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

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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 ailments¹. 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 products². Additionally, these herbs, spices and other naturally derived products find use in culinary, cosmetic and medicinal intentions³. They are used extensively around the world to enhance flavor, color and for conservation of food. Different phytoconstituents 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 diseases⁵.

The phytoconstituent, ursolic acid (URSO), is a pentacyclic triterpenoid carboxylic acid found abundantly in medicinal plants, herbs and foods⁴⁻⁷. 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-diabetic⁴, anti-hyperlipidaemic⁹, hepatoprotective¹⁰, neuroprotection¹¹ and neuro-regeneration¹² and antibacterial¹³ among many others. It has a complex mechanism of action, which includes: attenuation of H₂O₂ and MPP (1-Methyl-4-phenylpyridinium) induced impairment in catalase and superoxide dismutase (SOD) activity¹⁴; prevention of lipid peroxidation¹⁴; protection of DNA against oxidative damage¹⁵ and inhibition of nitric oxide (NO) production¹⁶.

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 potential¹⁹. CYP is an alkylating anticancer drug that acts by cross-linking DNA²⁰. Cyclophosphamide causes acute cardio-toxicity with signs and symptoms of myopericarditis, which manifests into fatal complications, like cardiac

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tamponade, congestive heart failure, arrhythmias, etc.^{19,21}. In the body, CYP is broken down into Phosphoramidate mustard and Acrolein²². 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-induced²⁴ and doxorubicin-induced²⁵ oxidative stress and isoproterenol-induced myocardial ischemia²⁶. Studies have also found URSO to act through uncoupling of antioxidants and mitochondria in isolated hearts²⁷. It is also proven to inhibit nuclear factor-kappaB activation induced by ROS produced from metabolism of carcinogenic agents and thus preventing tumorigenesis²⁸. 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-kappaB⁵. 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 metabolism²⁹.

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 drugs³⁰⁻³². 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 Animals³³ and CPCSEA Guidelines³⁴. 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 \pm 5°C 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/kg⁴¹.

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 nm⁴². 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 nm⁴³. Catalase was estimated by method of Aebi⁴⁴, 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 ($\times 10^{-4}$) |
|----------------|-------------------|-------------------------|----------|-----------------------|--|
| | Day 1 | Day 10 | % Change | | |
| Normal Control | 220.17 \pm 2.76 | 259.74 \pm 2.64 | 15.24 | 0.72 \pm 0.01 | 27.33 |
| CYP | 225.73 \pm 2.78 | 190.83 \pm 3.19*** | -18.35 | 1.16 \pm 0.03*** | 60.26*** |
| URSO | 222.51 \pm 2.91 | 259.00 \pm 2.18 | 14.08 | 0.74 \pm 0.02 | 28.18 |
| CYP+URSO | 217.83 \pm 2.37 | 196.00 \pm 2.80***### | -11.21 | 0.90 \pm 0.01***### | 53.06***### |

Results are expressed as mean \pm 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 \pm 6.17 | 191.33 \pm 5.23 | 143.66 \pm 4.09 | 195.66 \pm 3.18 | 82.66 \pm 4.33 |
| CYP | 289.66 \pm 3.18*** | 289.66 \pm 3.18*** | 213.66 \pm 3.75*** | 297.33 \pm 4.05*** | 199.33 \pm 5.48*** |
| URSO | 184.00 \pm 3.21 | 190.66 \pm 4.63 | 150.33 \pm 1.45 | 203.00 \pm 4.35 | 77.33 \pm 6.36 |
| CYP+URSO | 247.66 \pm 4.09***### | 247.66 \pm 4.09***### | 176.66 \pm 4.41***### | 250.00 \pm 2.88***### | 131.66 \pm 6.17***### |

