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Ursolic acid prevents cyclophosphamide induced myocardial oxidative tissue damage in wistar rats

Laxit Bhatt*[,1,2,+], Priya Singh[1], Binu Sebastian[2] & Viraj Joshi[3]

[1]Biologic Research Toxicology, Sun Pharma Advanced Research Company, Vadodara, India
[2]Department of Pharmacology, Shree Devi College of Pharmacy, Mangalore, India
[3]Department of Chemical Analysis, Zydus Research Centre, Ahmedabad, India
E-mail: [+]bkltox@gmail.com

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Herbs and spices of natural origin have been used traditionally for culinary and therapeutic purposes. Ursolic acid, a pentacyclic triterpenoid, is a major component of various foods, herbs and spices utilized in daily life. The purpose of this study was to investigate the cardio-protective action of ursolic acid against cardio-toxicity induced by cyclophosphamide. Animals were randomly divided into control group (1% dimethyl sulfoxide, s.c.), toxic control (Cyclophosphamide 200 mg/kg, i.p.), ursolic acid treated (40 mg/kg, s.c.) and ursolic acid-cyclophosphamide combination treatment. Animals were sacrificed at the end of the treatment period and biochemical, electrocardiographic and histopathological changes were measured. Cyclophosphamide treatment showed marked increase in biomarker enzymes (CKNAC, CKMB, LDH, AST, and ALT), decreased tissue antioxidants (SOD, catalase & GSH), hyperlipidemia (TC, TG) and abnormal electrocardiographic (HR, RR, QRS, QT & PR intervals) and histopathological changes. Ursolic acid treatment restored tissue antioxidants, normalised biochemical and electrocardiographic parameters and prevented structural damage of the myocardium. Findings from this study indicate the protective role of ursolic acid against cardiotoxicity caused by cyclophosphamide.

Keywords: Cardiomyopathy, Cardioprotection, Chemotherapy adverse effects, Oxidative stress, Phytoconstituents

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Herbs and natural products have been used since ancient times in treatment of different ailments[1]. Natural products and products derived from them possess a wide array of biological activities. Flavonoids, triterpenoids and polyphenolic compounds are the primary anti-oxidants of a number of natural products[2]. Additionally, these herbs, spices and other naturally derived products find use in culinary, cosmetic and medicinal intentions[3]. They are used extensively around the world to enhance flavor, color and for conservation of food. Different phytoconsitutents of therapeutic interest can be found in herbs and spices[3,4]. It is believed that the most effective approach to discovery and development of newer drugs is to explore the biological sources for new active molecules that treat multitude of diseases[5].

The phytoconstituent, ursolic acid (URSO), is a pentacyclic triterpenoid carboxylic acid found abundantly in medicinal plants, herbs and foods[4–7]. Major sources of the phytoconstituent are apple peels, rosemary, thyme, ocimum species and various different herbs and spices[5,6,8]. It possesses multiple beneficiary effects including anti-diabetic[4], anti-hyperlipidaemic[9], hepatoprotective[10], neuroprotection[11] and neuro-regeneration[12] and antibacterial[13] among many others. It has a complex mechanism of action, which includes: attenuation of H_2O_2 and MPP (1-Methyl-4-phenylpyridinium) induced impairment in catalase and superoxide dismutase (SOD) activity[14]; prevention of lipid peroxidation[14]; protection of DNA against oxidative damage[15] and inhibition of nitric oxide (NO) production[16].

Occurrence of cardio-toxicity during chemotherapy with cytotoxic drugs is of high prevalence and is a dose limiting factor in cancer treatment[17,18]. Cyclophosphamide (CYP) is widely used for treatment of various leukemias, myelomas, and lymphomas. However, its use is limited due to its cardiotoxic potential[19]. CYP is an alkylating anticancer drug that acts by cross-linking DNA[20]. Cyclophosphamide causes acute cardio-toxicity with signs and symptoms of myopericarditis, which manifests into fatal complications, like cardiac

*Corresponding author

tamponade, congestive heart failure, arrhythmias, etc.[19,21]. In the body, CYP is broken down into Phosphoramide mustard and Acrolein[22]. These metabolites cause formation of free radicals that cause irreversible myocardial tissue injury by damaging mitochondria and its capacity to detoxify reactive oxygen species (ROS)[23], causing for the need of an anti-oxidant therapy.

