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Evaluation of antigenotoxic effects of diosgenin in mice exposed to cyclophosphamide

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ABSTRACT

Diosgenin is a steroidal saponin found in a variety of plants including fenugreek and roots of wild yam and is widely used in traditional medicine systems. Diosgenin is shown to have anti-invasive, anti-proliferative and proapoptotic activities on wide range of cancer cells and a well known precursor of various synthetic steroidal drugs that are extensively used in the pharmaceutical industry. The present investigation explores the genotoxic and antigenotoxic properties of diosgenin in experimental mice exposed to cyclophosphamide. CP increases the formation of micronucleated PCEs in the bone marrow of mice significantly and lowers the total WBC. In mice pre-treated with diosgenin, the formation of mnPCEs is lowered in a dose dependent manner and the immune parameters are restored. Diosgenin also reduces the lipid per oxidation levels indicated by MDA assessment and restores the antioxidant GSH in the liver tissues of the mice, counteracting the effects of CP. In our study, Diosgenin did not exhibit inherent genotoxic properties nor had a synergistic effect with CP. These results indicate the potential of diosgenin as an antigenotoxic agent with a possibility to be used as an adjuvant, to counteract the side-effects of chemotherapeutic drugs.

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INTRODUCTION

Environmental pollutants, industrial chemicals and effluents, heavy metals, cytotoxic drugs dyes and colours used for different purposes can have genotoxic effects. Genotoxicity is the ability of an agent to inflict damage on the DNA molecule which in turn can lead to cancer (Olinski *et al.*, 2002; Klaunig and Kamendulis, 2004). Chemopreventive constituents occurring in plant and animal products are known to strengthen our innate defences against the adverse effects of genotoxins and carcinogens (Ramel *et al.*, 1986; Ferguson, 1991; Álvarez-González *et al.*, 2004; Salwa and Lina, 2010). These natural substances act by diverse mechanisms in counteracting the actions of genotoxic agents (De Flora, 1998; Marchand, 2002; Galati and O'Brien, 2004; Heitzman *et al.*, 2005; Collins, 2005). Plants and plant products that have been used in traditional medicine systems, like ayurveda and folk medicine, form the basis for using these natural products or their active constituents in prevention and treatment of diseases (Ribiero and Salvadaro, 2003). In this scenario, it is important to understand the genotoxic and antigenotoxic properties of these natural compounds.

Diosgenin is a naturally occurring steroid saponin found in a variety of plants including fenugreek (*Trigonella foenum graecum*), roots of wild yam (*Dioscorea villosa*), *Solanum incanum* Lloydia, *Solanum xanthocarpum* (Raju and Mehta, 2009) and *Costus speciosus* (Singh *et al.,*2013). Diosgenin and plant extracts containing diosgenin have shown a wide range of biological activities including antioxidant (Mirunalini *et al*.,2011), anticholesterolemic (Kondeva-Burdina *et al*.,2007), antiproliferative (Li *et al.*, 2010), anticancer and antidiabetic (Moalic *et al.*, 2001; Raju and Bird, 2007) effects in experimental models.

The current study aims at studying the genotoxic and antigenotoxic properties of Diosgenin in experimental animals against cyclophosphamide induced toxicity by assessing the formation of micronucleated PCEs, Blood parameters, and levels of lipid peroxidation and reduced glutathione in the liver tissues.

MATERIALS AND METHODS

Chemicals

Cyclophosphamide (CP) was procured from local licensed outlets (Brand name-Uniphos 500, United Biotech, India (Batch No.UPDJ3B7-Oct, 2013). Diosgenin was procured from Natural Remedies Ltd., Bangalore (batch No.T12K015- 2013). All other chemicals and stains used in the assay were of analytical grade.

Animals

Eight week-old healthy Swiss albino mice of either sex, weighing 25±5 g were used. Animals were maintained under conventional laboratory conditions, at temperature 25±2°C, and a 12 h natural light period. Commercial pellet diet and drinking water were provided *ad libitum*.

Micronucleus Assay

Micronucleus test was performed by counting the number of micronuclei present in the bone marrow of control and pretreated mice, according to modified method of Hayashi *et al*., (1994). The mice were formed into five groups, with six animals in each. The various treatments were shown in Table 1.

