

EFFECT OF DIOSGENIN - A PLANT STEROID ON LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN HUMAN LARYNGEAL CARCINOMA CELLS (HEP2)

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ABSTRACT

Carcinoma is a major public health burden in all countries. Out of all carcinomas laryngeal carcinoma accounts for 25% of head and neck carcinoma and 2% of all human malignancies. In this study, we made an attempt to investigate the pro-oxidant activity of diosgenin on Hep2 cell line by adopting biochemical approaches. Diosgenin is a steroidal sapogenin with estrogenic and anti tumor properties. Cell viability was assessed via an MTT assay. Intracellular ROS generation was estimated spectrophotometrically. The effect of diosgenin on lipid peroxidation and the activities of antioxidants status were also analysed. Diosgenin inhibited Hep2 cell growth. The IC50 cytotoxic dose of diosgenin enhances lipid peroxidation status and decreases antioxidant levels in Hep2 cells, thereby decreasing cell malignancy or transformation and inhibits tumor promotion. Our data demonstrates that diosgenin is a potent inhibitor of Hep2 carcinoma cells, by growth inhibition, enhanced ROS generation and lipid peroxidation and decreased antioxidant activity in Hep2 cells which shows that diosgenin possess prooxidant properties.

Keywords: Diosgenin, Laryngeal carcinoma, Lipid peroxidation, Antioxidant.

INTRODUCTION

Laryngeal cancer may also be called cancer of the larynx or laryngeal carcinoma. Most laryngeal cancers are squamous cell carcinomas, reflecting their origin from the squamous cells which form the majority of the laryngeal epithelium. Cancer can develop in any part of the larynx, but the cure rate is affected by the location of the tumor. Laryngeal cancer is the second most common respiratory cancer after lung cancer. Its incidence is increasing over time in much of the world and this increase is generally accepted to be related to changes in tobacco and alcohol consumption. It is a relatively common cancer in men, but rarer in women¹.

Reactive oxygen species (ROS) is an entire class of highly reactive molecules derived from the metabolism of oxygen and is often generated as byproducts of biological reactions or from exogenous factors. Reactive oxygen species, such as H₂O₂ and oxygen radicals, are highly reactive chemical species that can result from oxidative metabolism and cause oxidation of cellular components including DNA, protein and lipids. Reactive oxygen species (ROS) have been identified as the responsible agents in the pathogenesis of various diseases² and normal physiological phenomena such as aging³. ROS can also induce cell death directly or act as intracellular messengers to induce various kinds of stimuli during apoptosis. Living systems have evolved endogenous antioxidant systems as a response to oxidative stress caused by ROS⁴. Antioxidants can inhibit or delay oxidative stress induced apoptosis. Enzymatic antioxidants and non enzymatic antioxidants and oxidant detoxifiers have the ability to inhibit tumor initiation and promotion in vivo and in vitro⁵.

Reactive oxygen species (ROS) formed within the cells can oxidize biomolecules such as carbohydrates, proteins, lipids and DNA which may lead to cell death and tissue injury. Of all the biomolecules, lipids appear to be more susceptible to the damaging effects of ROS. Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It mostly affects the polyunsaturated fatty acids (PUFA) because they contain multiple double bonds in between which lies methylene -CH₂- groups that possess especially reactive hydrogens⁶. Scavengers of ROS have therefore evolved an effective chemopreventive agent. Several dietary phytochemicals are an alternative due to their various degrees of antiproliferative and immunostimulatory effects on various types of human cancers. Epidemiological studies have shown an inverse relationship between dietary practices and the incidence of cancer. Phytochemicals are an integral part of the human diet due to their wide abundance in fruits and vegetables and have attracted

considerable interest as potential anticancer agents. They prevent oxidative damage as a result of their ability to scavenge reactive oxygen species. By contrast the prooxidant properties of these compounds could contribute to tumor cell apoptosis and anticancer action. Chemoprevention using drugs is a promising approach to control the mortality and occurrence of cancer that accounts for millions of death worldwide⁷. Recent researches revolve round the urgency to evolve suitable chemotherapy consistent with new discoveries in cell biology for the treatment of cancer with no toxic effect^{8,9}.