Research suggests that URSO actively prevents ethanol-induced[24] and doxorubicin-induced[25] oxidative stress and isoproterenol-induced myocardial ischemia[26]. Studies have also found URSO to act through uncoupling of antioxidants and mitochondria in isolated hearts[27]. It is also proven to inhibit nuclear factor-kappaB activation induced by ROS produced from metabolism of carcinogenic agents and thus preventing tumorigenesis[28]. Ursolic acid prevents activation of cyclooxygenase-2 (COX-2), lipoxygenase, and inducible nitric oxide synthase (iNOS), major factors of inflammation and tissue injury by down-regulating of nuclear factor-kappaB[5]. URSO is also known to alter the expression of lipid metabolism genes and for enhancing the binding of PPAR-α receptors, leading to alterations in hepatic lipid metabolism[29].

The occurrence of fatal cardio-toxicity even on a single dose of CYP warrants the development of cardio-protective regimens that would not only prevent the generation of immediate toxic changes but also not produce adverse effects of their own. Products such as isolated phytoconstituents of natural origins play an important part in this setting. These phytoconstituents are more potent and induce less toxic effects than their synthetic chemically produced counterpart drugs[30–32]. In this study, we have used isolated pure ursolic acid to evaluate the cardio-protective effect of the pure compound against cardiomyopathy induced by alkylating anticarcinogenic agents like cyclophosphamide.

Materials and methods

Experimental Protocol

All experiment protocols involving animals were conducted according to the principles stated in the Guide for the Care and use of Laboratory Animals[33] and CPCSEA Guidelines[34]. The protocol was approved by the Institutional Animal Ethics Committee (certificate number: SDCP/IAEC-09/2012-13). Twenty-four male wistar rats weighing between 200-300g and approximately of 7-9 weeks of age were procured from and housed at Shree Devi College of Pharmacy, Mangalore, India. The cages had paddy husk bedding and were maintained at 25±5ºC under 12 h light-dark cycles. The animals had access to purified water and feed. Animals were acclimatized to laboratory conditions for at least one week before starting the experiment. They were then divided into four groups of six animals each:

- Group I *(Normal Control)*: 1% dimethyl sulfoxide (s.c.) for 10 days.
- Group II *(CYP)*: CYP (200 mg/kg, i.p.) on Day 1.
- Group III *(URSO)*: Ursolic acid dissolved in 1% DMSO (40 mg/kg body weight, s.c.) for 10 days.
- Group IV *(CYP+URSO)*: Cyclophosphamide (200 mg/kg, i.p.) on Day 1 and ursolic acid dissolved in 1% DMSO (40 mg/kg body weight, sc) for 10 days.

Chemical and reagents

Pure ursolic acid was purchased from Yucca Enterprises, Mumbai, India. Commercially available cyclophosphamide was purchased from Zydus Oncosciences, Ahmedabad, India. Commercial kits for estimation of biomarkers were procured from Crest Biosystems, Goa, India (CK-MB, CK-NAC, LDH, AST, and ALT) and from Robonik India Pvt. Ltd., Thane, India (TG and TC). Other chemicals and reagents were of highest analytical grade.

Dose selection

Several studies conducted on URSO were taken into consideration during dose selection for the present study. Two studies conducted by Saravanan R. and others[24,35] utilized three doses: 10, 20 and 40 mg/kg of URSO in wistar rats and evaluated the protective effect after sub-chronic administration of the phytoconstituent. Other study by Radhiga T.[36] proved protective effect of the phytoconstituent at 40 mg/kg after 7 days of administration. Acute toxicity studies were deemed not necessary due to availability of abundant data from previously conducted studies[37,38]. Considering the present experimental model of 10 days, 40 mg/kg by sc route was selected. The dose of 200 mg/kg ip for CYP was selected on the basis of similar experimental models in published literature[39,40]. Dose volume for subcutaneous administration was 5 ml/kg and for intraperitoneal administration was 10 mL/kg[41].

General observations and mortality

Animals were observed once daily for occurrence of any clinical signs and twice daily for morbidity or mortality. Cage-side observation was performed daily during clinical signs observation and if any abnormalities were found, the animals were removed from the cage and observed individually.

