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Protective Effect of Esculetin against Cyclophosphamide Induced Chromosomal Aberration, Micronuclei Formation and Oxidative Stress in Swiss Albino Mice

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ABSTRACT

Escueltin is a coumarin derivative with wide range of biological activity. In the present study we investigated the protective effects of esculetin against cyclophosphamide induced oxidative stress and DNA damage**.** Following parameters were evaluated: (a) chromosomal aberration and mitotic index; (b) micronuclei formation and polychromatic erythrocyte frequency, and (c) malondialdehyde, glutathione and superoxide dismutase levels in liver homogenates. CP (50 mg/kg intraperitopeanlly) treatment significantly increased the different types of aberrant cells and micronuclei formation in bone marrow cells of mice. It also increase the lipid peroxidation and decreased glutathione and superoxide dismutase activity in liver. Whereas, pretreatment with esculetin (50, 75 and 100 mg/kg, per orally) alleviated aberrations in chromosome and lessened micronuclei formation. Esculetin pretreatment also shielded the liver of mice against cyclophosphamide induced oxidative stress as the levels of oxidative stress markers where near normal levels. Protective effect of esculetin correlated well with its genoprotective activity. It can be concluded that esculetin offsets cyclophosphamide induced oxidative stress and resulting DNA damage and can be useful as a chemopreventive agent against cyclophosphamide induced toxicity.

Keywords: Esculetin, cyclophosphamide, oxidative stress, chromosomal aberration and micronuclei formation.

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INTRODUCTION

Cyclophosphamide (CP) is a cytotoxic bifunctional alkylating agents belonging to nitrogen mustards class. It is widely used in the treatment of various malignant and non-malignant tumors. It is also used in organ transplant rejection and autoimmune diseases due to its immunosuppressant activity[1-3]. CP has been classified as known human carcinogen by the International Agency for Research on Cancer (IARC)[4]. Acrolein, a metabolite of CP, is responsible for its carcinogenic activity. Acrolein causes damage to normal cell DNA and toxicities to various target organs by inducing oxidative stress[5].

Esculetin (ESC), a coumarin derivative, is contained in plants like Artemisia scoparia, Artemisia capillaries (Compositae), Ceratostiggmawillmottianum (Plumbaginacease), and in leaves of Citrus limonia (Rutaceae)[6-7]. ESC (6,7-dihydroxycoumarin) has shown a wide range of pharmacological activities[8]. These activities are: ESC decrease the activity of ferric soybean lipoxygenase 1[9] and 5 lipoxygenase[10], inhibits xanthine oxidase activity[11] inhibits the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism; and protects DNA against oxidative stress[12], promotes analgesic[13], immunomodulatory[14] and anti-tumoral effects in hepatic, oral, leukemia and lung[15-18], decreases neutrophil infiltration[19], subepithelial fibrosis and TGF-1 levels in the lung[20]; inhibits synthesis of leukotriene B4, thromboxane B2[21], platelet aggregation[22], matrix metalloproteinases production[23-24], growth of human leukemia cells[25] and the production of IL-6 and IL-8[26]; and anti-inflammatory[27].

Effective cancer chemotherapy as well as immunosuppressive therapy with CP is severely limited due to its unwanted toxicity to normal tissue. Thus, it is necessary to defend normal cell DNA from cyclophosphamide induced damage for improving clinical efficacy of cyclophosphamide. In the present study, we have made an attempt to evaluate the beneficial effects of ESC against CP-induced mutagenesis and oxidative stress in the bone marrow cells.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (20-25g) were procured from Central Animal Facility, Gauhati Medical College and Hospital (GMCH), Guwahati, Assam. Animals were divided into 6 groups of ten mice each. The animals were kept at room temperature (22±2^{o} C), and controlled cycle of 12h light and 12h dark. All the animals were given standard mice feed pellets and tap water *ad libitum*. The animals were acclimatized to the experimental conditions for one week before the commencement of the experiment. The study protocol was approved by the Institutional Animal

Ethics Committee of GMCH, Assam (CPCSEA Registration No. 351, 3/1/2001). The study was performed in accordance to the Indian National Science Academy Guidelines for the care and use of animals in scientific research.