Before sacrifice the animal's blood samples were collected for the estimation of WBCs, RBCs and hemoglobin content. The animals were sacrificed after 24h of cyclophosphamide administration by cervical dislocation under light anesthesia. The liver samples were collected for the estimation of LPO and GSH. The femur and tibia were also excised. Bone marrow micronucleus slides were prepared and coded to avoid observed bias. Smears were stained for 5 min in May-Grunwald solution and for 10 min in Giemsa respectively. Micronucleus were identified in one form of RBCs i.e., polychromatic erythrocytes. About 1000 PCEs per animals were scanned for the presence of micronucleus. The blood parameters namely total WBC count, RBC and Hemoglobin were measured by standard procedures.

Measurement of LPO

Thiobarbituric acid reactive substance (TBARS) or Malondialdehyde (MDA) in the liver homogenate was estimated by using standard protocol. The level of LPO was determined by using extinction coefficient of MDA-TBA complex which is $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ for calculation of the concentration of MDA and expressed as nmol/g of wet tissue weight (Okhawa *et al*., 1979).

Determination of GSH

Estimation of reduced glutathione in the liver homogenate was based on the formation of a relatively stable yellow product when sulphydryl groups react with 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB). The results were expressed as nmol/g of wet tissue weight (Moron *et al*., 1979).

Statistical analysis

Data were expressed as mean±SEM for each group and subjected to the statistical analysis by one-way ANOVA followed by Tukey's post-test. $p<0.05$ was considered as the level of significance.

RESULTS

The treatment of mice with cyclophosphamide, intraperitonially, at the rate of 50mg/kg body weight resulted in a statistically significant increase of the number of micronucleated PCE's (mnPCEs) per 1000 PCE as compared to the normal control. Rise in the number of mnPCEs induced by CP was significantly reduced by oral pretreatment with diosgenin at the rate of 100 and 200µg/kg body weight, in a dose dependent manner, the higher dosage of diosgenin being more effective in lowering the mnPCE frequency (Figure 1 and Figure 2). Treatment of mice with diosgenin alone, at a concentration of 200µg/kg body weight, did not result in any significant change in the number of mnPCEs. Further, the study also evaluated the effect of diosgenin on various blood parameters namely total count of WBC, RBC and HB were analysed. Treatments with cyclophosphamide alone, diosgenin alone or in combination did not alter the total count of RBC and haemoglobin content as compared to normal control (Data not presented here). CP administration resulted in significant reduction of total WBC as compared to normal control group, whereas diosgenin alone did not cause a reduction

in the total count of WBC. Treatment with diosgenin was significant in replenishing the WBC loss caused by CP. (Figure 3).

The study also estimated the level of Lipid peroxidation in the liver homogenates using MDA as an indicator. The results showed a significant increase in the level of lipid peroxidation in liver tissue of animals exposed to CP. Diosgenin pretreatment lead to a significant lowering of the CP induced lipid peroxidation. In addition, estimation of the influence of different treatments on the level of cellular antioxidant, reduced Glutathione (GSH) was done. CP caused a considerable fall in the cellular GSH level which was restored by Diosgenin pretreatment (Table 2). Diosgenin did not lead to a fall in the level of GSH.

DISCUSSION

There has been a global trend in recent years towards the application of natural substances present in fruits, vegetables and herbs as antioxidants and anticancer agents as well. Diosgenin is widely used in various traditional herbal medicine systems (Raju and Rao, 2012). It is used in the pharmaceutical industry extensively, as a precursor of various synthetic steroidal drugs. The oestrogenic effects of diosgenin on mammalian glands are well documented and employed as a constituent of oral contraceptive pills (Djerassi, 1992). This natural plant saponin is shown to have anticancer properties in research conducted by several workers (Moalic *et al*., 2001; Raju and Bird, 2007; Raju and Mehta, 2009). Diosgenin is shown to act by diverse mechanisms such as elevation of free radical scavenging enzymes (Das and Bharali, 2014) and elevation of cyclooxygenase activity (Moalic *et al.*, 2001; Son *et al.*, 2007). Diosgenin suppressed PAS expression and modulate AKt, mTOR and JNK phosphorylation in HER-2 overexpressing cancer cells, indicating the potential to act as a unique chemotherapeutic modulator in the prevention or treatment of HER 2- overexpressing cancer (Chiang *et al.*, 2007).