Body weight and heart weight measurement

Body weight was measured on Day 1 and Day 10 of the treatment period and percentage change in body weight was reported. Heart weights were immediately recorded from sacrificed animals after cleaning the organs with saline and tissue paper. A ratio of heart weight/body weight (terminal) was calculated and reported.

Electrocardiographic studies

Animals were anesthetized with the combination of ketamine (75 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) prior to ECG measurement on Day 10. A digital physiograph (Model: DI-2, INCO, Ambala, India) was used to record the cardiogram. Heart rate, QRS, QT, PR and RR intervals were determined from Lead II of the cardiogram.

Biochemical Analysis

Animals were fasted overnight prior to blood collection and necropsy. Blood was collected under anaesthesia from the retro-orbital plexus. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase-MB (CK-MB), creatine kinase-NAC (CKNAC) and lactate dehydrogenase (LDH), serum cholesterol and triglyceride levels were estimated by using commercial kits with the help of a semi auto analyzer (Model: Prietest TOUCH, Robonik (India) Pvt. Ltd.).

Tissue antioxidant enzymes

Hearts were collected from each animal and weighed after cleaning with saline and tissue paper. They were homogenized with 0.9% buffered KCl (pH 7.4) solution and centrifuged at 5000 rpm for 15 min. The supernatant was decanted and used for the estimation of superoxide dismutase (SOD), catalase and reduced glutathione (GSH) by colorimetric methods. SOD activity was determined on the capacity of the enzyme to reduce nitroblue tetrazolium. The absorbance was measured at 560 nm[42]. Ellman's method was followed for the estimation of GSH. The method is based on the reaction between GSH and 2-nitrobenzoic acid to form a yellow colored compound that has absorbance at 412 nm[43]. Catalase was estimated by method of Aebi[44], based on the decomposition of hydrogen peroxide and the change in absorbance was measured at 240 nm.

Histopathological analysis

After weighing the hearts, representative parts from the organs were removed and fixed in 10% formalin. Sections were 5µm thick and H&E stained before evaluation. Damage and severity to the myocardium was evaluated and scored as none, mild, moderate and severe.

Statistical analysis

InStat data analysis program was used for statistical analysis (GraphPad Software, USA). Data were analysed by one-way analysis of variance (ANOVA) and presented as means±standard deviation (SD). Individual differences were determined by Tukey multiple comparison tests. A value of less than 0.05 was considered statistically significant. Types of Comparisons:

1. Normal control group vs CYP control/CYP+URSO group marked by ***/**/*****
2. CYP control group vs CYP+URSO group marked by **# /## /###**

Results

General observations and mortality

All animals were observed daily for occurrence of clinical signs. No physical abnormal changes or mortality was observed in control and treated groups.

Body weight, heart weight and heart to body weight ratio

In the CYP treated group, body weight of the animals was found to be significantly decreased when compared to control group. Treatment with ursolic acid reverted most of the changes and produced a moderately significant increase in body weight. CYP treated group showed a remarkable increase in heart weight, believed to be due hypertrophy of the heart. Treatment with URSO normalised the heart weight and heart weight/body weight ratio when compared to CYP Control group (Table 1).

Electrocardiographic parameters

The CYP control group demonstrated a significant increase in Heart Rate, RR segment, QT segment, PR interval and QRS interval compared with the normal control. Ursolic acid pre-treatment almost normalised all of the parameters (Table 2).

Biochemical analysis

CYP Control group displayed rise in serum biomarker values compared to concurrent normal control group. Treatment group CYP+URSO showed a marked reduction in AST, ALT, CK-MB, CK-NAC and LDH values (Fig. 1).

Lipid profile assay

TG and TC level showed marked increase in toxic control group in comparison to normal. Ursolic acid treated group showed normalization in serum vales of TC and TG (Table 3).

Tissue antioxidant enzymes

CYP treatment caused reduction in heart tissue homogenate (HTH) levels of SOD, Catalase and GSH. Ursolic acid treated animals displayed significantly higher values compared to CYP control group animals (Table 3).

Histopathological analysis

Myocardial cells of the animals in normal control and URSO groups showed normal texture and intact cell membranes. Animals in the CYP Control group showed changes such separation of myocardial tissue, vacuolization of myocardial cells, and accumulation of inflammatory cells and loss of myofibril. Treatment with Ursolic acid in the CYP+URSO group, showed decreased infiltration of inflammatory cells, lesser defragmentation, vacuolization and myofibril loss (Fig. 2).