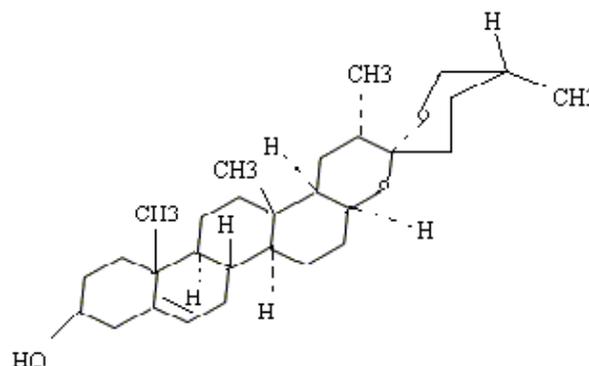


Fig. 1: Structure of Diosgenin

Diosgenin [25R-spirost-5-en-3 β -ol], is an important steroidal metabolite found in various plant species. It is a hydrolysate of dioscin contained in the rootstock of yam and it exists widely in the natural plants, as glucoside¹⁰. The discovery of diosgenin has made it one of the most researched and studied herbal product. It has been reported to have wide spectrum of biological properties such as anti inflammatory, anticarcinogenic, antithrombosis, antiaging and anti HIV properties^{11,12}. Diosgenin is an important steroidal metabolite, used as a starting material for the synthesis of steroidal drugs, as it exhibits estrogenic activity¹³. It has been shown to reduce the level of serum cholesterol^{14,15}. Diosgenin has received considerable attention because of the variety of their promising pharmaceutical properties^{16,17}. The consumption of diosgenin has positive actions on stress and inflammatory conditions. Previous studies have demonstrated that diosgenin belonging to spirostanol saponins have notable antitumor activities. They can induce direct cytotoxicity,¹⁸ cell cycle arrest and apoptosis^{19,20,21}. From various literature cited above shows that diosgenin possess a potent antitumor activity and if diosgenin works by increased oxidative damage in cancer cells, it

might be useful for effective cancer chemotherapy, Therefore, the present study was investigated to evaluate the prooxidant activity of diosgenin against human laryngeal cancer cells by adopting biochemical approach.

MATERIALS AND METHODS

Chemicals

Diosgenin was purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade.

Cell culture

Hep2 cell line were obtained from National Center for Cell Science (NCCS), Pune, India and were cultured in MEM supplemented with 10% heat inactivated fetal calf serum, L-glutamine 3%, 100 IU penicillin and 100 g/mL of streptomycin at 37°C in 5% CO₂.

Experimental Design

The cells were divided into four equal groups of six samples each.

Group I	: Control (untreated Hep2 cells)
Group II	: Hep2 + Diosgenin (0.125mg/mL)
Group III	: Hep2 + Diosgenin (0.25mg/mL)
Group IV	: Hep2 + Diosgenin (0.5mg/mL)

Cell growth inhibition test

MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay was first proposed by Mossman²². After incubation, the medium was removed from the wells carefully for MTT assay. Each well was washed with MEM (w/o) FCS for 2 – 3 times and added 200µL of MTT at a concentration of 5mg/mL. Incubated for 6-7hrs in 5% CO₂ incubator for cytotoxicity. After incubation, added 1mL of DMSO in each well, mixed by pipette and left for 45seconds. The viable cells showed the purple color formation. The suspension was transferred into the cuvette of spectrophotometer and O.D values were read at 595nm by taking DMSO as a blank. Graph was plotted by taking concentration of the drug on X axis and relative cell viability on Y axis.

$$\text{Cell viability (\%)} = \text{Mean OD/Control OD} \times 100$$

Measurement of intracellular ROS in cancer cells by spectrofluorimetric method

ROS was measured based on the method of Philip Jesudason *et al.*²³. The percentage ROS was estimated in the control and diosgenin treated cancer cells. Briefly, an aliquot of the above mentioned isolated cells 8x10⁶ cells/ml were made upto a final volume of 2mL in normal phosphate buffered saline (pH 7.4). An 1mL aliquot of cells were taken, to which 100 µL DCFH-DA(10µM) was added and

incubated at 37° C for 30 min. Fluorescence measurements were made with excitation and emission filters set at 485 + 10 nm and 530 + 12.5nm respectively. All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the formula [(Ft 30 - Ft 0) / (Ft0 x 100)] and the fluorescence intensities were measured at of 0 and 30 min. We have also observed the cells microscopically using blue filter.

Biochemical estimations

The cancer cells were harvested by trypsinization. The trypsinised cells were washed with PBS. The cells were suspended in 130mM KCl plus 50mM PBS containing 10µM dithiothreitol and centrifuged at 20000 x g for 15min (4°C). The supernatant was collected and used for further enzymic and other estimations.

