Mangiferin protects rat myocardial tissue against cyclophosphamide induced cardiotoxicity

Laxit Bhatt a, *, Binu Sebastian a, Viraj Joshi b

a Department of Pharmacology, Shree Devi College of Pharmacy, Airport Road, Kenjar Village, Malavoor Panchayat, Mangalore, 575412 Karnataka, India
b Department of Quality Assurance, Shree Devi College of Pharmacy, Airport Road, Kenjar Village, Malavoor Panchayat, Mangalore, 575412 Karnataka, India

1. Introduction

Evolution of cancer therapy has made cancer a manageable disease today than ever before. However, chemotherapy-induced cardiac complications are an important cause of morbidity and mortality. Patients are likely to suffer from a cardiac disease rather than recurrent cancer. It is therefore necessary to manage both, the neoplasm and associated toxicities of anti-neoplastic drugs, especially cardiotoxicity [1,2].

Cyclophosphamide (CYP) is perhaps the most widely used anti-neoplastic agent [3]. It is used for the treatment of chronic and acute leukemias, myelomas, lymphomas, and for bone marrow transplantation [4]. CYP is attributed to also possess highly potent immunosuppressant activity [3]. Apart from having tumor selective action, it also possesses many highly toxic side-effects. Dose-mediated cardiotoxicity is one of the most important toxic effects [5]. The incidence of fatal cardiomyopathy due to a single high dose of CYP is up to 17%, depending on the different regimens and patient populations [6]. In contrast to the delayed cardiotoxic effects of other anti-neoplastic drugs, CYP causes lethal cardiomyopathy within 1–10 days after first administration of the dose (180–200 mg/kg) [7,8]. The cardiotoxic effects of CYP consists of acute, dose-dependent cardiac damage, morphologically characterized by necrosis, hemorrhage and later development of fibrosis [4,9].

The anti-neoplastic activity of CYP is due to phosphoramide mustard, the therapeutically active metabolite, which possesses significant DNA-alkylating activity [10,11]. The other metabolite, acrolein interferes with antioxidant system producing highly reactive oxygen-free radicals – superoxide radicals and hydrogen peroxide [12]. These Reactive Oxygen Species (ROS) cause damage to the inner mitochondrial membrane of the heart, diminishing the oxygen radical detoxifying capacity of cardiac mitochondria [13].

Natural products are known to possess wide range of biological activity. Flavonoids and polyphenolic compounds are the active antioxidant principles found in large number of natural products.
Apart from strong antioxidant activity, they also demonstrate a number of other biological activities like, hepatoprotective, antidiabetic, anti-bacterial, and anti-cancer to name a few [14–16]. These phenolic compounds have the ability to suppress lipid peroxidation, prevent DNA oxidative damage, and scavenge free radicals. Free radicals cause depletion of the immune system antioxidants, change in gene expression, and induce abnormal proteins resulting in degenerative diseases and aging [17].

*Mangifera indica* L. (Mango) is an important plant of the Ayurvedic and other indigenous systems of medicine. Different parts of the mango tree are known to possess different bioactivities and have been extensively used in Ayurvedic system of medicine. The fruit and its juice are used as a restorative tonic, while the seeds are used in asthma. The bark finds use in diphtheria and rheumatism and the smoke of the dried leaves in prevention of hiccups and throat infections [18]. Leaves of *M. indica* L. are a rich source of phenolic compounds. Mangiferin, obtained from the leaves and bark of the tree, is a xanthone with wide range of pharmacological effects [19]. Bioactivity of mangiferin is attributed to its ability to decrease localized O2 concentration and generation of mangiferin phenoxyl radicals and metal–ligand complexes with iron, that prevent the formation of OH radicals and o xo-ferryl groups that cause tissue damage. It also helps in maintaining the oxidant–antioxidant balance necessary for normal cellular function [20].