Table 1 — Effects on physical parameters

Treatment	Body Weight (g)			Heart Weight (g)	Heart/Body Weight Ratio ($x10^{-4}$)
	Day 1	Day 10	% Change		
Normal Control	220.17±2.76	259.74±2.64	15.24	0.72±0.01	27.33
CYP	225.73±2.78	190.83±3.19***	-18.35	1.16±0.03***	60.26***
URSO	222.51±2.91	259.00±2.18	14.08	0.74±0.02	28.18
CYP+URSO	217.83±2.37	196.00±2.80***##	-11.21	0.90±0.01***###	53.06***###

Results are expressed as mean ± SD, n=6. ***p<0.001 when compared to normal, ###p<0.001, ## p<0.01, when compared to CYP treated. CYP – Cyclophosphamide, URSO – Ursolic Acid

Table 2 — Effects on Heart Rate and Electrocardiographic parameters

Treatment	Heart Rate (bpm)	RR (ms)	QRS (ms)	QT (ms)	PR (ms)
Normal Control	182.33 ±6.17	191.33 ±5.23	143.66 ±4.09	195.66 ±3.18	82.66 ±4.33
CYP	289.66 ±3.18***	289.66 ±3.18***	213.66 ±3.75***	297.33 ±4.05***	199.33 ±5.48***
URSO	184.00 ±3.21	190.66 ±4.63	150.33 ±1.45	203.00 ±4.35	77.33 ±6.36
CYP+URSO	247.66 ±4.09***###	247.66 ±4.09***###	176.66 ±4.41***###	250.00 ±2.88***###	131.66 ±6.17***###

Results are expressed as mean ± SD, n=6. ***p<0.001 when compared to normal, ###p<0.001 when compared to CYP treated. CYP: Cyclophosphamide, URSO: Ursolic Acid.

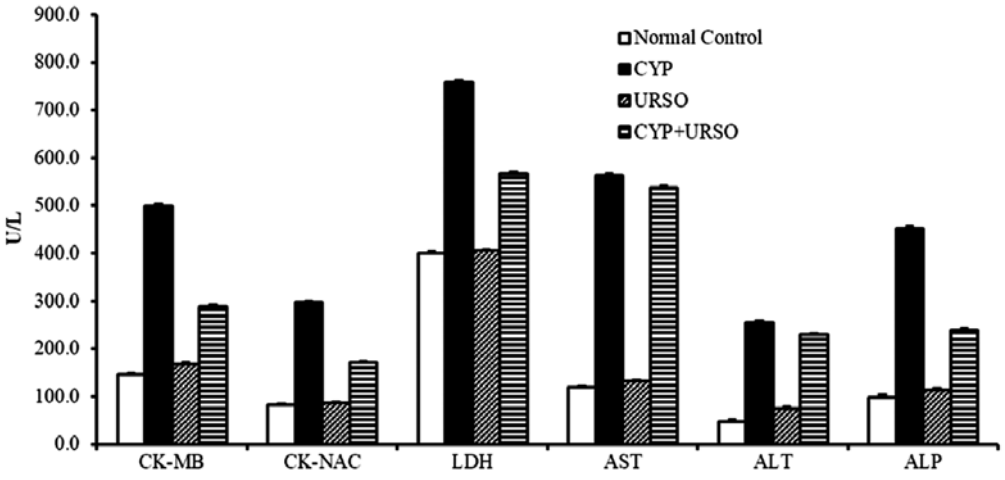

Fig. 1 — Effect on serum biomarker levels

Table 3 — Effects on total cholesterol & triglycerides and heart tissue homogenate levels of SOD, catalase and GSH

Treatment	Blood Serum Level (mg/dL)		Heart Tissue Homogenate (U/L)		
	TC	TG	SOD	Catalase	GSH
Normal Control	21.76±1.36	79.61±3.81	87.46 ±3.98	57.78 ± 5.20	87.90 ± 4.78
CYP	74.46±3.6***	205.57±7.15***	17.99 ± 0.01***	16.24 ± 0.35***	24.49 ± 0.49***
URSO	33.14±3.37	83.41±2.03	61.63 ± 3.99	45.25 ± 2.61	69.56 ± 5.29
CYP+URSO	42.85±1.94***###	157.52±6.94***###	55.17 ±0.47**###	38.81 ± 0.27**###	54.06 ± 0.86***##

Results are expressed as mean ± SD, n=6. **p<0.01***p<0.001 when compared to normal control group, ##p<0.01, ###p<0.001 when compared to CYP control group. CYP –Cyclophosphamide, URSO: Ursolic Acid.