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

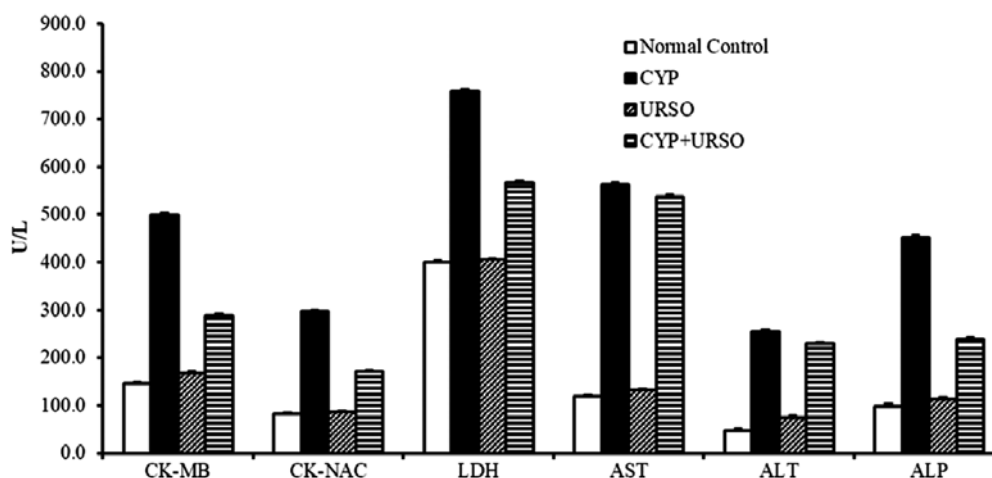


Fig. 1 — Effect on serum biomarker levels

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.

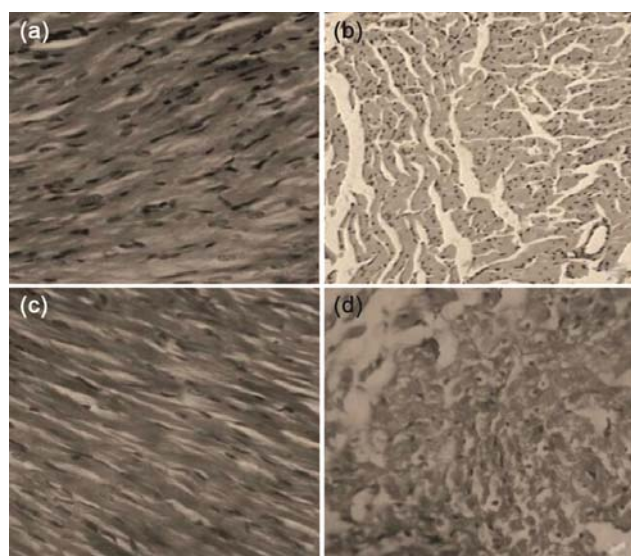


Fig. 2 — Photomicrographs (X400) of heart tissues in (A) Normal control (B) CYP control (C) Ursolic acid control (D) CYP+URSO treatment groups

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, etc⁴⁶⁻⁵⁰. 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 prunes⁵³.

Various studies have demonstrated the efficacy of these principles in preventing ailments like cardiomyopathy caused by oxidative stress⁵⁴. 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 toxicity³⁹. Increased heart weight can be reasoned to edema and necrotic myocardial tissues⁵¹. 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 cells⁵². Damaged cell membrane allows biomarkers: CKMB, 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 blood⁵⁵. 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 blood³⁶. 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 tissue⁵¹. 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 glutathione⁵⁶. Free radicals such as superoxide ion, hydroxyl radical (OH[•]), alkoxy and peroxy radicals, and hydrogen

peroxide (H_2O_2) oxidizes polyunsaturated fatty acids (PUFA), proteins, cellular DNA and sterols⁵². 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 functions⁵⁸. These tissue antioxidants remove oxygen (O_2) and hydrogen peroxide (H_2O_2) before they can interact to form more reactive hydroxyl radicals⁵⁹. 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 state⁵⁶. 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 groups⁶⁰. 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) enzymes⁵¹. 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 glycerol⁵¹. CYP-treatment causes an increase in total cholesterol, free cholesterol, free fatty acids and triglycerides⁵¹. 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 neuromuscular 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 interval⁶¹⁻⁶³. Ursolic acid successfully prevents CYP induced bradycardia and normalizes the intervals⁴ 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 previously⁶⁴. It is known that upregulation of NF- κ B mediates inflammation in the tissues through pro-inflammatory cytokines like TNF- α , IL-6, and IL-1 β 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.

