymes (AST, ALT & LDH), creatinine, TC, LDL, VLDL & TG and increased the levels of HDL & tissue antioxidants (SOD, GSH and CAT), which observed similar protection as K.A.H.A.W against Doxorubicin induced cardiac damage, while individual ingredients (i.e. saffron extract, olive oil and CoQ10) showed grade-2 cardiac damage (slight decrease in inflammation, redness, capillary dilatation and scar formation), reduction in heart/body weight ratio, decreased the levels of marker enzymes (AST, ALT & LDH), creatinine, TC, LDL, VLDL & TG and increased the levels of HDL & tissue antioxidants (SOD, GSH and CAT) which was less significant as compared to FDC.

Compliance with ethical standards

Ethical statement The study protocols was approved by Institutional Animal Ethical Committee (IAEC), Faculty of Pharmacy, Integral University, Lucknow (Reg No. 1213/PO/Re/S/08/CPCSEA, 5th June 2008) having Approval No. IU/IAEC/18/17.

Conflict of interest This manuscript described has not been published before; not under consideration for publication anywhere else; and has been approved by all co-authors.

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