Effect of Aqueous Extract of Phyllanthus Fraternus Leaf Against Cyclophosphamide Induced Dyslipidemia and Aortitis in Wistar Albino Rats

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ABSTRACT

Introduction: Cyclophosphamide is an alkylating agent used in cancer chemotherapy and immunosuppression. It induces hyperlipidemia and causes damage to the arterial wall that can result in vascular diseases. *Phyllanthus fraternus* is an Indian medicinal plant with good antioxidant content. The present study was done to evaluate the potential role of the plant against cyclophosphamide induced atherosclerotic changes in rats.

Material and Methods: 20 adult Wistar rats were divided into four treatment groups of 5 each. Group I served as normal control receiving *i.p.* injection of 0.9 % normal saline. While group II, III and IV received two doses (100mg /kg b/w) of cyclophosphamide through *i.p.* injection on day 1 and 5. Group III and IV received 200mg/kg and 400mg/kg of aqueous extract of *Phyllanthus fraternus* daily p.o. for 10 days. Blood samples were collected on 11th day for estimation of Triglycerides, Total cholesterol and HDL-choslesterol. Histological sections of aorta were made.

Results: The lipid profile showed significant improvement in the extract treated groups III and IV compared to cyclophosphamide control group II after 10 days treatment. However, histological analysis revealed variable reversal of the damage.

Conclusion: Extract of *Phyllanthus fraternus* showed ameliorative effects on vascular complications caused by Cyclophosphamide in rats.

Keywords: Aorta, Cyclophosphamide, Lipid profile, *Phyllanthus fraternus*.

INTRODUCTION

Cyclophosphamide (CP) is an alkylating anticancer agent widely used clinically in cancer chemotherapy of leukemias, multiple myeloma, lymphomas and solid organ tumours, immunosuppressive agents in rheumatic arthritis, lupus and in preparation for bone marrow transplantation.[1,2] It is a prodrug metabolised to active radicals by liver microsomal cytochrome P450 mixed function oxidase.[3] Apart from high tumor selectivity, it also has a number of toxic effects.[4] CP is a genotoxic agent as its metabolites cause DNA damage and apoptotic cell death.[5,6] Acrolein, a metabolite of CP, has been known to initiate a form of chemical atherogenesis by inducing oxidative stress and damage to the vessel wall endothelium and smooth muscles.[7] Hyperlipidemia is known as a risk factor for atherosclerosis, which is a major cause of cardiovascular morbidity and premature death globally.[8] Cholesterol and lipoprotein levels have a direct link with vascular diseases.[9] Several studies have established that CP chemotherapy induces hyperlipidemia and atherosclerosis. Hypercholesterolemia, hypertriglyceridemia and impaired secretion of heart lipoprotein lipase were reported in CP treated rabbits.[10,11]

Phyllanthus fraternus is a weed and a medicinal herb widely distributed in most tropical and subtropical countries. The plant belongs to Euphorbiaceae family.[12] In India, it is known as 'Bhumyamlaki' in Ayurveda and is widely used in traditional herbal medicine for treatment of various diseases like hepatitis, infections, fever, asthma, etc. Phytochemical analysis of plant extract contains alkaloids, tannins, saponin, terpenoid and steroid which are medicinally important bioactive compounds.[13,14] Aerial part of the plant shows greater antioxidant property by virtue of its higher polyphenolic content.[15]

The study was conducted to see whether aqueous extract of *Phyllanthus fraternus* (AEPF) could protect against the dyslipidemia and damaging effects on aorta wall by the active metabolites of cyclophosphamide.

MATERIAL AND METHODS

Drugs and chemicals

Cyclophosphamide (CYPHOS™) was purchased from Getwell Pharmaceuticals, Haryana, India. Biochemical estimation and analyzing kits (BeneSphera™) were obtained from Avantor Performance Materials India Ltd, Uttarakhand. All other chemicals and solvents used were of highest purity and analytical grade. Standard pellet diets were procured from Amricon Agrovet Pvt. Ltd. marketed as Amrit feeds.

Plant materials

The fresh plant of *P. fraternus* were harvested from the Lamphel area, Imphal in the month of August. They were identified and authenticated by Professor P. K. Singh of the Life Sciences Department, Manipur University, Imphal. A voucher sample is kept in the University herbarium for reference (Voucher no. 000874).