The lipid peroxidation markers such as TBARS was estimated by the method of Niehaus and Samuelson²⁴, conjugated dienes (CD) by the method of Recknagel and Rao²⁵, lipid hydro peroxide by the method of Jiang *et al.*²⁶. The enzymatic antioxidants such as superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) were estimated by the methods of Kakkar *et al.* Sinha, Rotruck *et al.* respectively^{27,28,29}. The reduced glutathione in cancer cells was estimated by the method of Ellman³⁰. The proteins were also estimated by Lowery *et al.*³¹.

Statistical analysis

All quantitative measurements were expressed as means + SD for untreated and diosgenin treated cells. The data were analysed using one way analysis of variance (ANOVA) on SPSS/PC (statistical package for social sciences, personal computer) and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the p<0.05.

RESULTS

Effect of diosgenin on cell proliferation in Hep2 cells (MTT assay)

The cell line cultures were exposed to different concentration of diosgenin (0.007-1mg/mL) for 24hours and observed for cytotoxicity microscopically shown in fig. 2 and the result was further confirmed by MTT assay as shown in Table (1). The IC₅₀ value was plotted by taking the concentration of diosgenin on (x-axis) versus percentage of cell viability on (y-axis). The result showed that Hep2 cells proliferation were significantly inhibited by diosgenin with an IC₅₀ value of 0.125mg/mL as shown in fig. 3.

The changes in the levels of % cell viability of 0.01, 0.03, 0.06 mg/mL of diosgenin treatment have not showed significant (P < 0.05) proliferation inhibition. 0.125, 0.25, 0.5 mg/mL of diosgenin treatment significantly inhibits Hep2 cells. Hence for further experiment we have chosen these concentrations of diosgenin.

Table 1: Measurement of cell viability

S. No	Concentration (mg/mL)	Dilution	Absorbance	Cell viability
1	1	Neat	0.07	11.11
2	0.5	1:1	0.12	19.04
3	0.25	1:2	0.25	39.68
4	0.125	1:4	0.31	49.20
5	0.0625	1:8	0.39	61.90
6	0.03125	1:16	0.43	68.25
7	0.0156	1:32	0.52	82.53
8	0.0078	1:64	0.59	93.65
9	Cell control	-	0.63	100

Diosgenin enhances ROS level in Hep2 cancer cells

Cytotoxic drugs induce oxidative stress through the generation of reactive oxygen species (ROS). The levels of ROS was measured by using a non-fluorescent probe, 2,7- diacetyl dichlorofluorescein (DCFH-DA) that can penetrate into the intracellular matrix of cells where it is hydrolysed by cellular esterases to form dichlorofluorescein (DCFH). The non- fluorescent DCFH is oxidized

by intracellular ROS and forms the highly fluorescent DCF which is measured spectrofluorometrically at emission filters set at 485 ± 10nm and 530 ±12.5nm respectively.

Levels of ROS in control and diosgenin treated cells were depicted in fig 4 and 5. Diosgenin treatment significantly increased ROS level in Hep2 cells. Among all the doses tested 0.5mg/mL of diosgenin showed maximum generation of ROS in Hep2 cells.