References

- 1 Zhan C-D, Sindhu RK, Pang J, Ehdai A, & Vaziri ND, Superoxide dismutase, catalase and glutathione peroxidase in

- the spontaneously hypertensive rat kidney: effect of antioxidant-rich diet, *J Hypertens*, 22 (10) (2004) 2025–33.
- 2 Scartezzini P & Speroni E, Review on some plants of Indian traditional medicine with antioxidant activity, *J Ethnopharmacol*, 71 (1–2) (2000) 23–43.
 - 3 Dini I, Spices and Herbs as Therapeutic Foods, In: *Food Quality: Balancing Health and Disease*, (Academic Press), 2018, 433–469.
 - 4 Liu J, Pharmacology of oleanolic acid and ursolic acid, *J Ethnopharmacol*, 49 (2–1) (1995) 57–68.
 - 5 Cargin ST & Gnoatto SB, Ursolic acid from apple pomace and traditional plants: A valuable triterpenoid with functional properties, *Food Chem*, 220 (2017) 477–489.
 - 6 Silva MG, Vieira I, Mendes F, Albuquerque I, Dos Santos R, *et al.*, Variation of Ursolic Acid Content in Eight *Ocimum* Species from Northeastern Brazil, *Molecules*, 13 (10) (2008) 2482–2487.
 - 7 Liu J, Oleanolic acid and ursolic acid: Research perspectives, *J Ethnopharmacol*, 100 (1–2) (2005) 92–94.
 - 8 Liu J, Liu J, & Jie L, Pharmacology of oleanolic acid and ursolic acid, *J Ethnopharmacol*, 49 (2–1) (1995) 57–68.
 - 9 Somova LO, Nadar A, Rammanan P, & Shode FO, Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension, *Phytomedicine*, 10 (2–3) (2003) 115–121.
 - 10 Gutiérrez-Rebolledo GA, Siordia-Reyes AG, Meckes-Fischer M, & Jiménez-Arellanes A, Hepatoprotective properties of oleanolic and ursolic acids in antitubercular drug-induced liver damage, *Asian Pac J Trop Med*, 9 (7) (2016) 644–651.
 - 11 Li L, Zhang X, Cui L, Wang L, Liu H, *et al.*, Ursolic acid promotes the neuroprotection by activating Nrf2 pathway after cerebral ischemia in mice, *Brain Res*, 1497 (2013) 32–39.
 - 12 Liu B, Liu Y, Yang G, Xu Z, & Chen J, Ursolic acid induces neural regeneration after sciatic nerve injury, *Neural Regen Res*, 8 (27) (2013) 2510–9.
 - 13 Fontanay S, Grare M, Mayer J, Finance C, & Duval RE, Ursolic, oleanolic and betulinic acids: Antibacterial spectra and selectivity indexes, *J Ethnopharmacol*, 120 (2) (2008) 272–276.
 - 14 Balanehru S & Nagarajan B, Protective effect of oleanolic acid and ursolic acid against lipid peroxidation, *Biochem Int*, 24 (5) (1991) 981–990.
 - 15 Ramos AA, Pereira-Wilson C, & Collins AR, Protective effects of Ursolic acid and Luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells, *Mutat Res - Fundam Mol Mech Mutagen*, 692 (1–2) (2010) 6–11.
 - 16 Tsai SJ & Yin MC, Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells, *J Food Sci*, 73 (7) (2008) H174–8.
 - 17 Meinardi MT, Gietema JA, van Veldhuisen DJ, van der Graaf WT, de Vries EG, *et al.*, Long-term chemotherapy-related cardiovascular morbidity, *Cancer Treat Rev*, 26 (6) (2000) 429–47.
 - 18 Grenier MA & Lipshultz SE, Epidemiology of anthracycline cardiotoxicity in children and adults, *Semin Oncol*, 25 (4 Suppl 10) (1998) 72–85.
 - 19 Goldberg MA, Antin JH, Guinan EC, & Rapoport JM, Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor, *Blood*, 68 (5) (1986) 1114–8.