Preparation of extract

The plant leaves were cleansed and dried under shade, powdered by mixer grinder and stored in airtight container for future use. Preparation of aqueous extract was done by the method described by Verma SCL and Agrawal SL.[16] The powdered

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leaves of *Phyllanthus fraternus* were extracted using soxhlet apparatus. The greenish brown extract obtained was filtered, evaporated, shade-dried, scraped out, weighed and stored in glazed porcelain jar for use in the experiment. The yield was 13.5%.

Phytochemical Studies

The AEPF was subjected to preliminary qualitative phytochemical standard screening tests and the phytoconstituents like steroids, alkaloids, flavonoids, tannins and saponins were found to be present.

Acute Toxicity Studies

Acute toxicity studies were conducted as per OECD guideline by 425 method.[17] The mice did not show any mortality at the dose of 2000 mg/kg and hence its 1/10th dose i.e., 200 mg/kg and 1/5th dose i.e., 400 mg/kg were used as the therapeutic doses in the study.

Experimental protocol

Prior approval from the Institutional Animal Ethics Committee (RegNo: 1596/GO/a/12/CPCSEA) was obtained for the study. Wistar albino rats of either sex (140- 210 g) procured from the Animal House, RIMS, Imphal, India were used for the study. Animals were fed with standard pelleted diet and water was provided ad libitum. The rats were housed under conditions of controlled temperature (21 ± 2°C) and acclimatized to 12h light:dark cycle. Animal experiments were conducted according to the guidelines of Institutional Animal Ethics Committee. Rats were divided into four groups, each consisting of five animals, a total of 20 rats. Group I served as normal control receiving *i.p.* injection of 0.5ml of 0.9 % normal saline. While group II, III and IV received two doses (100mg /kg b/w) of cyclophosphamide through *i.p.* injection on day 1 and 5. Groups I and II received p.o. 0.5ml of 2% gum acacia suspension while Groups III and IV were fed 200mg/kg and 400mg/kg of AEPF daily P.O. for 10 days. Blood samples were collected on 11th day from retro-orbital venous sinus by capillary puncture after ether anesthesia. Serum was separated by centrifugation and used in biochemical estimation of Triglycerides, Total cholesterol and HDL-choslesterol.

Sacrifice of the animals

At the end of the experimental period, all the animals were physically observed and euthanized with high dose ether. A midline incision was performed at the thoracic region. The vessels (aorta and pulmonary trunk) were dissected out, fixed in 10% neutral buffered formalin for 48 hours.

Biochemical estimation

Serum cholesterol was estimated using CHOD/PAP method[18] and Triglyceride by GPO/PAP method.[19] HDL-cholesterol required prior precipitation using polyethylene glycol.[20] These methods use direct colorimetry for quantitation.

The atherogenic indices were calculated as described by Ikewuchi and Ikewuchi[21,22] using the following formulae:

Cardiac Risk Ratio = Total Cholesterol ÷ HDL-Cholesterol

Percentage of protection were determined based on the formula described by Dhandapani et al.[23]

Protection (%) = [(AI of experimental control - AI of treated group) ÷ AI of experimental control] × 100

CP treated group was taken as experimental control group.

STATISTICAL ANALYSIS

The results were expressed as mean±standard deviation (S.D.) for five animals in each group. Differences between groups were assessed by one way analysis of variance (ANOVA) using the SPSS 21 software package for Windows. Post hoc testing was performed with Tukey for inter-group comparisons; $P<0.05$ was considered significant for all comparisons.

RESULTS

Intraperitoneal administration of CP resulted in a significant decrease ($P<0.05$) in the weights of the Negative control group II rats compared to Normal group I that showed increase at the end of the experiment (Table-1). AEPF treatment groups III and IV showed lesser weight loss compared with CP only treated group ($P<0.05$).

The serum levels of triglycerides and total cholesterols (TC) in CP treated rats were significantly increased ($P<0.05$) when compared to controls (Table-2). In contrast to this, a significant decrease ($P<0.05$) in the levels of HDL-cholesterol (HDL-C) was observed in group II animals. Supplementation of AEPF to group III and IV animals prevented the increase in serum lipid levels emphasizing its hypolipidemic role. The extract treated groups showed increasing HDL-C levels and thereby counteracted the abnormal dyslipidemia and restored the levels of serum lipid to near normal.