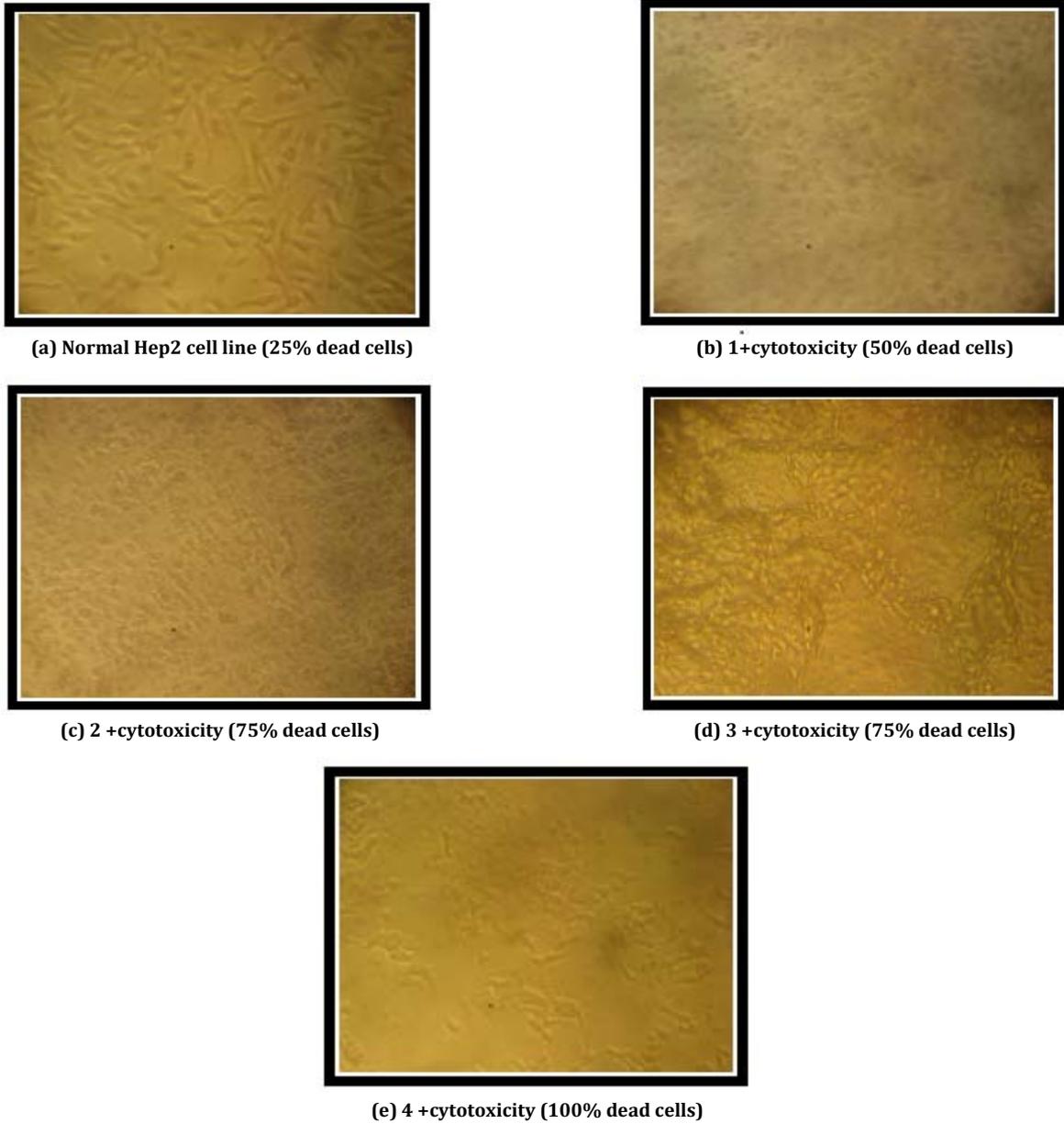


Fig. 2: Cytotoxicity of Hep2 cell lines

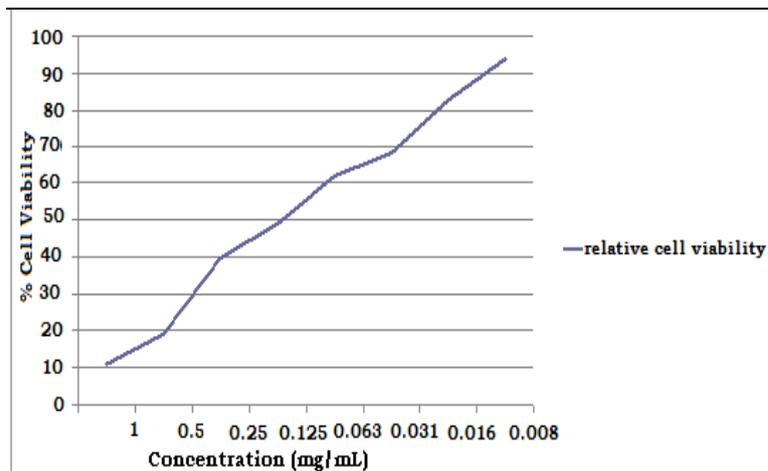


Fig. 3: MTT Assay

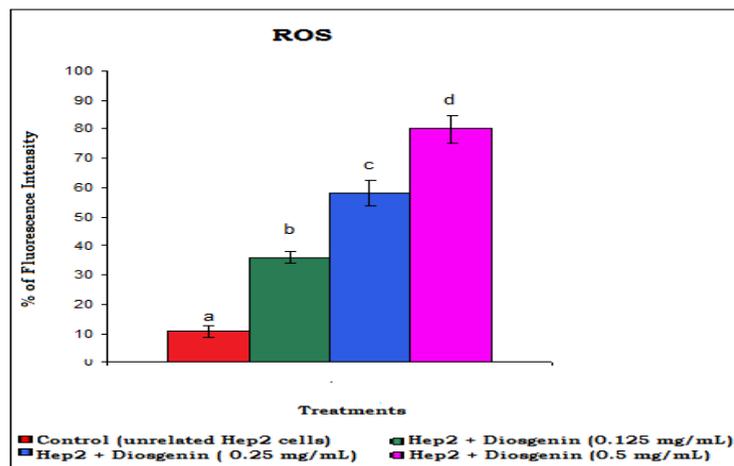


Fig. 4: Diosgenin enhances ROS levels in Hep2 cells

Values are given as mean + S.D. of six experiments in each group. Values not sharing a common superscript differ significantly at P<0.05 (DMRT).

