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CARDIOPROTECTIVE ACTIVITY OF AQUEOUS EXTRACT OF *TAGETES PATULA* FLOWERS AGAINST CYCLOPHOSPHAMIDE INDUCED CARDIOTOXICITY IN RATS: A BIOCHEMICAL, AND HISTOPATHOLOGICAL STUDY.

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Abstract

Background: Cyclophosphamide (CP) is commonly used as an anticancer and immunosuppressive agent. It is a potent cardiotoxic agent associated with acute cardiotoxicity such as fatal cardiomyopathy, cardiomegaly, and severe congestive cardiac failure. The drug is often used to induce cardiotoxicity models in experimental animals. The present study is to evaluate the effect of aqueous extract of *Tagetes patula* flowers (AETP) against cyclophosphamide induced cardiotoxicity in albino rats.

Methods: 20 healthy wistar albino rats were divided into 4 groups. Group I (Normal) received normal saline intraperitoneal on day 1 and 2% gum acacia suspension p.o for 10 days. Group II (CP treated) received cyclophosphamide 150 mg/kg i.p on day 1 and 2% gum acacia suspension p.o for 10 days. Group III (AETP 200 mg/kg) and Group IV (AETP 400 mg/kg) were treated with cyclophosphamide 150 mg/kg i.p on day 1 and AETP 200 mg/kg and 400 mg/kg per orally respectively for 10 days. Cardiac biomarkers like creatinine kinase (CK), CK isoenzyme MB, and lactate dehydrogenase were assessed. Histopathological examination of the myocardium was done.

Results: CP treated group demonstrated significant elevation of cardiac biomarkers along with damage of myocardium. However, treatment with aqueous extract of *Tagetes patula* flowers revealed potential protective effect on myocardial tissue and improved cardiac biomarkers.

Conclusion: The study demonstrated the protective role of aqueous extract of *Tagetes patula* flowers against CP-induced myocardial injury.

Keywords: *Tagetes patula*, Cyclophosphamide, Cardiotoxicity, Cardiac biomarkers.

Introduction

Cyclophosphamide (CP), a commonly used oxazaphosphorine alkylating agent, has been extensively used an anticancer and as an immunosuppressive agent.[1,2,3] It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, rheumatic arthritis and in preparation for bone marrow transplantation.[4] High dose cyclophosphamide used in transplant regimens is associated with acute cardiotoxicity within 10 days such as fatal cardiomyopathy, cardiomegaly, severe congestive heart failure etc.[1] It is metabolized in liver to form the active cytotoxic metabolite phosphoramide mustard. Increased intracellular phosphoramide mustard causes increased generation of free oxygen radicals which causes cardiotoxicity.[5] Phosphoramide mustard also alkylates and binds to DNA causing cross-linking and inhibition of protein synthesis. It may cause hemorrhagic necrosis, interstitial haemorrhage and fibrin deposition, extensive capillary thrombosis and necrosis of myocardial fibres.[6]

Globally herbal medicines are extensively used due to their therapeutic efficiency and minimum side effects. *Tagetes patula*, also known as French marigold is commonly used for preparing ethnobotanical remedies against rheumatism, stomach and intestinal problems, kidney and hepatic disorders, fever, and pneumonia.[7] However to the best of our knowledge there is no scientific report demonstrating the cardioprotective activity of *Tagetes patula*. Therefore, in the present study we have focused on the cardioprotective activity of *Tagetes patula* flowers in CP induced cardiotoxicity model in albino rats.

Materials and Methods

The study was conducted in the Department of Pharmacology, Regional Institute of Medical Sciences, Imphal after getting approval of the Institutional Animal Ethics committee, RIMS, Imphal (No.1596/GO/a/12/CPCSEA).

Requirements

Albino rats, polypropylene cages, feeding tubes, distilled water, soxhlet apparatus, plant extracts, mixer grinder, evaporating dish, gum acacia, cyclophosphamide injection, semi-autoanalyzer, centrifuge, enzyme (CK,CK-MB, LDH) estimation kit, diethyl ether, dissecting instruments,

formaldehyde solution, alcohol in different grades, xylene, paraffin wax, drying oven, brass mould, egg albumin, glass slides, haematoxylin and eosin stain, microtome and microscope.