Research suggests that mangiferin possesses anti-diabetic effect on streptozotocin induced diabetes in mice and rats, possibly by reducing intestinal absorption of glucose [21,22]. It is known to prevent isoproterenol induced myocardial infarction in rats [23]. Previous studies have also revealed protective effects of mangiferin on rat brain by inducing peroxidation of phospholipids and prevention of DNA damage by bleomycin [24]. Immunomodulatory activity of mangiferin is evident in its action to inhibit TNF-induced activation of NF-κB in mice [25]. Apart from these bioactivities, mangiferin is also attributed to possess antimicrobial [26], anti-allergic [27], anti-inflammatory, analgesic [28], and hepatoprotective [29] activities among many others.

However, till now, there have been no studies carried out to demonstrate the protective effect of mangiferin against cardiotoxicity caused by anti-cancer drugs. This study is designed to evaluate the protective effect of mangiferin on cardiotoxicity caused by CYP.

2. Materials and methods

2.1. Animals

Wistar rats of both sexes, weighing between 200 and 250 g, were obtained from the animal facility of Shree Devi College of Pharmacy, Mangalore, India. The rats were maintained in an animal house with standard facilities. The animals were housed in clean cages and maintained at 25 ± 5 °C and humidity at 30–70% under 12 h light–dark cycles, and were fed with standard feed with free access to purified drinking water. Animals were acclimatized for one week to laboratory conditions before starting the experiment. All experiment protocols were conducted according to the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), the Ministry of Social Justices and Empowerment, Government of India. Prior to the commencement of the experiment Institutional Animal Ethics Committee (SDCP/IAEC-07/2012-13) approval was obtained.

2.2. Isolation of mangiferin

*M. indica* L. leaves were obtained from cultivated trees from Mangalore area. The plant was identified at the Herbarium, Department of Pharmacognosy, Shree Devi College of Pharmacy, Mangalore. The leaves were shade dried and powdered. The powdered material was defatted with petroleum ether (60–80 °C). Defatted powdered leaves were extracted in a Soxhlet apparatus with required quantity of ethanol for 21 h and concentrated under reduced pressure to yield semisolid mass. The ethanolic mass was then subjected to hydrolysis and followed by treatment with ethyl acetate. The ethyl acetate fraction was then precipitated with ethanol and crystallized. Briefly, the compound purity was confirmed through high performance liquid chromatography (HPLC) (Data not shown). The product was stored in a desiccator to prevent humidiﬁcation and the weighed dose was dissolved in Dimethyl sulfoxide (DMSO) and used for the present study.

2.3. Experimental protocol

After the end of 1 week acclimatization, animals were divided into 4 groups of 6 animals each.

- Group I (Normal Control) served as normal control and received 1% Dimethyl Sulfoxide (DMSO) (i.p.) for 10 days.
- Group II (CYP Control) served as toxic control, in which the animals received single injection of CYP (200 mg/kg, i.p.) on the first day of experimental period to induce cardiotoxicity [30].
- Group III (MANG) received mangiferin (100 mg/kg body weight i.p.) for 10 days, dissolved in 1% DMSO [23].
- Group IV (MANG + CYP) received CYP (200 mg/kg, i.p.) on the first day and mangiferin (100 mg/kg body weight, i.p.) for 10 days.

Acute & chronic toxicity studies [31] and pharmacological studies [23] conducted on mangiferin were used as reference in deriving the current dose of 100 mg/kg.

2.4. Biochemical analysis

At the end of the experimental period, all the rats were anaesthetized under light ether anesthesia and blood was collected by the retro-orbital route using microcapillaries. Serum was then separated from blood and used for the estimation of aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP), creatine kinase-MB (CK-MB), creatine kinase–NAC (CK-NAC) and lactate dehydrogenase (LDH). Then, the animals were sacrificed by mild ether anesthesia and four hearts from each group were homogenized with ice cold 0.25 M sucrose solution [32,33] for estimation of superoxide dismutase (SOD), catalase and Reduced Glutathione (GSH). SOD activity was determined on the capacity of the enzyme to reduce nitro blue tetrazolium [34]. The absorbance was measured at 560 nm. Ellman method was followed for the estimation of GSH [35], while method of Aebi was followed to estimate catalase [36].