The mouse bone marrow micronucleus assay *in vivo* allows a sensitive assessment of chromosomal damage and chromosome loss caused by chemical substances. The presence of micronuclei in cells is considered as a biomarker of clastogenic damage inflicted on the DNA (Kirsch-volders *et al*., 2000; Kirsch-volders & Vanhauwaert 2003; Álvarez-González *et al.*, 2004 and Celikler *et al.*, 2009). Genotoxicity was induced by Cyclophosphamide (CP), a standard genotoxicant, as per OECD guidelines (OECD, 2012). The results of the present study also show that, administration of cyclophosphamide caused significant increase in the number of mnPCEs. Pretreatment with diosgenin showed a significant decrease in the frequency of mnPCEs indicating the ability of diosgenin to lower the DNA damage. These results are in correlation with reports published earlier where Diosgenin had shown significant anticlastogenic effects against other cytotoxic drugs (Rajalingam *et al*., 2013).

CP is well known immunosuppressive agent (Shih *et al.*, 1983; Wójcik and Dąbkowska, 2010) causing reduction in the number of WBC as well as other immune parameters. In the present study, the administration of Diosgenin restored the total WBC counts, indicating its ability to restore immune functions. Diosgenin by itself did not show any adverse effect on WBC count. These results are in agreement with previous reports of certain natural substances capable of immunomodulatory effects on drug induced toxicity (Gupta *et al*.,2010; Merwid-Lad *et al.*, 2011). CP or diosgenin have not caused significant alteration of the other two blood parameters.

Lipid peroxidation is a free-radical-mediated chain of reactions that, once initiated, results in an oxidative deterioration of polyunsaturated lipids which are the important components of biological membranes. CP induces lipid peroxidation in the liver tissues indicated by rise in MDA levels as well as in blood of experimental animals (Berrigan *et al*.,1987; Premalatha *et al*., 1995; Ray *et al.*, 2011) and also was found to deplete hepatic glutathione. Hepatic GST activity was found to increase significantly in a dose dependent manner by diosgenin (Das and Bharali, 2014). Diosgenin oral pretreatment normalized the levels of lipid peroxidation induced by 7,12- DMBA in experimental animals (Rajalingam *et al.*, 2013). In the present study also, a significant increase in lipid peroxidation levels in CP treated mice was observed, which could be significantly lowered by pretreatment with diosgenin. This indicates the diosgenin has the capacity to protect membrane damage caused by cyclophosphamide. Reduced glutathione is strong cellular antioxidant, neutralizing hydroxyl radicals and singlet oxygen (Knotsky, 1990). It is low in cells under oxidative stress induced by drugs like CP. Natural antioxidants like vitamin E are capable of reversing these adverse effects (Premalatha *et al*., 1995; Collins *et al*., 2003). In the present study diosgenin pretreatment prevented the fall of GSH levels after exposure to CP and could be one of the protective mechanisms of diosgenin. Earlier studies have demonstrated a positive correlation between degree of lipid peroxidation and genotoxicity indicators like mnPCEs (Catiana *et al.*, 2008; Ramos *et al.*, 2008; Sinha *et al*., 2011 and Moretti *et al.*, 2012).

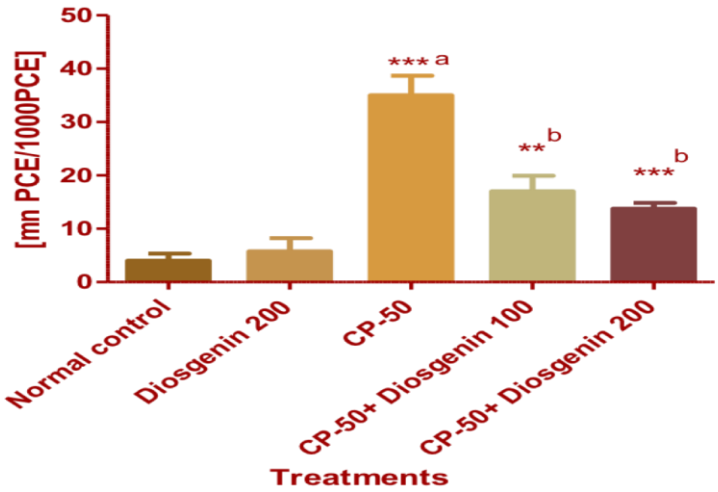

Figure 1: Effect of various treatments on frequency of mnPCEs in the experimental animals (Values expressed are mean±SEM n=06) ** p<0.01, ***p<0.001 a-when compared with normal control b- when compared with drug (CP) control)