Preparation of plant extract

Tagetes patula flowers were collected from Imphal valley during November - December 2016. The plants were identified and authenticated by Dr. PK Singh, Professor, Department of Life Sciences, Manipur University (Acc.no.MUMP-003631). Aqueous extract of *Tagetes patula* (AETP) was prepared by soxhlet extraction method described by Verma SCL and Agrawal SL.[8] The leaves were cleaned, air dried under shade and powdered by a mixture grinder. 50 grams of the coarse powder was extracted at a time. A brownish crude dry extract was obtained. The dried extract was scraped out, weighed and stored in airtight container. The yield was 14%.

Phytochemical screening

The preliminary phytochemical tests of the plant extract was carried out using standard procedures.[9,10]

Acute toxicity testing

The acute toxicity testing was carried out as per OECD guidelines 423[11] in albino mice. Three animals were used for each step. The plant extracts i.e. AETP (aqueous extract of *Tagetes patula)* was administered to the fasted mice at a dose of 300 mg/kg p.o. and observed once in every 30 min during the first 24 h and thereafter, daily for 14 days. As there was no mortality, the procedure was repeated with a higher dose of 2000 mg/kg, and the animals were observed for mortality and toxic symptoms. It was observed that the dose of 2000 mg/kg p.o. of the plant extract caused no mortality or toxic symptoms in the tested animals and the dose was considered safe. Two doses of 200 mg/kg ($1/10^{th}$ of the maximum test dose) and 400 mg/kg ($1/5^{th}$ of the maximum test dose) of the plant extract were selected as working doses for the experiment.

Selection of animals

Healthy adult albino wistar rats of either sex weighing 100-210 g were procured from the Animal house, RIMS, Imphal, India. The animals were kept in polypropylene cages at the room temperature under 12:12 h light and dark cycle in the well-ventilated animal house in the Department of Pharmacology, RIMS, Imphal. Animals were acclimatized for seven days. All the rats were provided with commercially available standard pellet diet and water ad libitum. The animals were maintained under standard conditions in the animal house as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).[12]

Inclusion and exclusion Criteria:

Healthy adult wistar albino rats of either sex with baseline serum levels of CK, CK-MB, and LDH within the range of 100-140 IU/L, ≤80 IU/L, 115-192 IU/L respectively were included in the study.[13] The pregnant and lactating albino rats were excluded from study.

Experimental design

In this study, two doses of AETP i.e. 200 and 400 mg/kg were selected as working dose based on acute toxicity data to assess the protection against myocardial toxicity associated with CP administration. The animals were divided into 4 groups (I, II, III, and IV) of 5 animals each. On day 1, group I animals received normal saline (NS) 0.5 ml/100 g intraperitoneally (i.p) as a single injection. Similarly group II, III, and IV animals were administered CP (150 mg/kg) mixed in NS at a volume of 0.5 ml/100 g i.p. Animals in group I and II received 2% gum acacia in distilled water at a volume of 1ml/100 g orally for 10 days. Animals in group III and IV received AETP 200 and 400 mg/kg respectively suspended in 2% gum acacia in distilled water at a volume of 1ml/100 g orally for 10 days (Table1). Both control and treated animals were observed for 10 days after the last i.p. injection for general appearance, behaviour and mortality.

Table 1: Allotment of animals to different groups and treatments

Groups	Drugs given by i.p. route (Single dose)	Drugs given by oral route (Single daily dose for 10 days)
I. Normal	0.5 ml/100 g of 0.9% NS	2% gum acacia at 1 ml/100 g
II. CP treated	Cyclophosphamide -150 mg/kg	2% gum acacia at 1 ml/100 g
III. AETP 200 mg/kg	Cyclophosphamide- 150 mg/kg	AETP- 200 mg/kg
IV. AETP 400 mg/kg	Cyclophosphamide -150 mg/kg	AETP- 400 mg/kg

Collection of blood

To assess the baseline biochemical parameters, blood samples were drawn before any drug was given to the animals, and again on the 11^{th} day after 24 h of the last day treatment. Under ether anaesthesia, blood samples were collected from the retro-orbital venous sinus using glass capillary tube.[14] About 2 ml of blood from each animal of all groups were collected in a vacutainer and allowed to clot. The blood was then centrifuged at a speed of 3000 rpm for 10 minutes. The serum separated was stored in the refrigerator at 4°C to be used for biochemical estimation of CK, CK-MB, and LDH. Ciprofloxacin eye drops were then applied to the eyes to prevent the development of infection after washing off the blood on the eye with cold saline.