2.5. Electrocardiographic studies

Twenty-four hours after the last treatment, the animals were anesthetized with the combination of ketamine (75 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The leads were attached to the dermal layer of both the front paws and the hind legs and recordings were made with the help of a digital physiograph (Model number – DL-2, INCO, Ambala, India). The changes in heart rate, QRS, QT, PR and RR intervals were determined.

2.6. Lipid profile assay

Serum cholesterol and triglyceride levels were measured by commercial kits with the help of a semi-autoanalyzer.
Effect of Mangiferin on body weight

| Treatment   | Body weight (g) | Day 1  | Day 10 (Terminal) | Relative weight gain |
|-------------|-----------------|--------|-------------------|---------------------|
| Normal control | 244.75 ± 4.23    | 255.00 ± 4.99 | 10.25 ± 3.36 |
| CYP control  | 243.14 ± 7.17    | 191.00 ± 4.72 | 52.14 ± 8.83 "***" |
| MANG         | 225.43 ± 3.49    | 235.33 ± 4.20 | 9.90 ± 1.61 |
| MANG + CYP   | 240.74 ± 3.15    | 225.00 ± 6.45 | 15.74 ± 6.78 "****" |

All the values are in Mean ± SD, n = 6. "**p < 0.001 when compared to normal control group. "***p < 0.001 when compared to CYP control group. CYP – Cyclophosphamide; MANG – Mangiferin; g – grams.

2.7. Histopathological analysis

Hearts were immediately removed from the sacrificed animals and were fixed in 10% formalin before being processed for histopathological analysis. Histological sections of the heart were stained with hematoxylin and eosin. Myocardial damage and its severity were reported for specimen. The sections were given scores as follows: No Changes = 0; Mild = + (myocytes damage or small multifocal degeneration with slight depth of inflammation); Moderate = ++ (extensive myofibrillar degeneration); Marked = +++ (necrosis with diffuse inflammatory process).

2.8. Statistical analysis

The results are expressed as mean ± SD. Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison tests. The p < 0.05 was considered significant.

3. Results

Effect of mangiferin on CYP induced cardiomyopathy was evaluated by measuring the cardiac biomarker enzymes and antioxidant enzymes levels as well as evaluating electrocardiographic parameters and myocardial tissue histopathology. The observations recorded were terminal and inter-group comparison was performed.

3.1. General observations and mortality

General observations were recorded daily for the study period in all the animals. Abnormal changes like watery faeces, scruffy hair coat with slight alopecia and a pink tinge were observed in the CYP-treated group. However, these changes were remarkably reduced in mangiferin-treated group. Mortality was found in 3 animals (50%) of the CYP-treated group (Table 2).

3.2. Body weight and heart weight

Effect of CYP on heart weight, body weight and ratio of heart weight to body weight is shown in Table 1. In the CYP-treated group, heart weight and heart weight to body weight ratio significantly increased compared to normal control group. The increase may be due to hypertrophy of the heart. In the MANG + CYP group, the heart weight and heart weight to body weight ratio was significantly less compared to CYP-treated group. Body weight was significantly reduced in the CYP-treated group animals, which was found to increase in the MANG + CYP treated group animals (Tables 1 and 2).

3.3. Effect on electrocardiographic parameters

The CYP control group demonstrated a significant increase in RR segment, QT segment, PR interval and QRS interval and significant decrease in heart rate compared with the normal control. Mangiferin pre-treatment normalized almost all of the parameters (Table 3).

3.4. Serum enzyme biomarkers

The CYP-treated group demonstrated significant increase in serum AST, ALT, ALP, CK-MB, CK-NAC and LDH values compared with the normal control. Treatment group MANG + CYP showed a significant decrease in AST, ALT, ALP, CK-MB, CK-NAC and LDH values compared with toxic control (Table 4).