Atherogenic index (Cardiac risk ratios) values of TC/HDL-C ratio also significantly increased ($P<0.05$) in CP group as compared to normal control rats. Extract treatment lowered down the ratios to near normal value. The protection percentage

Groups	Initial Wt (gm)	Final Wt (gm)	Wt Difference (gm)
I	174.60 ± 21.40	190.80 ± 19.00	16.20 ± 4.54
II	182.00 ± 24.60$^{n.s}$	126.25 ± 20.61*	-55.75 ± 6.29*
III	182.50 ± 16.05$^{n.s}$	141.00 ± 18.70*	-41.50 ± 18.28*
IV	179.00 ± 23.12$^{n.s}$	149.75 ± 25.53	-29.25 ± 5.37$^{*\#}$

Table-1: Changes in the body weight of normal, negative and treated groups of rats.

Groups	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)
I	109.41 ± 7.89	81.81 ± 9.09	42.80 ± 2.18
II	191.17 ± 24.72*	206.81 ± 8.7*	16.34 ± 7.41*
III	179.70 ± 19.43$^{*\#}$	136.36 ± 12.85$^{*\#}$	26.25 ± 2.84$^{*\#}$
IV	138.23 ± 7.59$^{\#}$†	122.72 ± 11.73$^{*\#}$	35.41 ± 4.17$^{\#}$

Table-2: Effect of aqueous extracts of *Phyllanthus fraternus leaves* on lipid profile in cyclophosphamide induced hyperlipidemic rats after 10 days treatment.

Groups	Atherogenic Index (Cardiac risk ratio)	Protection percentage (%)
I	1.91	--
II	12.65*	--
III	5.19#	58.97
IV	3.47#	72.57

Values are expressed as mean±S.D. (n=5) One way ANOVA (SPSS 21), n.s = not significantly different, *$P < 0.05$ with respect to Normal gr; #$P < 0.05$ with respect to CP gr; †$P < 0.05$ with respect to III gr

(N.B: **Group I** – Control group administered distilled water, **Group II** – CP toxic group administered CP @ 100 mg/ kg b/w i.p. injection on day 1 and 5, **Group III and IV**– Test 1 and 2 groups administered CP as in group 2 along with AEPF @ 200 and 400 mg/ kg body wt. respectively for 10 days)

Table-3: Comparision of Atherogenic index groupwise and estimated protection percentage.

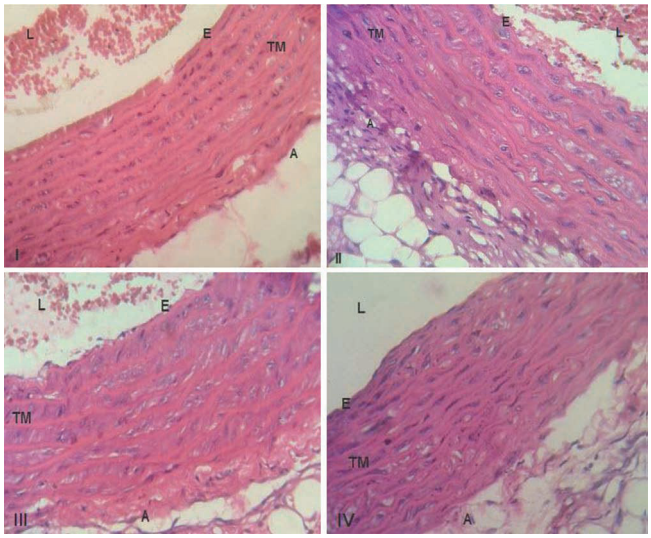

Figure-1: Photomicrograph of the transverse section of aorta of rats depicting effects of CP on aorta wall histopathological features of cyclophosphamide control (II) and aqueous extract of Phyllanthus fraternus leaves treated (200 and 400mg/kg) (III and IV) rats after 10 days of treatment. Note the normal alignment of the three tunicas (L= Lumen, E= Endothelium, Tunica intima, TM=Tunica media, A=Tunica adventicia) in the aorta of normal control (I). H and E x40 magnification.

showed favourable increment dose dependently.