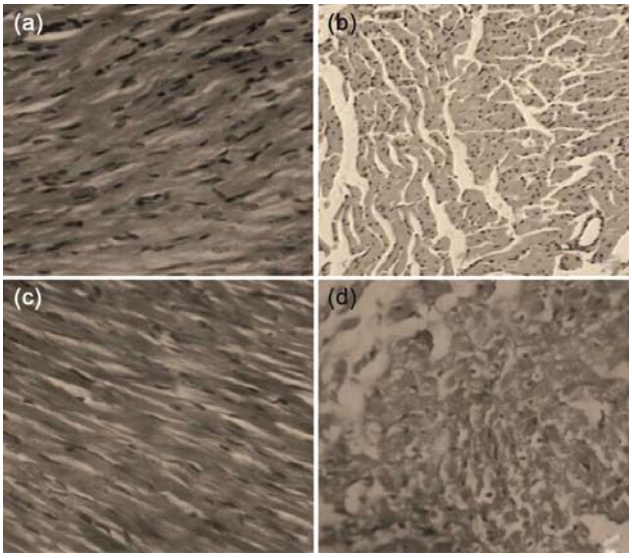

Fig. 2 — Photomicrographs (X400) of heart tissues in (A) Normal control (B) CYP control (C) Ursolic acid control (D) CYP+URSO treatment groups

Discussion

Traditionally, herbs, spices and medicinal plants have been used since ages for purpose of cosmetic, culinary and therapeutic intentions. Polyphenols and triterpenoids are the active antioxidant principles of most fruits, herbs and spices used in daily life. There has been a keen interest in utilizing them for therapeutic purposes[2,45]. In addition to antioxidant effects, these active principles, alone or in combination with other constituents, display a magnitude of pharmacological activities such as hepatoprotective, antidiabetic, antibacterial, anticancer, etc[46–50]. These active principles inhibit oxidative damage of DNA through free radicals. ROS induce changes in gene expression causes generation of abnormal proteins resulting in diseases and aging[51,52]. Hence, they prove to be highly effective means to treat and prevent disorders of such origin. Ursolic acid is found in abundant quantities in apple peels. Other major sources of the phytoconstituent include: basil, cranberries, peppermint, and prunes[53]. Various studies have demonstrated the efficacy of these principles in preventing ailments like cardiomyopathy caused by oxidative stress[54]. We have evaluated the possible protective effect of ursolic acid against chemotherapy-induced cardio-toxicity in this study.

Single dose of cyclophosphamide caused marked rise in heart weight and decrease in body weight of the animals indicating that the normal metabolic functions were disordered, a sign of cyclophosphamide toxicity[39]. Increased heart weight can be reasoned to edema and necrotic myocardial tissues[51]. Treatment with ursolic acid improved overall metabolic functions, normalized body weight, and heart weight in the treated animals.

Cyclophosphamide causes direct damage to the myocardial endothelium by causing extravasation of plasma proteins and infiltration of erythrocytes into myocardial interstitium and muscle cells[52]. Damaged cell membrane allows biomarkers: CKNAC, isoenzyme CKMB (cardiac muscle fraction of CK), LDH, AST and ALT to leak into systemic circulation. Increased cellular permeability and damage to functional integrity of the cardiac tissue leads to myocardial necrosis. An estimate of damage to the myocardium can be assessed by quantification of these biomarker enzymes from blood[55]. CYP control group showed higher serum levels of these biomarkers, confirming the cardiotoxicity induced by CYP. Administration of ursolic acid resulted in reduction of levels of biomarkers in blood[36], This effect can be attributed to the membrane stabilizing action of the phytoconstituent.