3.5. Effect on SOD, catalase and GSH

The SOD, catalase and GSH activities were significantly reduced in the CYP control group compared to normal control group. However, the activities of these enzymes were significantly increased in the mangiferin treated (MANG + CYP) group compared to CYP control group, indicating the tissue protecting activity of mangiferin (Table 5).

3.6. Effect on lipid profile

Significant incremental values were found for triglycerides and cholesterol levels in the CYP control group compared to normal control. Treatment MANG + CYP group showed significant decreased values of both triglyceride and cholesterol (Table 5).

3.7. Effect on histological score

Heart tissue of normal control group and MANG group showed healthy myocardial cells with normal texture. CYP control group showed vacuolization of the cardiomyocytes, infiltration of inflammatory cells, myocardial tissue separation and myofibril loss. MANG + CYP group demonstrated protective efficiency of mangiferin. The group showed decreased infiltration of leukocytes and lesser defragmentation of myofibrils. Intracellular spaces within the myocardium also decreased (Table 6; Fig. 1).

4. Discussion

Plants containing flavonoids and polyphenolic compounds possess strong antioxidant activity. Mangiferin, the major bioactive constituent of M. indica L., is a glycosyl xanthone. Epidemiological studies have found that antioxidants and similar polyphenolic compounds can successfully prevent cardiomyopathy [19]. In the present study, we studied the possible role of mangiferin from mango leaves as a cardioprotective compound in CYP-induced cardiomyopathy.

CYP treatment caused an increase in heart weight and decrease in body weight of the animals. There was also a significant rise in mortality rate. This indicates that the general state of the animals was deranged, an indication of CYP toxicity. Increase in heart weight and heart weight to body weight ratio significantly increased compared to normal control group. The increase may be due to hypertrophy of the heart. In the MANG + CYP group, the heart weight and heart weight to body weight ratio was significantly less compared to CYP-treated group. Body weight was significantly reduced in the CYP-treated group animals, which was found to increase in the MANG + CYP treated group animals (Tables 1 and 2).
Photomicrographs were used to evaluate the damage in the heart tissue: (0) no changes; (+) mild changes; (+++) moderate changes; (++++) marked changes.

Table 3
Effect of Mangiferin on electrocardiographic parameters

| Treatment          | Heart rate (beats/min) | RR interval (ms) | QT interval (ms) | QRS interval (ms) | PR interval (ms) |
|--------------------|------------------------|------------------|------------------|-------------------|-----------------|
| Normal control     | 182.33 ± 15.12         | 191.33 ± 12.81   | 195.66 ± 7.79    | 143.66 ± 10.02    | 82.66 ± 10.61   |
| CYP control        | 87.66 ± 7.79***        | 289.66 ± 7.79*** | 297.33 ± 9.26*** | 213.66 ± 9.19***  | 199.33 ± 13.43*** |
| MANG               | 182.66 ± 10.02         | 194.00 ± 16.29   | 196.00 ± 9.26    | 148.66 ± 9.19     | 84.66 ± 11.86   |
| MANG + CYP         | 150.33 ± 9.92****      | 256.33 ± 9.92**** | 263.66 ± 12.03*** | 184.66 ± 8.37**** | 150.33 ± 14.16**** |

All the values are in Mean ± SD, n = 6. ***p < 0.001 when compared to normal control group. **p < 0.01, ***p < 0.001 when compared to CYP control group. CYP – Cyclophosphamide; MANG – Mangiferin; min – minutes; ms – milliseconds.