Normal rat aorta histology showed distinct and intact layers of the vessel wall. Aortic section of group II CP treated rats showed endothelial cell layer disruption in tunica intima, cytoplasmic vacuolations in smooth muscle cells of tunica media and minor increase in aorta wall thickness. Extract treated groups showed lesser damaging features compared with the negative control rats.

DISCUSSION

Hyperlipidemia or dyslipidaemia is the presence of elevated or abnormal levels of lipids and lipoproteins in the blood. Serum lipids and lipoproteins abnormalities are regarded as a highly modifiable risk factor for cardiovascular diseases.

Among the small animal models of atherosclerosis study, rats served as a potentially useful *in-vivo* model for evaluating hypercholesterolemia and hypertension. Rats are generally resistant to the development of vascular atherosclerotic changes.[24] The lipid laden sclerotic lesions in arteries of rats are assumed to be residual lesions following an acute arterial inflammation.[25] They developed augmented thrombosis and atherosclerotic lesions under hypertensive and hyperlipidemic conditions.[26-28] The sclerotic lesions in these animals showed good correlation in elevated plasma cholesterol level.[29] However, rats lack many pathophysiological resemblance with humans that are important clinically.[30]

CP is known to reduce body weight due to intensive antimitotic activity and increase the rate of apoptosis.[31] CP treated rats showed significant weight loss compared to normal rats which showed increment at the end of experiment. AEPF treated groups III and IV also showed weight loss but to a lesser extent than CP only treated group. This may be probably due to anti-apoptotic effects of phytochemicals contained in AEPF.

Free radicals of CP may cause cellular cholesterol deposition by increasing cholesterol ester biosynthesis, decreasing cholesteryl ester hydrolysis and reducing cholesterol efflux.[32] Hence, rise in serum cholesterol levels is partly due to increase in biosynthesis and decrease in its utilization. Increase in serum triglyceride levels could be due to alterations in lipoprotein lipase activity.[11] Hyperlipidemia, dyslipidemia and vessel wall damage can lead to the development of atherosclerotic lesions.

From the results, it was observed that CP treatment induced hypertriglyceridemia, hypercholesterolemia and lower HDL-cholesterol level resulting in hyperlipidemic and dyslipidemic changes. Atherogenic index (Cardiac risk ratios) values of TC/ HDL-C ratio also increased in CP control rats compared to normal control rats. When the rats were supplemented with the two different doses of AEPF, the elevated levels of triglycerides, total cholesterol and atherogenic index have shown considerable decline. This decline was more detectable with higher dose of AEPF. Moreover, serum HDL-C level of extract treated animals was increased compared to negative control rats with CP only. Elevated HDL-C level is considered as protective against atherosclerosis as it facilitates reverse cholesterol transport back to liver to secrete into bile acids. This reduction in levels of cholesterol and triglyceride confirms the antihyperlipidemic action of AEPF and is responsible to alleviate CP induced lipid profile derangements.

Histopathologic findings in this study supported that chemotherapy with cyclophosphamide causes endothelial cell layer disruption, vacuolations of tunica media and thickening of the vessel wall could form atherosclerotic plaques that can block the blood flow. This anatomical derangements observed in the negative control group is caused by acrolein, an active metabolite of CP as it directly injures the endothelial and smooth muscle membrane of aorta resulting in the loss of function and integrity. AEPF ameliorates this damaging effect on aorta as evident from the study and suggests its vascular protective effect although total restoration to non-disease state could not be achieved.

Phytosterols, flavonoids, triterpenoids and tannins present in AEPF have antioxidant properties. Quercetin, a flavonoid has been reported for its lipid-lowering properties.[33] In the present study, lipid staining of aortic histology section was not used to analyse the result. So the amount of lipid accumulated cannot be commented. Antioxidant content of aorta tissue also was not determined. These will need further evaluation.

CONCLUSION

CP treatment causes hyperlipidemia, oxidative stress and tissue damage in the aorta wall. Administration of aqueous extract of *P. fraternus* partially reverses the CP induced hyperlipidemia and aortitis in dose dependent manner. Biochemical and histopathological studies confirm the protective role of AEPF. Thus, the present study showed that the AEPF has a promising ameliorative effects on vascular complications caused by CP in rats.

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