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Protective Effects Of Phyllanthus Emblica In Cyclophosphamide Induced Genetic Damage In *In Vitro* Human Lymphocytes

Zoology

KEYWORDS: flood hazard map, morphometric analysis, Karuvannur River

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ABSTRACT

Cyclophosphamide (CP) is a commonly used chemotherapeutic and immunosuppressive agent which is used in the treatment of wide range of cancers and autoimmune diseases. Besides that it is a well known carcinogen. In this study by using chromosomal aberrations (CA) s method, the modulatory effects exerted by the extract of phyllanthus emblica against the CP induced genotoxicity in the human lymphocyte various doses of PFE was used i.e 33, 78,114 ug/ml in human lymphocyte cultures. PFE has not induced any cytogenetic damage in human lymphocyte cell cultures. Hence it is found to be non mutagenic. However when cell cultures were treated with 100 µg/ml of CP induced the significant increase in the percentage of chromosomal aberrations but when primed with PFE to cell cultures there was a significant decrease in the frequency of chromosomal aberrations in invitro human lymphocytes. Thus the data clearly indicate that amla is a safer plant extract to be used along with CP in chemotherapy regimen.

Introduction:

A number of antineoplastic drugs are in common use to combat various types of cancers. These are shown to be mutagenic in different test systems and these antineoplastic drugs such as Cyclophosphamide, Cisplatin, Tamoxifen, Gemcitabine and Paclitxel etc., have shown clastogenic effects in various test systems. Potential genetic damage due to drugs and other chemicals is well recognized. Extensive studies have been carried out on mutagenicity of various drugs in microorganisms, insects, mammals and in exposed population [1-3]Cyclophosphamide (CPM) is a well-known bifunctional alkylating agent, widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated [4] It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythromatosis, multiple sclerosis, and other benign diseases [5,6]. According to the International Agency for Research on Cancer (IARC), CPM is widely used as reference mutagen and has been classified as carcinogenic for animals and humans [7].

According to believe in ancient Indian mythology, *Phyllanthus emblica* is the first tree to be created in the universe. It belongs to family Euphorbiaceae. It is also named as Amla, *phyllanthus Emblica* or Indian gooseberry. The species is native to India and also grows in tropical and subtropical regions including Pakistan, Uzbekistan, Srilanka, South East Asia, China and Malaysia. The fruits of PF are widely used in the Aryuveda and are believed to increase defense against diseases. It has its beneficial role in cancer, diabetes, liver, heart trouble, ulcer, anemia and various other diseases. Diet can modify the pathological processes, because certain naturally occurring substances known as antioxidants are present in plants and other sources have shown to be protective against mutagens or carcinogens or endogenous mutagens [9] Among the various phytonutrients, phyllanthus emblica posses good antioxidants. It was described in Indian Ayurvedic literature more than 200 years ago. It has been widely used by traditional medical practitioners for the treatment of various diseases. It exhibits many properties like antiviral, antimutagenic, hepato protective activity, hypoglycemic activity etc [10-13].

In the present investigation the protective effects of Phyllanthus embilica against cyclophosphamide induced cytogenetic damage were stidied in in vitro human lymphocytes.

MATERIALS AND METHODS: CHEMICALS;

PFE Extract preparation:

Cameron and Puling [14] suggested the daily intake of vitamin C is 1-10g/day for human being. Data based on maximum ascorbate concentrations in human body suggest a maximum body pool of around 5000mg, which is approximately 70mg/kg body weight in man In the present study, a corresponding amount of an aqueous extract of PFE containing the same amount of vitamin C was used for mice, as calculated from daily 1 g intake for a 60kg person. The fruits were procured in bulk, cut into pieces and dried in sunlight. Known quantities weighed and kept in distilled water for 24hr. The AA content of the decoction was estimated b the 2, 6-dichlorophenol indophenol method [15] and it amounted to 685mg/kg body weight.