Table 4
Effect of Mangiferin on serum enzyme biomarkers

| Treatment          | Blood serum level (IU/L) | Heart tissue homogenate (U/L) |
|--------------------|--------------------------|-------------------------------|
|                    | CK-MB | CK-NAC | LDH | AST | ALT | ALP | SOD | Catalase | GSH |
| Normal control     | 146.30 ± 5.24            | 82.18 ± 3.45                 | 399.64 ± 11.10 | 118.95 ± 7.03 | 46.81 ± 9.82 | 98.84 ± 11.32 |
| CYP control        | 499.09 ± 9.87***         | 296.70 ± 8.84***             | 757.75 ± 11.29*** | 562.77 ± 10.49*** | 254.66 ± 8.72*** | 452.21 ± 10.76*** |
| MANG               | 166.34 ± 4.90            | 86.93 ± 3.36                 | 405.21 ± 5.32 | 134.07 ± 6.84 | 78.09 ± 7.20 | 109.97 ± 6.39 |
| MANG + CYP         | 304.80 ± 6.30***         | 192.63 ± 4.04***             | 609.40 ± 11.49*** | 538.33 ± 3.83*** | 229.41 ± 4.58*** | 230.19 ± 16.46*** |

All the values are in Mean ± SD, n = 6. ***p < 0.001 when compared to normal control group. **p < 0.01, ***p < 0.001 when compared to CYP control group. CYP – Cyclophosphamide; MANG – Mangiferin.

Table 5
Effect of Mangiferin 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 ± 3.33              | 79.61 ± 9.33                 | 87.46 ± 9.75 | 57.78 ± 12.74 | 87.90 ± 11.71 |
| CYP control        | 74.46 ± 8.82***           | 205.54 ± 17.52***            | 17.99 ± 4.92*** | 16.24 ± 5.76*** | 24.49 ± 8.55*** |
| MANG               | 33.58 ± 6.64              | 75.94 ± 9.19                 | 63.34 ± 7.47 | 40.39 ± 7.99 | 65.29 ± 8.04 |
| MANG + CYP         | 43.81 ± 6.88****          | 177.75 ± 10.63****           | 51.09 ± 14.01**** | 39.34 ± 4.53**** | 52.97 ± 10.12**** |

All the values are in 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; MANG – Mangiferin; mg – milligrams; dl – deciliter.

Histopathological evaluation of heart tissue in (A) Normal (B) CYP Control (C) MANG (D) MANG + CYP.

Of the many actions of CYP, one is direct damage to myocardial endothelium, leading to death of myocardial cells. Enzymes like CK-NAC, CK-MB, LDH, ALT, AST and ALP, otherwise trapped inside the myocardial cells, are released into the blood stream due to damage to the endothelium. Quantitative estimation of these enzymes (hence called, biomarker enzymes) can be associated with the extent of damage to the myocardial tissue [38]. Administration of mangiferin showed decrease in the levels of these biomarker enzymes, indicating a reparative or membrane stabilizing action of the xanthone, preventing further damage to the myocardium.

As discussed earlier, CYP causes injury to the myocardial membrane. This injury is a result of generation of OH free radicals during CYP metabolism. These free radicals induced lipid peroxidation, resulting in loss of integrity of the myocardial membrane and thus its function. Reduced Glutathione (GSH), Catalase and Superoxide Dismutase (SOD) are important inhibitors of free radicals generated by lipid peroxidation; CYP induces decline in the amount of these enzymes leading to myocardial injury [39,40]. Our study showed marked increase in the values of these antioxidant enzymes after treatment with mangiferin, thus improving the antioxidant status and prevention of damage to the heart.
Mangiferin treated group also showed decreasing amounts of cholesterol and triglycerides. CYP induced free radicals cause accumulation of cholesterol by decreasing its uptake and increase in biosynthesis [40]. Triglycerides are converted to fatty acids in the body by lipoprotein lipase enzyme, alteration in the activity of which, results in increase in serum triglycerides levels [39]. Mangiferin shows antihyperlipidemic action, protecting against CYP induced hyperlipidemic cardiomyopathy.