Biochemical estimations

Serum creatine kinase (CK), CK isoenzyme MB (CK-MB), and LDH were estimated using commercially available kits as per the kit guidelines.[15-17]

Tissue preparation for histopathological study

Animals were sacrificed with high dose of ether. A middle abdomino-thoracic incision was performed. The myocardial

tissues were dissected out and washed in ice cold saline, and then fixed in 10% formalin for 24 hours. Then, the paraffin sections of ventricle were prepared and cut into 5 µm thick sections in a rotary microtome. The cut sections were stained with haematoxylin and eosin (H&E) dye[18] and observed under light microscope for histopathological changes among the different treated groups of animals. Different findings like haemorrhagic necrosis, interstitial haemorrhage and fibrin deposition, interstitial oedema, inflammatory cell infiltration, capillary thrombosis and necrosis of myocardial fibres were examined.

Statistical analysis

The results of serum biochemical parameters were analyzed using One way ANOVA followed by Dunnett's t-test using SPSS version 21, and $p<0.05$ was considered significant.

Disposal of animal carcasses:

The animal carcasses were buried deep in the ground covered with lime and disinfectants after the experiment.[19]

Results

Phytochemical screening

The preliminary qualitative phytochemical analysis of aqueous extract of *Tagetes patula* flowers revealed the presence of alkaloids, carbohydrates, flavonoids, saponins, tannins, gums and proteins.

General observations

During the treatment period, normal rats showed good activity, normal feed and water consumption. CP treated animals developed pink tinge in the pinna, foot paws and body. Body hair becomes scruffy because of the hair fall to the extent that the animals looked partially naked. The rats also had red exudates around the eyes and nose. There was gradual decrease in food intake, increasing weakness with lesser activity and reaction to external stimuli which were more severe in CP control group at the end of study period. However, rats treated with both AETP and CP developed less changes of the above parameters. No necrosis was observed at site of intraperitoneal injection in all the groups. There was no death in normal, extract treated and CP treated groups during experimental period.

Cardiac biomarkers

CP treated group demonstrated significantly elevated serum levels of CK, CK-MB, LDH compared with the normal control group. The levels of CK, CK-MB, and LDH were significantly ($p< 0.5$) decreased in the AETP treated groups i.e. group III and IV rats when compared with the CP treated group (Table 2).

Table 2: Serum levels of CK, CK-MB, LDH in different treated groups

Groups	CK (IU/L)	CK-MB (IU/L)	LDH (IU/L)
I. Normal	103.44 ± 14.72	42.4 ± 10.90	121.32 ± 14.99
II. CP treated	219.58 ± 11.96 *	164 ± 15.15*	299.26 ± 13.62†
III. AETP-200 mg/kg	156.56 ± 17.13‡	96.6 ± 9.15‡	240.04 ± 16.49‡
IV. AETP-400 mg/kg	135.77 ± 14.83‡	72.8 ± 11.41‡	207.12 ± 11.21‡
One way ANOVA			
F	11	27.58	27.3
df	19	19	19
p	<0.001	<0.001	<0.001

Values are expressed as mean ± SEM, n=5; *$p < 0.05$ and $^{\dagger}p < 0.001$ when compared with normal group; $^{\ddagger}p < 0.05$ when compared with CP treated group.

Histopathological observations

In normal control group, cardiac muscle fibres were uniform in size, shape and configuration (Figure 1A). CP control group showed loss of normal architecture of myocardium, necrosis and degeneration of myocardial fibres, inflammatory cell infiltration, increased interstitial space between cardiac muscle fibres, haemorrhages, vacuoles and fragmentation of cardiac muscle fibres (Figure 1B). The histological pictures of AETP treated groups showed more intact myofibrils, less vacuolization of the cytoplasm, less inflammatory cell infiltration when compared with the CP treated groups (Figure 1C & 1D).

Histopathological pictures of myocardial tissue in normal and different treated groups