Chemicals

Cyclophosphamide (CAS no. 6055-19-2), 6,7-dihydroxycoumarin (Esculetin) (CAS no. 305-01-1),2-thiobarbituric acid (CAS no. 504-17-6), 1,1,3,3-tetramethoxy propane (CAS no. 102-52-3),5,5'-dithio-bis(2-nitrobenzoic acid) (CAS no. 69-78-3), glutathione reduced (CAS no. 70-18-8) and colchicine (CAS no. 64-86-8) were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO, USA). All other chemicals and reagents were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany).

Experimental Design and animal treatment

Group I and group II animals were given 0.5% CMC per oral (p.o.). Group III, IV and V animals were given ESC 50, 75 and 100 mg/kg body weight (BW) suspended in 0.5% CMC (p.o.), respectively. Group VI animals were also given ESC 100 mg/kg, to check toxicity of ESC on bone marrow cells, if any. All above treatment was given for five days. On the fifth day, after 2 h of last dose, Group II, III, IV and V were given cyclophosphamide 50mg/kg dissolved in normal saline, intraperitoneally (i.p.) and group I and VI were given normal saline (i.p.). After 24 h of the last dose, animals where sacrificed and analysis of different parameters was carried out.

Bone marrow chromosomal aberrations test

Chromosomal aberration test was carried out as described by Preston (1987) with little modification[28]. On sixth day, 2 h prior to the animals sacrifice, five animals in each group were given colchicine 4 mg/kg BW (i.p.), to arrest the metaphase stage. After 2 h animals were sacrificed and both femurs were dissected out from each animal. Cytogenetic analysis was performed as per the protocol of Preston et al. The bone marrow was flushed out from both femurs using hypotonic solution of 0.56% (w/v) KCl and incubated (37 ^{0}C). After centrifugation (1000rpm, 5 min) the supernatant was discarded and the pellet was resuspended in ice-cold cornoy's fluid (3:1, methanol: glacial acetic acid). The suspension was dropped to ice-cold slides (previously kept in 50% alcohol in freezer) using pastures pipette and slides were immediately flamed for few seconds and allowed to dry at room temperature. On drying, slides were stained with phosphate-buffered 5% Giemsa solution. A total of 50 well-spread metaphase plates per animal in each group were analysed for chromosomal aberrations at a magnification of 100X using compound microscope (DM750 Leica Microsystems). The chromosomal aberrations in cells were classified according to the most severe damage which had occurred as chromatid

breaks, fragments, rings and gaps. Gaps were defined as achromatic lesions in one or both chromatids not exceeding the width of a chromatid, and breaks as discontinuities greater than the width of a chromatid, irrespective of whether or not the distal fragment was dislocated. The incidence of aberrant cells was expressed as percentage of damaged cells (aberrant metaphases) in the total population of cells analysed. Gaps were not included in the calculation for incidence of aberrant cells. Mitotic index was also calculated.

Bone marrow micronuclei test

After 24 h of the last dose, five mice from all groups were sacrificed. Immediately after sacrificing, both femurs were dissected from each mice and bone marrow smear were prepared based on technique developed by Schmid (1975)[29]. Briefly, the bone marrow from both femurs was flushed out into centrifuge tubes containing 2 ml of Bovine Serum Albumin (5%) and centrifuged (1000 rpm, 10 min). The pellet was re-suspended in a drop of the supernatant and a smear was made on a grease-free clean slide. Remaining supernatant was discarded. The smear was air-dried and fixed with absolute methanol for 5 min. It was then air-dried and directly stained for 5 min with a freshly prepared 10% working solution of giemsa stain (pH 6.8). After staining the slides were rinsed twice in phosphate buffer (pH 6.8), dried at room temperature and at least 200 PCEs (polychromatic erythrocytes) per animals were scored to determine the the frequency of MNPCEs (MNPCEs/1000PCEs). Another 500 erythrocytes were counted to estimate the frequency of PCEs (PCEs/NCEs).