CYP treated group showed changes in ECG pattern such as decrease in heart rate, and prolongation of QT interval, QRS interval, PR interval and RR interval in this study [41]. Release of higher quantities of neurotransmitter, acetylcholine, during myocardial damage results in the bradycardia, as observed in the present study. Mangiferin prevents the CYP induced bradycardia. CYP also induces changes in parasympathetic tone and conduction system. This leads to an AV block causing PR interval elongation [42]. CYP is also associated with high-dose cyclophosphamide therapy. Arch Intern Med 1974;80:531–8.

Histopathologically, treatment with CYP showed vacuolization of the cardiomyocytes, infiltration of inflammatory cells, myocardial tissue separation and myofibril loss. Mangiferin treated group showed reversal of such toxic changes (Fig. 1), an effect of possible vasodilatation.

5. Conclusion
Cyclophosphamide induced oxidative stress and myocardial damage can be prevented to a greater extent by mangiferin. This finding is very important for cancer patients suffering from chemotherapy-induced complications as there are no established guidelines for prevention or treatment of cyclophosphamide-associated cardiomyopathy. Further clinical studies are needed to establish the protective effect of mangiferin.

Conflict of interest
None declared.

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References
[1] Schultz PN, Beck ML, Stava C, Vassilopoulou-Sellin R. Health profiles in 5836 long-term cancer survivors. Int J Cancer 2003;104:488–95.
[2] Katayama M, Inai Y, Hashimoto H, Kurata M, Nagai K, Tamita K, et al. Fulminant fatal cardiotoxicity following cyclophosphamide therapy. J Cardiol 2009;54:330–4.
[3] Gershwin ME, Goeltl EJ, Steinberg AD. Cyclophosphamide: use in practice. Ann Intern Med 1974;80:531–40.
[4] Goldberg MA, Antin JH, Guinan EC, Rappeport JM. Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor. Blood 1986;68:1114–8.
[5] Fraiser LH, Kanekal S, Kehrer JP. Cyclophosphamide toxicity: characterising and avoiding the problem. Drugs 1991;42:781–95.
[6] Taniguchi I. Clinical Significance of cyclophosphamide-induced cardiotoxicity. Intern Med 2003;44:89–90.
[7] Gottdiner JS, Appelbaum FR, Ferrans VJ, Deisseroth A, Ziegler J. Cardiotoxicity associated with high-dose cyclophosphamide therapy. Arch Intern Med 1981;141:758–63.
[8] Braverman AC, Antin JH, Plappert MT, Cook EF, Lee RT. Cyclophosphamide cardiotoxicity in bone marrow transplantation: a prospective evaluation of new dosing regimens. J Clin Oncol 1991;9:1215–23.
[9] Mills BA, Roberts RW. Cyclophosphamide-induced cardiomyopathy. A report of two cases and a review of the English literature. Cancer 1979;43:2223–6.
[40] Gesquière L, Loreau N, Minnich A, Davignon J, Blache D. Oxidative stress leads to cholesterol accumulation in vascular smooth muscle cells. Free Radic Biol Med 1999;27:134–45.

[41] Atlee JL. Perioperative cardiac dysrhythmias: diagnosis and management. Anesthesiology 1997;86:1397–424.

[42] Levine ES, Friedman HS, Griffith OW, Colvin OM, Raynor JH, Lieberman M. Cardiac cell toxicity induced by 4-hydroperoxycyclophosphamide is modulated by glutathione. Cardiovasc Res 1993;27:1248–53.

[43] McGowan GK, Walters G. Ventricular arrhythmias and hypokalemia. Lancet Lond Engl 1976;2:964.

[44] Nakamae H, Tsumura K, Hino M, Hayashi T, Tatsumi N. QT dispersion as a predictor of acute heart failure after high-dose cyclophosphamide. Lancet 2000;355:805–6.

[45] Defronzo RA, Colvin OM, Braine H, Robertson GL, Davis PJ. Cyclophosphamide and the kidney. Cancer 1974;33:483–91.