Materials and Methods

Chemicals: RPMI 1640 medium, new born fetal calf serum and phytohaemagglutinin – M were purchased from Gibco. 5 – Bromo – 2 – deoxyuridine and Hoechst 33258 stain (40 µg ml -1) from Sigma Aldrich, Colchicine from Loba-Chemie, 3% Giemsa stain solution in phosphate buffer (pH 6.8) from E. Merck, India, cyclophosphamide (cycloxan) from Biochem India were obtained. The garlic extract containing 2% allicin was purchased from Unicorn natural products. All other chemicals used were of analytical grade.

Dosage schedule: In the present study two experiments were conducted. Various doses of phyllanthus emblica and cyclophosphamide was added to the cell cultures in sterile conditions.

Group I : Controls with 0.5ml of physiological saline.
Group II: 38 g/ml PFE extract
Group III: 76 g/ml PFE extract
Group IV: 114 g/ml PFE extract

In the second experiment for modulation studies all the three groups as follows:
Group I : controls with 0.5ml of physiological saline.
Group II: Cyclophosphamide 100 µg/kg
Group III: CP 100+ 38 g/ml PFE extract
Group IV: CP 100+76 g/ml PFE extract
Group V: CP 100+114 g/ml PFE extract

Analysis of chromosomal aberrations in Human Lymphocytes:

Intravenous blood of healthy human adults was collected aseptically using an anticoagulant heparin. Lymphocyte cultures were prepared

and harvested according to the standard method prescribed by Moorhead *et al.,* [16]. Lymphocyte cultures were initiated in RPMI 1640 medium containing 25% human AB serum, 0.5% PHA & 0.25% antibiotic Dicrysticin from Sigma Aldrich. The concentration of CP was150 µg/ml and the ascorbic concentrations were calculated based on therapeutic dose. The doses were 3.33µg/ml, 8.33µg/ml and 16.33µg/ml. For priming experiments 150+3.33, 150+8.33 and 150+16.33 µg/ml were added to the cell cultures and at an interval of &Simultaneously the control group cultures were also maintained. The cultures were grown at 37°C for 72 hrs. Later all cultures were terminated by adding colchicine (0.02%) to arrest the cell cycle at metaphase. After 2 h. of treatment the material was centrifuged at 1000 rpm for 10 min. The cells were treated with prewarmed hypotonic solution (KCI 0.08 M) for 20 min. After hypotonic treatment the cultures were centrifuged and the supernatant was removed, cells were fixed by adding chilled fixative (Methanol: Acetic acid 3:1). Later the slides were examined for various types of chromosomal aberrations such as chromatid gaps, breaks, acentric fragments. Isochromatid gaps, breaks and polyploids. Metaphases were scored per culture and the data was analyzed statistically using 2x2 contingency Chi-square test.

Results:

The results showed that the percentage of chromosomal aberration at 24 hrs treatment was increased from 2.00 in control to 1.25, 2.5 and 3.75 in cultures treated with 38, 76 and 114 g/ml Phyllanthus fruit extract (Table 51, 52). Similarly at 48 hrs the frequency of chromosomal aberrations in cultures treated with 38, 76 and 114 g/ml Phyllanthus fruit extract as 2, 3.25 and 3.75 as against 2.25 in control cultures. At 72 hrs treatment the frequencies of chromosomal aberrations were 1.5 in control cultures and it has increased to 4.0, 4.0 and 5.00 in 38, 76 and 114 g/ml Phyllanthus fruit extract treated cultures (Table-1). The overall incidences of frequencies were found to be statistically significant at all dose levels and all time intervals. (P>0.05, Table-1).