Oxidative parameters

Estimation of lipid peroxidation: The lipid peroxidation assay was carried out using the method of Ohkawa et al. with some modifications[30]. After the animal was sacrificed, liver was dissected out and was rinsed in ice-cold physiological saline. It was subsequently minced and 10% homogenate was prepared in cold phosphate buffer (pH 7.4). The homogenate was centrifuged (1000xg, 10mins) to yield a pellet that was discarded, and supernatant (S1) containing mainly water, proteins and lipids (cholesterol, galactolipid, individual phospholipids and gangliosides) was kept for lipid peroxidation assay. Briefly the colour reaction was developed by adding 200 µl 8.1%sodium doudecyl sulphate to 100 µl supernatant (S1); this was subsequently followed by the addition of 600µl of acetic acid/HCl (pH3.4) mixture and 600µl 0.8% thiobarbituric acid (TBA). This mixture was incubated at 95^{o}C on a water bath for 60 min. Thiobarbituric acid reactive species (TBARS) produced were measured at 532nm and the absorbance was compared with that of a standard curve using malondialdehyde (MDA).

Estimation of reduced glutathione (GSH): GSH was estimated based on method of Moron et al[31]. The supernatant (S1) obtained from the tissue homogenate was used for estimation of reduced glutathione. The supernatant (40µl) was mixed with 400µl Tris buffer (pH 7.4) and 3360µl water. Then 0.2mL (200µl) DTNB solution was added and absorbance was measured at 412 nm. The interpretation of data was done using the standard curve of GSH, which was prepared using reduced glutathione. The level of GSH was expressed as nmol/mg of protein. Total protein content was estimated by method described by lowry et al[32].

Estimation of SOD: Superoxide dismutase (SOD) Assay kit (Cat. No. CS 19160) supplied by Sigma-Aldrich was used and the manufacture's protocol was used for the quantitative in vitro determination of Superoxide dismutase(SOD) in liver. The method used for estimation was the colorimetric method. The SOD activity was quantified by measuring color development at 450nm.

Statistical Analysis

Results were shown as mean ± SEM for each group. Statistical analysis was performed using Graph Pad Prism 5 statistical software. For multiple comparisons, one-way analysis of variance (ANOVA) was used. In cases where ANOVA showed significant differences, post-hoc analysis was performed with Tukey's test. A, $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In the present study, protective effect of esculetin against CP induced MN formation, chromosomal aberrations and oxidative stress was evaluated. CP is well known agent for cancer chemotherapy and immunosuppressive therapy for organ transplant rejection and autoimmune diseases like systemic lupus erythmatoses, multiple sclerosis, rheumatoid arthritis[33]. Despite its wide spectrum of clinical uses, CPA also possesses a wide spectrum of cytotoxicity to normal cells in humans and experimental animals[34]. The acute toxicities of CPA are associated primarily with its genotoxicity and it is believed to be due to its metabolite acrolein. In somatic cells, CPA has been shown to produce gene mutations, DNA-strand breaks, chromosomal aberrations (CA), micronuclei and sister chromatid exchanges in a variety of cultured cells in the presence of metabolic activation as well as sister chromatid exchanges without metabolic activation[35-37].

Bone marrow chromosomal aberration assay is broadly accepted test to gauge the clastogenic/aneugenic potential of chemicals. CP treatment (50 mg/kg) led to significant increase in incidence of % of aberrant cells (32±0.17) as compared to control (1.5 ± 0.72). Further, CP treatment also significantly decreases the mitotic index (3.3 ± 0.51) as compared to control (5.1 ±

0.42). Pre-treatment with ESC significantly and dose dependently decreased the CP-induced increase in the incidence of % of aberrant cells (Table 1).