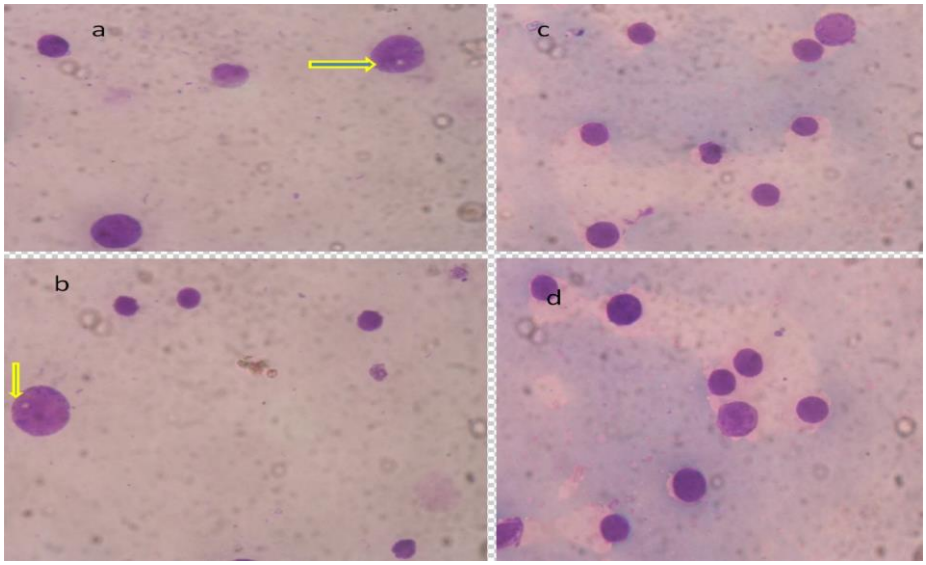

Figure 2: Bonemarrow smears of the different treatments; a, b -Presence of micronuclei in poly-chromatic erythrocytes in bonemarrow of CP 50mg/kg bodyweight treated mice; c-Normal control; d-Diosgenin 200µg/kg bodyweight

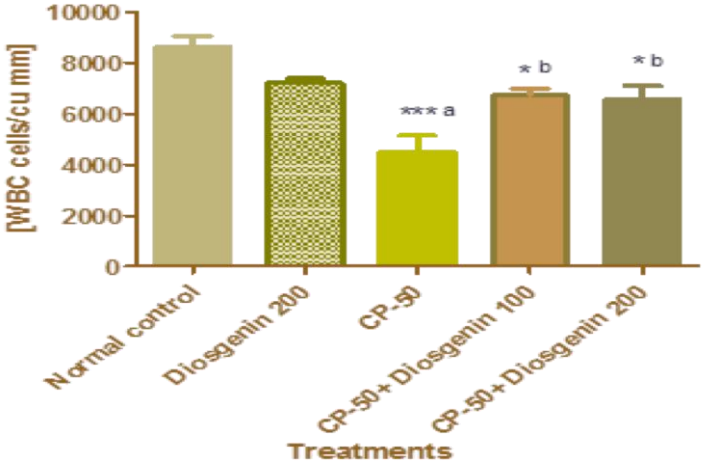

Figure 3: Effect of various treatments on total count of WBC (Values expressed are mean±SEM n=06) ** p<0.01, ***p<0.001 a-when compared with normal control b- when compared with drug (CP) control)

Table 1: Micronucleus test

Group	Treatment
Normal control	Vehicle 10 ml/kg
Test control	Diosgenin 200 µg /kg p.o.
Drug control	Cyclophosphamide alone 50mg/kg i.p.
Treatment 1	CP 50mg/kg i.p. +Dios 100 µg/kg p.o.
Treatment 2	CP 50mg/kg i.p. +Dios 200 µg/kg p.o.

Table 2: Effect of various treatments on Lipid peroxidation and GSH levels in different experimental animals

Treatment	LPO (nmol/gm wet tissue)	GSH (nmol/gm wet tissue)
Normal control	2.29± 0.19	14.83±1.76
Diosgenin 200 µg /kg p.o. (test control)	1.21± 0.61	12.46±1.65
CP 50mg/kg i.p. (drug control)	9.02± 2.15***a	4.51±2.16***a
CP 50mg+Dios 100 µg/kg p.o.	5.21±0.94**b	8.42±2.32**b
CP50mg+Dios 200 mg/kg p.o.	4.05±0.58**b	10.54±3.16**b

(Values expressed are mean±SEM n=06) ** p<0.01, ***p<0.001 a-when compared with normal control b- when compared with drug (CP) control)

Natural substances which can suppress drug induced lipid peroxidation and genotoxicity can prove to be valuable in reducing drug induced toxic side effects. The results of present study indicate the scope of diosgenin as an antigenotoxic agent against drug induced genotoxic effects. Further studies can reveal the potential of this natural compound as a chemopreventive and chemoprotective agent.

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