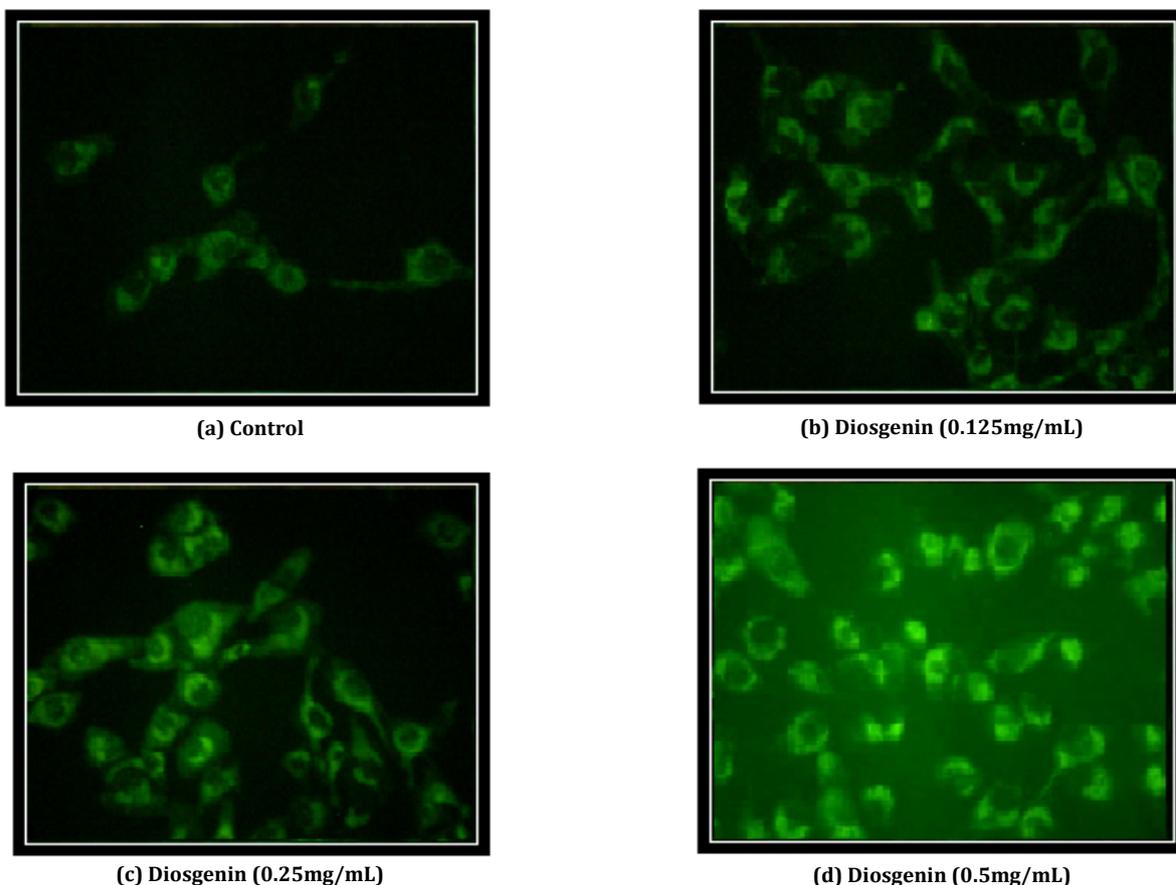


Fig. 5: Effect of Diosgenin on ROS generation in Hep2 cells

Diosgenin enhances lipid peroxidation in Hep2 cancer cells

To study the effect of diosgenin on oxidative damage, the lipid peroxidation indices i.e. TBARS, CD and LHP in Hep2 cell line with and without diosgenin treatment were measured. Diosgenin treatment increased the levels of lipid peroxidation in Hep2 cells.