Table 1: Protective effect of esculetin (5 days pre-treatment) on CP (100 mg/kg) induced chromosomal aberrations in bone marrow cells of mice.

Group (n=5)	Chromosomal aberrations				Incidence of aberrant cells (%)	Mitotic index (%)
	Gap	Break	Fragments	Others		
I	0.6 ± 0.25	1.1 ± 0.4	1.5 ± 0.3	0 ± 0.0	1.5 ± 0.72	5.1 ± 0.42
II	8.2 ± 0.14	20 ± 0.11	11.6 ± 0.11	2.0 ± 0.3	32±0.17^{***,a}	3.3 ± 0.51^{**,a}
III	2 ± 0.20	12.2 ± 0.45	5.6 ± 0.60	1.0 ± 0.6	19 ±0.86^{**,b}	3.7 ± 0.40
IV	3.8 ± 0.37	6.9 ± 0.25	5.8 ± 0.30	1.2 ± 0.14	12±0.47^{***,b}	3.9 ± 0.42^{*,b}
V	3.2 ± 0.31	5.2 ± 0.13	4.6 ± 0.32	1.0 ± 0.23	9 ± 0.45^{***,b}	4.3 ± 0.36^{**,b}
VI	0.9 ± 0.52	1.2 ± 0.51	1.2 ± 0.345	0 ± 0.0	1.8 ± 0.60	4.8 ± 0.37

All values are expressed as mean ± SEM (n=5), ***P<0.001, **p<0.01,*p<0.05, a vs control, b vs CP 100.

Micronuclei are chromatin masses that arise from chromosome fragments of intact whole chromosomes lagging behind at the anaphase[38]. MN frequencies have been considered to be a reliable index for detecting chromosome breakages and loss[39].Bone marrow MN assay is extensively used tool for the assessment of clastogenic/aneugenic potential of chemicals. In CP (50mg/kg) alone treated animals (group II) significant micronuclei formation in the bone marrow cells was observed as compared to vehicle treated animals (Group I). ESC pre-treatment in group III, IV and V significantly and dose dependently reduced the CP induced increase in micronuclei formation in comparison to CP treated group II. Furthermore, the cytotoxicity induced by CP as assessed by PCEs-to-NCEs ratio in the bone marrow returned to near normal level in dose dependent manner (Table 2).The present in vivo studies showed that pre-treatment of ESC can protect against CP induced mutagenesis in dose-dependent manners as evident from MN assay and chromosomal aberration assay of the bone marrow cells. These results indicate the anti-mutagenic efficacy of ESC.

Table 2: Protective effect of esculetin (5 days pre-treatment) on CP (50mg/kg) induced MN frequency in bone marrow PCEs.

Group (n=5)	Treatment	MNPCEs / 1000 PCEs	PCE / NCE
I	Vehicle	0.5±0.25	1.19±0.02
II	CP 50 mg/kg, i.p.	29.5±0.85^{***,a}	0.72±0.05^{***,a}
III	ESC 50 mg/kg, p.o.+ CP 50 mg/kg, ip	20.36±0.64^{***,b}	0.82±0.07^{**,b}
IV	ESC 75 mg/kg, p.o.+ CP 50 mg/kg, ip	12.64±53^{***,b}	0.91±0.03^{***,b}
V	ESC 100 mg/kg, p.o.+ CP 50 mg/kg, ip	13.80±0.40^{***,b}	0.94±0.05^{***,b}
VI	ESC 50 mg/kg, p.o.	1.0±0.30	1.20±0.06

All values are expressed as mean ± SEM (n=5). ***P<0.001,**p<0.01,*p<0.05, a vs control, b vs CP 50