In the present study, the frequency of total chromosomal aberrations of Cyclophosphamide + Phyllanthus fruit extract treated in vitro human lymphocyte cultures were 2.00 in control 8.2 in control and 100 g/ml of cycophosphamide treated cultures where as it decrease to 4.80, 4.20 and 2.20 was observed in cyclophosphamide + Phyllanthus fruit extract primed cultures i.e. 100+38, 100+76 and 100+114 g/ml respectively at 24 hrs exposure (Table 56-58). Similarly the total number of aberrations were 10.4 in mitomycin C cultures. Similarly at 48hrs exposure, the frequency of total chromosomal aberrations of Cyclophosphamide + Phyllanthus fruit extract treated invitor human lymphocytes cultures were 1.60 and 7.8 in control and 100 g/ml cyclophosphamide treated cultures where as a decrease to 5, 4.4 and 2.6 was observed in Cyclophosphamide + Phyllanthus fruit extract primed cultures i.e., 100+38, 100+76 and 100+114 g/ml respectively at 48 hrs exposure. Similarly the total number of aberrations were 11.2 in mitomycin C' cultures. The frequency of total chromosomal aberrations at 72 hrs of Cyclophosphamide + Phyllanthus fruit extract treated invitor human lymphocytes cultures were 1.80 and 7.60 in control and 100 g/ml cyclophosphamide treated cultures where as a decrease to 5.40, 5 and 3 was observed in cyclophosphamide + Phyllanthus fruit extract primed cultures i.e., 100+38, 100+76 and 100+114 g/ml respectively at 48 hrs exposure. Similarly the total number of aberrations were 10.2 in mitomycin C' cultures (Table 2). The data was found to be statistically significant (p<0.05 Table – 2).

DISCUSSION

For studying cytogenetic effects induced by suspected agent in human beings the suitable method adopted is the micro culturing of human peripheral blood lymphocytes. It is an essentially important and sensitive indicator for both in vivo induced structural and numerical chromosomal aberrations. But in vitro studies play an important role than in vivo, test systems have all the advantages of the experimental situation and are valuable tools for obtaining dose response information and relation between the agent and cell cycle. The advantage of this method is that cell cycle is well defined and easy to locate the divisional stages, which are more sensitive to the action of the test compounds 17.

Among the alkylating agents used for the treatment of wide range of cancers, CP is one of the widely used drugs. Acrolein and phosphoramide are the active compounds of CP. These active compounds of the CP slow down the growth of cancerous cells by interfering with the actions of DNA within those cells. The mutagenicity of CP in particular is related to formation of the ultimate cytotoxic metabolite phosphoramide mustard through the intermediate agentsine C cause gene mutations, CA and rearrangements and aneuploidy in somatic cells as well as an increased frequency of secondary treatment-related tumors in human cancer survivors18,19. Hence the development of effective modulatory hydroxycyclophosphamide and deschloroethylcyclophosphamide20,21 which is capable of inducing DNA crosslinks and strand lesions 22. It has been tested extensively for its genotoxic effects both in vitro and in vivo in different test systems giving consistently positive results23. Several lines of studies have demonstrated that the CP and many other chemotherapeutic agentscause gene mutations, CA and rearrangements and aneuploidy in somatic cells as well as an increased frequency of secondary treatment-related tumors in human cancer survivors18,19. Hence the development of effective modulatory strategies for CP induced toxicity will be of great importance for the chemotherapy for cancer.

During the recent years much focus has been given for the search for natural compounds which modulates the drug/chemical induced toxicity24-27. Several studies have clearly established the protective effects of various phytonutrients upon drug-induced toxicity28-31.

Phyllanthus emblica enjoyed a hallow position in Ayurveda an Indian system o medicine. It is a first tree to be created in the universe. Its fruit juice contains highest vitamin C contains as 478.56mg/100ml. It is used in the preparation of Indian pickles. The fruit when blended with other fruits boosted their nutritional quality in terms of vitamin C content. It is often used as Triphala which is a herbal formulation containing fruit of Terminalia chebula and Terminlia belerica in equal proportions. It has important medicinal value against various diseases. In vitro and in vivo animal studies suggested wide range of potential therapeutic or preventive effects has been reported. Such effects in humans have not conformed so far. PFE when prepare in the Triphala delayed the development of fore stomach Papillomagness, breast cancer, skin tumors, liver fibrosis, diabetic cataract, Alzheimer's diseases. 32-36