Acrolein, the toxic metabolite of CYP generates ROS that causes damage to the surrounding tissue[51]. This is mediated by mitochondrial dysfunction and increased intracellular calcium that in turn activates xanthine oxidase, which stimulates production of more ROS and depletion in glutathione[56]. Free radicals such as superoxide ion, hydroxyl radical (OH^-), alkoxyl and peroxyl radicals, and hydrogen

peroxide (H_2O_2) oxidizes polyunsaturated fatty acids (PUFA), proteins, cellular DNA and sterols[52]. These free radicals generated through CYP metabolism cause increase in lactoperoxidase activity and decrease in activity of cellular antioxidants[57,58]. Protective antioxidant enzymes such as SOD and CAT are present in lower levels in the heart compared to other body tissues making it more vulnerable to oxidative stress as H_2O_2 inhibits SOD and superoxides inhibit CAT functions[58]. These tissue antioxidants remove oxygen (O_2) and hydrogen peroxide (H_2O_2) before they can interact to form more reactive hydroxyl radicals[59]. Inhibition of SOD and CAT leads to increased free radicals induced damage in cardiac tissues. In this study, marked decrease in levels of SOD and CAT was observed in heart tissue homogenate, a result of CYP toxicity. Treatment with ursolic acid resulted in increase in amount of these enzymes, thereby improving the antioxidant status and preventing cardiac damage. GSH (reduced glutathione) is another important regulator of cellular redox state[56]. CYP-induced decrease in tissue GSH was observed in this study which maybe a result of binding of CYP metabolites to free or protein bound tissue –SH groups[60]. These conjugates hinder the normal functions of the antioxidant. Acrolein is known to deplete GSH, GSH-linked glutathione-S-transferases, and aldose reductase and thereby preventing detoxification of reactive aldehydes[20,60]. In this study, marked decrease in HTH levels of GSH was observed. Treatment with ursolic acid resulted normalized the HTH levels of GSH.

Cyclophosphamide-induced hyperlipidemia is caused by inhibition of lecithin cholesterol acyl transferase (LCAT) and lipoprotein lipase (LPL) enzymes[51]. LCAT esterifies free cholesterol in serum and tissues and is vital in remodeling of HDL lipoproteins. LPL mostly found in adipose tissue and myocardial capillaries clears triglycerides from plasma, turning into free fatty acids and glycerol[51]. CYP-treatment causes an increase in total cholesterol, free cholesterol, free fatty acids and triglycerides[51]. Our observations were consistent with previous findings on CYP toxicity. Ursolic acid treatment reversed the toxic increase of TC and TG in serum. This effect of the phytoconstituent may be attributed to its anti-lipase, lipolytic, and PPAR-α agonist activity[8,29].

Cyclophosphamide administration diminished the heart rate and increased QT, QRS, PR and RR intervals. This effect on heart rate maybe due to increased secretion of acetylcholine at the neuro-muscular junctions, which results in bradycardia. Elongation of QT interval is due to hypokalemia caused by blockade of the potassium channels. Release of acetyl choline causes change in parasympathetic tone and conduction system of the heart, leading to AV block causing elongation of PR interval[61–63]. Ursolic acid successfully prevents CYP induced bradycardia and normalizes the intervals[4] indicating its protective effect.

Cyclophosphamide treated group showed vacuolization of myocytes, infiltration of leukocytes and other inflammatory cells, disruption of myocardial tissue and myofibril as reported previously[64]. It is known that upregulation of NF-κB mediates inflammation in the tissues through pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β which lead to fibrosis and myocardial disruption[65,66]. Ursolic acid treatment showed normalization of those pathological changes possibly by vasodilatation and restoration of the antioxidant enzymes.

Conclusion

Patients that suffer from cancer are at a risk of developing heart diseases like cardiomyopathy, IHD, and arrhythmias due to chemotherapy. Management of drug-related cardiomyopathy calls for an interdisciplinary approach. In present study, a single, high dose of cyclophosphamide caused marked myocardial tissue damage mediated through oxidative stress causing deleterious effects on biochemical and histopathological parameters in rats. Ursolic acid ameliorated the compromised myocardium by scavenging free radicals, reversing drug induced hyperlipidemia and strengthening antioxidant defense systems of tissues. These observations support the hypothesis of using the phytoconstituent, ursolic acid, as an adjunct therapy in management of cyclophosphamide-induced cardio-toxicity.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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