Among all the concentration (0.125, 0.25 and 0.5) tested, 0.5mg/mL of diosgenin showed maximum lipid peroxidation (fig 6).

Diosgenin treatment increases the levels of lipid peroxidation in Hep2 cells. Among all the concentration (0.125, 0.25, 0.5) tested, 0.5 mg/mL of diosgenin showed maximum lipid peroxidation.

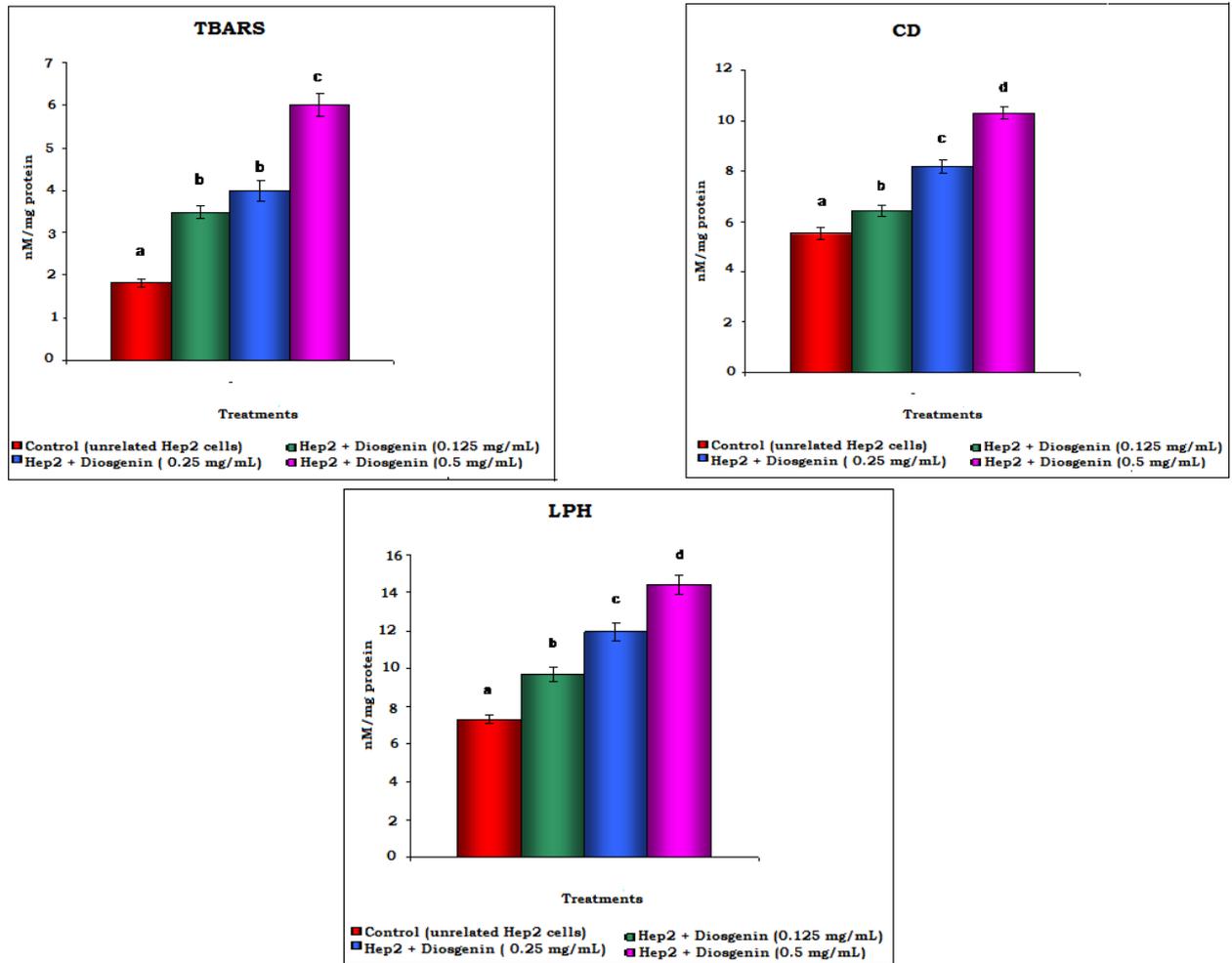


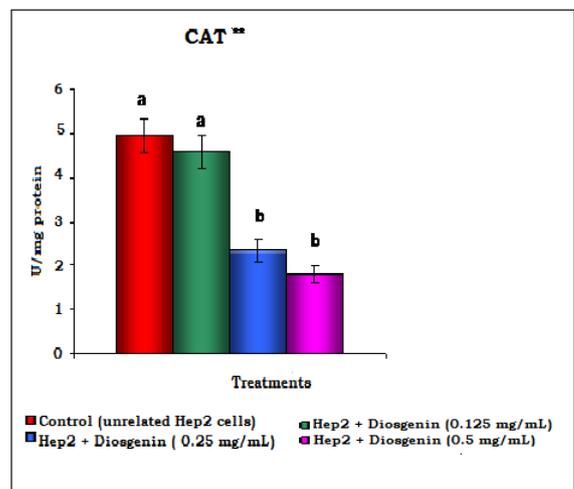
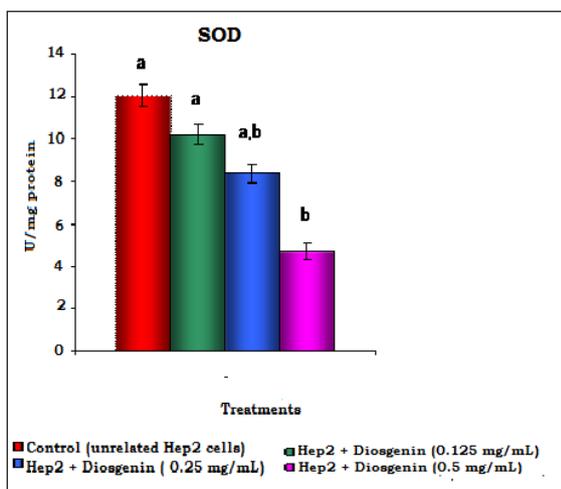
Fig. 6: Effect of Diosgenin on lipid peroxidation markers in Hep2 cancer cells.

Values are given as mean + S.D. of six experiments in each group. Values not sharing a common superscript differ significantly at P < 0.05(DMRT).

Changes in the levels of antioxidants