The present results are comparable with the reports of other investigators. When cadmium chloride administered orally 3mg/kg in a single dose, co-treatment with phyllanthus fruit extract at dose of 500mg/kg showed decreased mortality in rats. Further there are histopathological changes reduced peroxidation in liver, kidney and testis after acute cadmium exposure 37. The protective effects of phyllanthus fruit extract aganist adriamycin and chromium induced genotoxicity in bone marrow cells of mice has been reported 38,39. Earlier the protective effect of garlic extract against cyclophosphamide induced genotoxicity has been reported17. In another study, chromium (VI) induced apoptosis, DNA fragmentation and immunosuppressive effects on lymphocyte proliferation has been ameliorated following treatment with amla and it also restored the altered levels IL-2 and -IFN40.

In the present study pretreatment of phyllanthus fruit extract was shown to be more effective in reducing the genotoxicity of cyclophosphamide. The protective nature of phyllanthus emblica is because of presence of Vitamin C, tannins, polyphenolic compounds and elligic acid 41 .Ascorbic acid (vitamin c) polyphenolic compounds such as elligic and tannic acids are inhibitors and blocking agents aganist carcinogens on direct acting N- Nitroso compounds. Elligic acid protects DNA attack of electrophyilic

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species of free radicals by binding to nitcleophyilic sites 42,43 Earlier the protective effects of phyllanthus emblica against Chromium.

Table -1: Frequency of Chromosomal aberrations recorded in invitro human peripheral lymphocytes with various doses of Phyllanthus fruit extract for 24, 48 and 72 hrs interval.

Dose (µg/ml) and duration of treat	24hrs	48 hr	72 hr			
	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)
Control	392	8	395	5	394	6
	(98.00)	(2.0)	(98.75)	(2.25)	(98.5)	(1.5)
38µg/ml	395	5	392	8	389	11
	(98.75)	(1.25)*	(98.0)	(2.0)*	(97.25)	(4.0)*
76 µg /ml	390	10	387	13	384	16
	(97.50)	(2.5)*	(96.75)	(3.25)*	(96.00)	(4.0)*
114µg ml	385	15	385	15	380	20
	(96.25)	(3.75)*	(96.25)	(3.75)*	(95.00)	(5.00)*

P<0.05

Table -2: Frequency of Chromosomal aberrations recorded in invitro human peripheral lymphocytes analysed after 24, 48 and 72 hrs in cycophosphamide treated cultures primed with Phyllanthus fruit extract

Dose Time	Non primed	Primed with **Phyllanthus fruit extract**						
		3 8 µg/ml	76 µg/ml	114 µg/ml				
	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)
24 hours								
Control	490 (98.00)	10 (2.00)						
Mitomycin	448 (89.6)	52 (10.40)*						
100 µg/ml	459 (92.80)	41 (8.20)*	477 (95.20)	24 (4.80)*	479 (95.80	21 (4.20)	489 (97.8	11 (2.20)
48 hours								
Control	492 (98.40)	8 (1.60)						
Mitomycin	444 (88.80)	56 (11.20)*						
100 µg/ml	461 (92.20)	39 (7.80)*	475 (95.00)	25 (5.00)*	478 (95.60)	22 (4.40)*	487 (97.40)	13 (2.60)*
72 hours								
Control	491 (98.2)	9(1.80)						
Mitomycin	449 (89.80)	51 (10.20)*						
100 µg/ml	462 (92.40)	38 (7.60)*	473 (94.60)	27 (5.40)*	485 (95.0)	20 (5.00)*	485 (97.00)	15 (3.00)*

CONCLUSIONS

At this point of time more investigation is needed to delineate the down regulation pathways of modulatory actions of extract of phyllanthus emblica and which component of it is exerting effect on CP induced genotoxicity in human lymphocytes in in vitro. We here predict that this finding of ours give the directions for the future research possibilities for the design and development of PFE related modulatory drugs in combination with the CP. Such drugs might minimize the side effects caused by the widely used chemotherapeutic agent CP.

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