Acrolein is a highly reactive aldehyde that covalently binds to cellular macromolecules and subsequently disrupts the function and causes organ toxicity[40-41]. Acrolein interferes with the tissue antioxidant defense system, produces highly reactive oxygen free radicals and is mutagenic to mammalian cells[42]. It is detoxified by conjugation with GSH *via* GSTs in hepatocytes and this may cause intracellular GSH depletion and injuries to the hepatocytes[43-44]. CP also decrease SOD activity by either inhibition of enzyme synthesis and/or direct effect of hydrogen peroxide[45-46]. Acrolein and lipid peroxidation product-MDA belongs to carbonyl compounds, which can bind with sulphydryl, amino and carboxyl groups of amino acids, proteins and peptides, and with the primary phosphate, hydroxyl and amino groups of the nucleic acids[47].

To discover whether ESC renders protection against CP induced oxidative, we assessed oxidative markers like lipid peroxidation, SOD level and GSH level in liver homogenates of all treatment groups. CP (50 mg/kg) treatment led to significant increase in malondialdehyde (MDA) level as compared to vehicle control group. ESC (50 and 75 mg/kg) pretreated groups IV and V showed significant restoration in MDA levels in a dose dependent manner as compared to CP alone treated group II (Figure 1). Further, significant reduction in the GSH and SOD levels in the liver were observed in CP (50 mg/kg) treated group as compared to control group. Animals pretreated with ESC (12.5, 25 and 50 mg/kg) showed significant restoration of GSH and SOD levels in liver in a dose-dependent manner compared to CP treated animals (group II) (Figure 2 and 3).These results suggest that protection by ESC may be mediated by modulation through cellular oxidative levels.

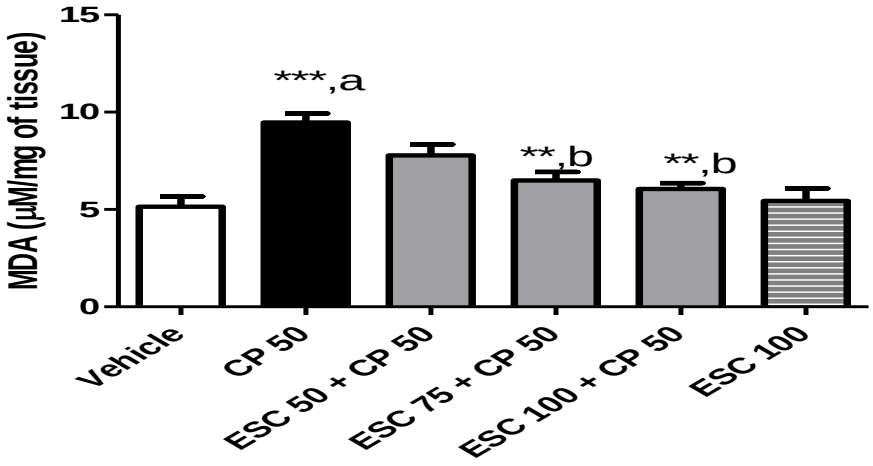

Figure 1: Effect of esculetin (5 day treatment) on MDA level in liver.

All the values are expressed as mean SEM (n=5), ***p<0.001,**p<0.01,*p<0.05, a vs control, b vs CP 50

Recently plant extract of *buchananialanzan*bark[48] and many chemicals like ellagic acid[49], carnosine[50], astaxanthin[51]etc. possessing antioxidant activity have shown to provide protection against cyclophosphamide induced genotoxicity and oxidative stress. In the present study ESC restored the oxidative stress, decreased MN formation and chromosomal aberrations induced by CP in mice.