 - 20 Ewer MS & Ewer SM, Cardiotoxicity of anticancer treatments: what the cardiologist needs to know, *Nat Rev Cardiol*, 7 (10) (2010) 564–75.
 - 21 Dow E, Schulman H, & Agura E, Cyclophosphamide cardiac injury mimicking acute myocardial infarction, *Bone Marrow Transplant*, 12 (2) (1993) 169–72.
 - 22 Clarke L & Waxman DJ, Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxygenase catalysts of drug activation, *Cancer Res*, 49 (9) (1989) 2344–50.
 - 23 Souid A-K, Tacka KA, Galvan KA, & Penefsky HS, Immediate effects of anticancer drugs on mitochondrial oxygen consumption, *Biochem Pharmacol*, 66 (6) (2003) 977–87.
 - 24 Saravanan R & Pugalendi V, Impact of ursolic acid on chronic ethanol-induced oxidative stress in the rat heart, *Pharmacol Rep*, 58 (1) (2006) 41–7.
 - 25 Chakraborty M, Bhattacharjee A, & Kamath J V., Cardioprotective effect of ursolic acid against doxorubicin induced cardiotoxicity, *Indian Drugs*, 53 (11) (2016) 65–71.
 - 26 Radhiga T, Rajamanickam C, Sundaresan A, Ezhumalai M, & Pugalendi KV, Effect of ursolic acid treatment on apoptosis and DNA damage in isoproterenol-induced myocardial infarction, *Biochimie*, 94 (5) (2012) 1135–1142.
 - 27 Senthil S, Sridevi M, & Pugalendi K V., Protective Effect of ursolic acid against myocardial ischemia induced by isoproterenol in rats, *Toxicol Mech Methods*, 17 (1) (2007) 57–65.
 - 28 Shishodia S, Majumdar S, Banerjee S, & Aggarwal BB, Ursolic acid inhibits nuclear factor-kappaB activation induced by carcinogenic agents through suppression of IkkappaB α kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9 and cyclin D1, *Cancer Res*, 63 (15) (2003) 4375–83.
 - 29 Jia Y, Bhuiyan MJH, Jun H, Lee JH, Hoang MH, *et al.*, Ursolic acid is a PPAR- α agonist that regulates hepatic lipid metabolism, *Bioorg Med Chem Lett*, 21 (19) (2011) 5876–5880.
 - 30 Song H-P, Wu S-Q, Qi L-W, Long F, Jiang L-F, *et al.*, A strategy for screening active lead compounds and functional compound combinations from herbal medicines based on pharmacophore filtering and knockout/knockin chromatography, *J Chromatogr A*, 1456 (2016) 176–186.
 - 31 Kinghorn AD, Chai H, Sung CK, & Keller WJ, The classical drug discovery approach to defining bioactive constituents of botanicals, *Fitoterapia*, 82 (1) (2011) 71–79.
 - 32 Sasidharan S, Chen Y, Saravanan D, Sundram KM, & Yoga Latha L, Extraction, isolation and characterization of bioactive compounds from plants' extracts, *African J Tradit Complement Altern Med*, 8 (1) (2011) 1–10.
 - 33 National Research Council (US) Institute for Laboratory Animal Research, *Guide for the Care and Use of Laboratory Animals*, (National Academies Press (US), 1996).
 - 34 Government of India Ministry of Fisheries, Animal Husbandry and Dairying, *Breeding of and Experiments on Animals (Control and Supervision) Rules*, 2006.
 - 35 Saravanan R, Viswanathan P, & Pugalendi KV, Protective effect of ursolic acid on ethanol-mediated experimental liver damage in rats, *Life Sci*, 78 (7) (2006) 713–718.
 - 36 Radhiga T, Rajamanickam C, Senthil S PK, Effect of ursolic acid on cardiac marker enzymes, lipid profile and

- macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats, *Food Chem Toxicol*, 50 (11) (2012) 3971–7.
- 37 Cornejo Garrido J, Chamorro Cevallos GA, Garduno Siciliano L, Hernandez Pando R, & Jimenez Arellanes MA, Acute and subacute toxicity (28 days) of a mixture of ursolic acid and oleanolic acid obtained from *Bouvardia ternifolia* in mice, *Bol Latinoam y del Caribe Plantas Med y Aromat*, 11 (1) (2012) 91–102.
- 38 Toxicology Data Network, Ursolic Acid - National Library of Medicine HSDB Database, (<https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+3157>).
- 39 Viswanatha Swamy AHM, Patel UM, Koti BC, Gadad PC, Patel NL, *et al.*, Cardioprotective effect of *Saraca indica* against cyclophosphamide induced cardiotoxicity in rats: a biochemical, electrocardiographic and histopathological study, *Indian J Pharmacol*, 45 (1) (2013) 44–8.
- 40 Bhatt L, Sebastian B, & Joshi V, Mangiferin protects rat myocardial tissue against cyclophosphamide induced cardiotoxicity, *J Ayurveda Integr Med*, 8 (2) (2017) 62–67.
- 41 Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, *et al.*, A good practice guide to the administration of substances and removal of blood, including routes and volumes, *J Appl Toxicol*, 21 (1) (2001) 15–23.
- 42 Kakkar P, Das B, & Viswanathan PN, A modified spectrophotometric assay of superoxide dismutase, *Indian J Biochem Biophys*, 21 (2) (1984) 130–132.
- 43 Ellman GL, Tissue sulfhydryl groups, *Arch Biochem Biophys*, 82 (1) (1959) 70–77.
- 44 Aebi H, Catalase in vitro, In: *Oxygen Radicals in Biological Systems*, edited by Nathan Kaplan & Nathan Colowick, (Academic Press), 1984, 121–126.
- 45 Kuhn M, Bathaie SZ, & Tamanoi F, Chapter One - Introduction, In: *Natural Products and Cancer Signaling: Isoprenoids, Polyphenols and Flavonoids*, edited by Bathaie SZ, & Tamanoi F, (Academic Press), 2014, 1–6.
- 46 Lay MM, Karsani SA, Mohajer S, & Abd Malek SN, Phytochemical constituents, nutritional values, phenolics, flavonols, flavonoids, antioxidant and cytotoxicity studies on *Phaleria macrocarpa* (Scheff.) Boerl fruits, *BMC Complement Altern Med*, 14 (1) (2014) 152.
- 47 Hossain MA, AL-Raqmi KAS, AL-Mijizy ZH, Weli AM, & Al-Riyami Q, Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown *Thymus vulgaris*, *Asian Pac J Trop Biomed*, 3 (9) (2013) 705–710.
- 48 Chakraborty M, Rahila K, Bhatt L, & Kamath J, Hepatoprotective activity of *crotalaria juncea* against paracetamol intoxicated rats, *Int J Pharm Res Dev*, 5 (02) (2013) 37–41.
- 49 Rahila K, Bhatt L, Chakraborty M, Kamath J, & Rahila K, Hepatoprotective activity of *crotalaria juncea* against thioacetamide intoxicated rats, *Int Res J Pharm Appl Sci*, 3 (1) (2013) 98–101.
- 50 Bhatt L & Maithani S, Protective Effects of a Polyherbal Formulation, Freemodex, Against Acute Models of Pain, Inflammation, Arthritis, and Immunosuppression, *Int J Nutr Pharmacol Neurol Dis*, 7 (4) (2017) 88–93.
- 51 Motawi TMK, Sadik NAH, & Refaat A, Cytoprotective effects of DL-alpha-lipoic acid or squalene on cyclophosphamide-induced oxidative injury: An experimental study on rat myocardium, testicles and urinary bladder, *Food Chem Toxicol*, 48 (8–9) (2010) 2326–2336.