In normal healthy cells, enzymatic and non enzymatic antioxidants serve to balance the intracellular production of ROS, thereby delaying or inhibiting the destructive oxidation of molecular components. The susceptibility of tumor cells to drug may be

associated with decreased levels of antioxidants, which increases the cell sensitivity to pro-oxidant compounds. Activity of enzymatic antioxidants such as SOD, CAT and GPx and non enzymatic antioxidant, GSH were depicted in fig. 7. Among all the doses tested 0.5mg/mL of diosgenin significantly decreases the levels of antioxidants.



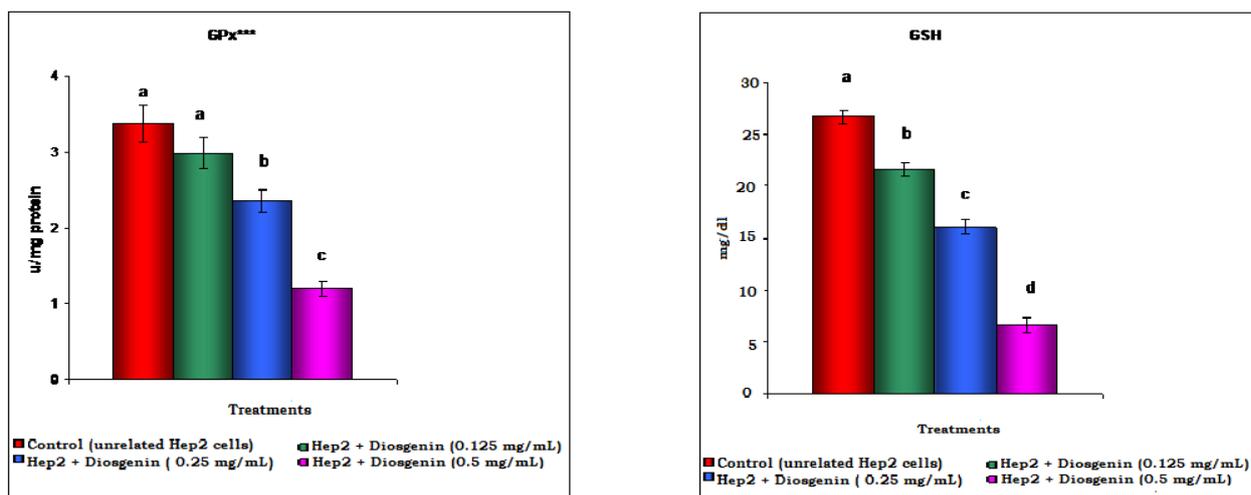


Fig. 7: Effect of Diosgenin on the activities of SOD, CAT, GPx and GPH in Hep2 cancer cells.