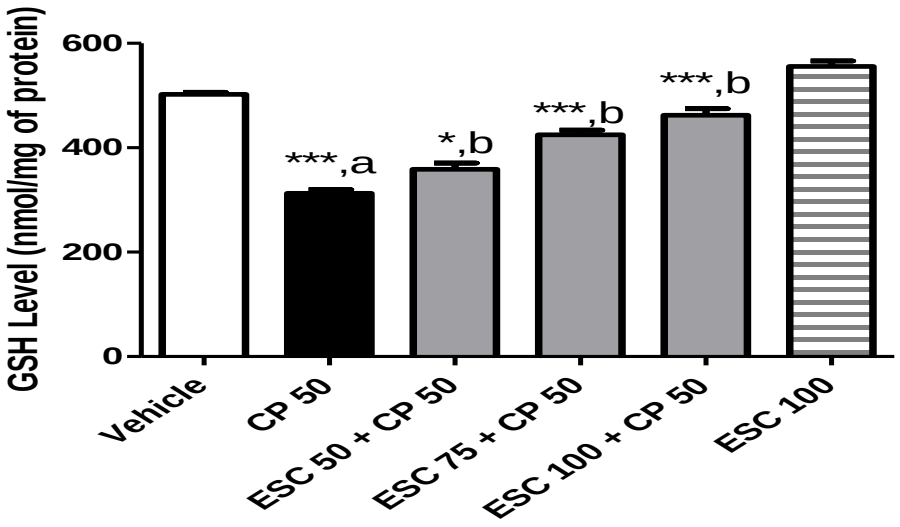

Figure 2: Effect of esculetin (5 day treatment) on GSH level in liver.

All the values are expressed as mean SEM (n=5), ***p<0.001,**p<0.01,*p<0.05, a vs control, b vs CP 50

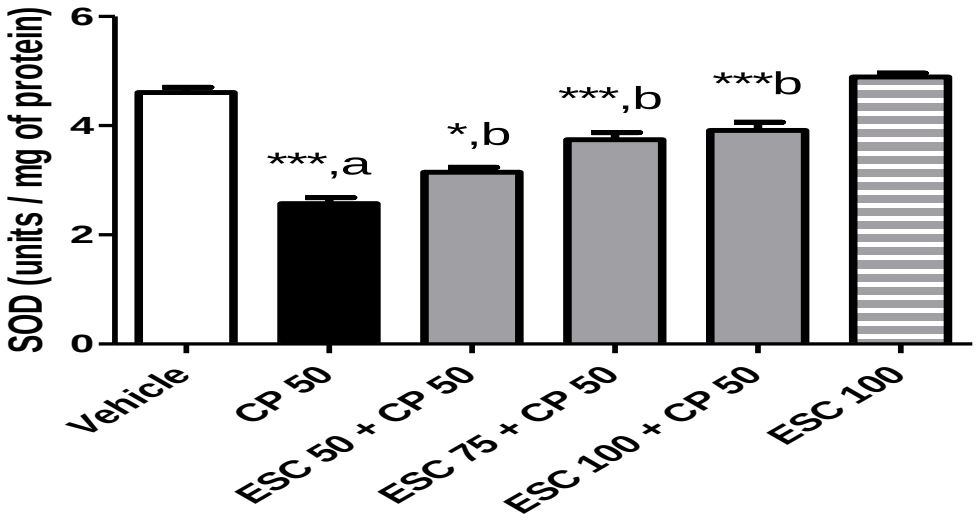

Figure 3: Effect of esculetin (5 day treatment) on SOD level in liver.

All the values are expressed as mean SEM (n=5), ***p<0.001,**p<0.01,*p<0.05, a vs control, b vs CP 50

CONCLUSION

In conclusion, the present study for the first time provides evidence that ESC pre-treatment offsets the CP-induced oxidative stress in liver; and micronuclei formations and chromosomal

aberrations in the bone marrow cells of mice. This anti-genotoxic, antimutagenic potential of ESC might be due to its antioxidant property. However, further studies using other end points with possible mechanistic evidence are required to elucidate the precise mechanism of protection offered by ESC.

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