- 52 Shanmugarajan TS, Arunsundar M, Somasundaram I, Krishnakumar E, Sivaraman D, *et al.*, Cardioprotective effect of *Ficus hispida* Linn. on cyclophosphamide provoked oxidative myocardial injury in a rat model, *Int J Pharmacol*, 4 (2) (2008) 78–87.
- 53 Kashyap D, Tuli HS, & Sharma AK, Ursolic acid (UA): A metabolite with promising therapeutic potential, *Life Sci*, 146 (2016) 201–13.
- 54 Radi R, Biological antioxidant defenses, *Toxicol Ind Health*, 9 (1–2) (1993) 53–62.
- 55 Christenson ES, James T, Agrawal V, & Park BH, Use of biomarkers for the assessment of chemotherapy-induced cardiac toxicity, *Clin Biochem*, 48 (4–5) (2015) 223–235.
- 56 Jeelani R, Khan SN, Shaeib F, Kohan-Ghadr HR, Aldaheri SR, *et al.*, Cyclophosphamide and acrolein induced oxidative stress leading to deterioration of metaphase II mouse oocyte quality, *Free Radic Biol Med*, 110 (2017) 11–18.
- 57 Shanmugarajan TS, Arunsundar M, Somasundaram I, Krishnakumar E, Sivaraman D, *et al.*, Cardioprotective effect of *Ficus hispida* Linn. on cyclophosphamide provoked oxidative myocardial injury in a rat model, *Int J Pharmacol*, 4 (2) (2008) 1–10.
- 58 Şekeroğlu V, Aydın B, & Şekeroğlu ZA, *Viscum Album L.* Extract and Quercetin Reduce Cyclophosphamide-Induced Cardiotoxicity, Urotoxicity and Genotoxicity in Mice, *Asian Pacific J Cancer Prev*, 12 (2011) 2925–2931.
- 59 Ahmed R, Tanvir EM, Hossen MS, Afroz R, Ahmmed I, *et al.*, Antioxidant Properties and Cardioprotective Mechanism of Malaysian Propolis in Rats, *Evidence-Based Complement Altern Med*, (2017) 1–11.
- 60 Raschi E, Vasina V, Ursino MG, Boriani G, Martoni A, *et al.*, Anticancer drugs and cardiotoxicity: Insights and perspectives in the era of targeted therapy, *Pharmacol Ther*, 125 (2) 2010 196–218.
- 61 Levine ES, Friedman HS, Griffith OW, Colvin OM, Raynor JH, *et al.*, Cardiac cell toxicity induced by 4-hydroperoxycyclophosphamide is modulated by glutathione, *Cardiovasc Res*, 27 (7) (1993) 1248–1253.
- 62 Atlee JL, Perioperative cardiac dysrhythmias: diagnosis and management, *Anesthesiology*, 86 (6) (1997) 1397–424.
- 63 Patel S, Bhatt L, Patel R, Shah C, Patel V, *et al.*, Identification of appropriate QTc formula in beagle dogs for nonclinical safety assessment, *Regul Toxicol Pharmacol*, 89 (2017) 118–124.
- 64 Dhesi S, Chu MP, Blevins G, Paterson I, Larratt L, *et al.*, Cyclophosphamide-Induced Cardiomyopathy: A Case Report, Review, and Recommendations for Management, *J Investig Med High Impact Case Reports*, 1 (1) (2013) 2324709613480346.
- 65 Song Y, Zhang C, Wang C, Zhao L, Wang Z, *et al.*, Ferulic Acid against Cyclophosphamide-Induced Heart Toxicity in Mice by Inhibiting NF- κ B Pathway, *Evidence-Based Complement Altern Med*, (2016) 1–8.
- 66 Liu Y, Tan D, Shi L, Liu X, Zhang Y, *et al.*, Blueberry Anthocyanins-Enriched Extracts Attenuate Cyclophosphamide-Induced Cardiac Injury, edited by Matsui T., *PLoS One*, 10 (7) (2015) e0127813.