* Enzyme activity required for 50% inhibition of nitroblue tetrazolium reduction in one minute.

** micromole of hydrogen peroxide consumed per minute.

*** microgram of glutathione consumed per minute.

Values are given as mean + S.D. of six experiments in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

DISCUSSION

In this present study, we evaluated the pro-oxidant effect of diosgenin in Hep2 cell line *in vitro*. The cytotoxicity of diosgenin in Hep2 cells was observed by MTT assay. Diosgenin treatment significantly decreased % cell viability. Low concentration of diosgenin was not significantly inhibits the growth of these cells. But high concentration of 0.5mg/mL of diosgenin treatment shows significant cytotoxicity. This might be due to prooxidant property of diosgenin which disrupt mitochondrial dehydrogenase activity. Previous study shows that diosgenin exhibits dose dependent cytotoxic effect in human leukemia K562 cells and in human osteosarcoma 1547 cell line^{32,33}. Moreover diosgenin caused an inhibition of the growth of fibroblast like synoviocytes from human rheumatoid arthritis, with apoptosis induction associated with cyclooxygenase-2 up-regulation²¹. Diosgenin is found to be effective in suppressing Fatty Acid Synthase (FAS) expression in HER 2-overexpressing breast cancer cells and preferentially inhibited proliferation and induced apoptosis in HER 2-overexpressing cancer cells³⁴. Our results along with the previous report suggest that diosgenin possess increased cytotoxicity effects.

Reactive oxygen species (ROS) are known to cause oxidative modification of DNA, proteins, lipids and small cellular molecules. Increased ROS levels are thought to constitute an essential step in cell death induction by many different cytotoxic drugs. ROS levels were assessed after 24 hours incubation with diosgenin. We noticed a significant increase in the ROS levels in 0.5mg/mL diosgenin treated cells. Peroxidases present in the cancer cells catalyses a one electron oxidation of phenols to form phenoxyl radicals, which in turn rapidly oxidize NADH to NAD. Then this NAD radical reduce O₂ to O₂. This might be the reason for increased ROS generation during diosgenin treatment. In our study, significant increase in lipid peroxidation indices in diosgenin treated cancer cells was observed. Diosgenin enhanced the resistant to lymphocyte DNA damage caused by an oxidant challenge with H₂O₂³⁵ which suggests that diosgenin possess potential pro-oxidant action when treated on cancer cells. Previously, prooxidant activities of phenolic compounds have been confirmed by several researchers^{36,37}. Thus it is inferred that diosgenin, in the same way enhances lipid peroxidation and thereby inducing cancer cell death.

Many studies suggested that antioxidant enzymes are critical in protecting against tumor promoting agents. Interestingly, cell malignancy or transformation is often accompanied by a decrease in activity of antioxidants like SOD, CAT, GPx which increases the cell

sensitivity to prooxidant compounds³⁸. In our study, decrease activity of antioxidants i.e., SOD, CAT, GPx were observed in diosgenin treated cancer cells. Previous study showed that phytochemicals depletes intracellular antioxidants thereby induced cancer cell death³⁹. Our results also correlate with their findings.

Prominent decrease of GSH levels in cancer cells was also noted. The phenol ring containing diosgenin is oxidized by peroxidase/ROS to phenoxyl radicals that co-oxidize GSH to form a thiyl radical (GS), which then reacts with GSH to form a disulfide radical anion. This might be the reason for decreased GSH levels in diosgenin treated Hep2 cells. Previous study shows that phytochemicals depletes intracellular antioxidants thereby induced cancer cell death³⁹.

Our results summarize that diosgenin decreases cell proliferation, antioxidant status and increases ROS generation and lipid peroxidation in Hep2 cancer cells. Due to its prooxidant activities, it can be considered as more potent, specific and effective chemotherapeutic agent. This study may provide a background for the introduction of this new type of promising therapeutic agent in the study of cancer chemotherapy.

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