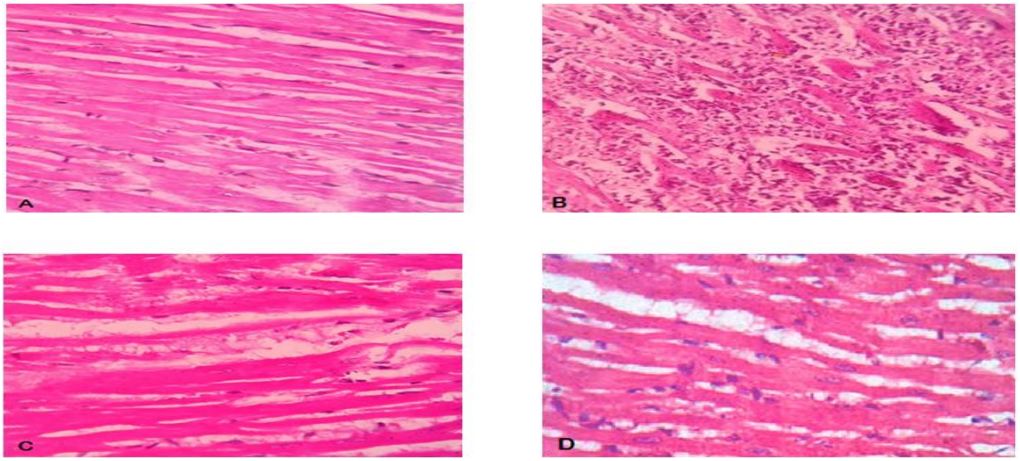

Figure 1: Photomicrograph of myocardial tissues: (A) Normal, (B) CP treated, (C) CP+AETP-200 mg/kg treated, (D) CP+AEPT-400 mg/kg treated. Stain: Hematoxylin and eosin, x40.

Discussion

A large number of animal models have been developed to mimic the clinical conditions of cardiotoxicity. Rats are commonly used rodents which are small, easily housed and maintained. Rats have been chosen for this experiment because of close resemblance of anatomy of viscera, physiology and biochemistry to that of human.[20] Rats are ideal choice for many labs due to their physiological similarity to humans and it is now known that almost all disease-linked human genes have counterparts in the rat. They are widely used in studying cardiovascular and metabolic disorders, as well as brain, digestive system, and various behavioural disorders.

Phytochemical analysis of aqueous extract of *Tagetes patula* flowers (AETP) revealed presence of active constituents like alkaloids, carbohydrates, flavonoids, saponins, tannins, gums and proteins. These phytochemicals have been reported to have different functional properties such as scavenging of reactive oxygen species, inhibition of free radicals generation.[21]

This experimental study demonstrated the increased levels of cardiac biomarkers like CK, CK-MB and LDH in CP treated group. Similar findings were also observed by different workers.[22,23] These findings suggest that CP induced oxidative stress causes leakage of cardiac enzymes due to its membrane damaging effects. Under normal conditions, 3–5% of the oxygen taken up by the cell undergoes univalent reduction leading to the formation of free radicals. Activated oxygen species, such as singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical produced by the partial reduction of oxygen are highly unstable and extremely reactive. In isolated perfused heart, brief exposure to oxygen radicals result in decrease in high energy phosphates, loss of contractile function and structural abnormalities. Oxygen radicals are capable of reacting with unsaturated lipids and of initiating the self-perpetuating chain reactions of lipid peroxidation in the membranes. Free radicals can also cause oxidation of sulfhydryl group in proteins and strand scission in nucleic acids. Tissue concentrations of these free radicals are limited by a system of enzymatic and non-enzymatic antioxidants. Among them most important cellular antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase. SOD catalyses reduction of superoxide anion to hydrogen peroxide, whereas catalase and GSHPx catalyse the reduction of hydrogen peroxide to water. At high dose, CP induces production of huge amount of free radicals which override the scavenging effects of antioxidants leading to a condition known as oxidative stress.[24] Serum CK, CK-MB and LDH enzymes are important markers of both early and late phase of cardiac injury. Evaluation of these enzymes gives a good correlation of myocardial injury.[25] Treatment with AETP reduces CP induced oxidative stress which leads to myocardial injury, which is evidenced by the reduction of serum cardiac biomarkers like CK, CK-MB and LDH. Phytochemicals like flavonoids and tannins present in the flower extract of the plant may be responsible for the antioxidant property.

In CP control group, there was enormous changes in the myocardial cell associated with degeneration of myocardial tissue, vacuolization of the cardiomyocytes, infiltration of leukocyte cells, myofibril loss and haemorrhages in pericardium. The histopathological findings in the AETP treated groups depicted marked reduction of myocardial injury compared to cyclophosphamide treated group.

In this study, there are some limitations. The effect of CP and AETP on electrocardiographic changes of heart has not been evaluated. Quantitative phytochemical analysis and endogenous antioxidant levels in various treatment groups have not been determined. Effect on other cardiovascular parameters such as blood pressure, heart rate, could have been undertaken. However, the cardiac enzyme markers and histopathology of myocardium provided substantial evidence of cardioprotection offered by the plant extract.

Conclusion

The study showed improvement in the biochemical parameters and histopathological pictures in treated groups with the aqueous extract of *Tagetes patula* flowers support its cardioprotective role. However, further studies are needed to elucidate the exact mechanism of cardioprotection offered by its active phytoconstituents.

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