

## PHARMACOLOGICAL EFFECT OF DIOSGENIN ON DIETHYLNITROSAMINE INDUCED HEPATOTOXICITY IN WISTAR RATS

Suraj G. Jagdale <sup>1</sup>, S. Arulmozhi \*<sup>1</sup>, Shrirang Jamadagni <sup>2</sup> and Kakasaheb R. Mahadik <sup>3</sup>

Department of Pharmacology <sup>1</sup>, Department of Pharmaceutical Chemistry <sup>3</sup>, Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune - 411038, Maharashtra, India.

Regional Ayurveda Institute for Fundamental Research (CCRAS) <sup>2</sup>, Ministry of Ayush, Government of India, Pune - 411038, Maharashtra, India.

**ABSTRACT: Background:** The liver is the major detoxification organ that deactivates and removes toxic chemicals. The oxidative stress-mediated toxicity of chemicals involves destruction primarily to liver tissue (Hepatotoxicity), which could lead to cancer. Diosgenin, a steroidal sapogenin, is reported to be an apoptosis inducer, antineoplastic and antioxidant. The present study is aimed to screen diosgenin for its effect on diethylnitrosamine induced hepatotoxicity in rats. **Materials and Methods:** 48 male Wistar rats were divided into 6 groups of 8 animals each. Group 1: the vehicle was given to the animals for 8 weeks. Group 2: den control, group 3 sorafenib, groups 4, 5, and 6 diosgenin (10, 20, and 40 mg/kg, respectively). Groups 2 to 6 were administered with 0.01% den in drinking water for 8 weeks. The respective treatment started from 4<sup>th</sup> week and continued till the 11<sup>th</sup> week. At the end of the study, animals were sacrificed for the determination of biochemical, antioxidant, and histological parameters. **Results:** Administration of den caused a significant increase in the levels of serum AST, ALT, ALP, LDH, and liver malondialdehyde as compared with control while the levels of glutathione, catalase, and superoxide dismutase were significantly decreased. Oral supplementation of diosgenin led to a significant decrease in the levels of AST, ALT, ALP, LDH, and malondialdehyde and increased the levels of glutathione, catalase, and superoxide dismutase. The liver histology of diosgenin administered groups was preserved. **Conclusion:** Diosgenin was found to prevent, slow, and treat the occurrence of hepatotoxicity.

Keywords:

Hepatotoxicity, Diethyl nitrosamine, Diosgenin, Antioxidant

**INTRODUCTION:** Hepatocellular carcinoma (HCC) is a malignant condition that is a multistep development that includes initial geno toxins insult, clonal expansions of hepatocytes from premalignant neoplasia, or foci lesions, and finally tumor progression. Hepatocellular carcinoma signifies one of the most common types of malignancies worldwide, with a significant increase in mortality rate <sup>1</sup>.

In developed countries, it is considered as the third cause of cancer-related death <sup>2</sup>. Hepatocellular carcinoma (HCC) is a widely documented danger to the liver. There are few successful corrective choices for this severe condition <sup>3</sup>. Worldwide, chronic hepatitis B and C virus infection is a firm etiological factor for HCC as it causes DNA damage *via* activation of inflammation and reactive oxygen species formation <sup>4</sup>.

Toxic exposure (alcohol abuse, aflatoxin B1 intake from contaminated food), liver cirrhosis, obesity, and nonalcoholic fatty liver disease also contribute to the development of HCC <sup>5</sup>. Alcohol consumption, foods contaminated with fungal toxins, toxic industrial chemicals, air/water pollutants, and hepatitis viral infection are the major risk factors associated with HCC. Approximately 7.5 L of fresh cases of HCC per year occurs universally, which makes HCC as the 5<sup>th</sup> common cause of cancer. Liver cancer is abundant more common

in countries in Sub-Saharan Africa and Southeast Asia than in the US. More than 800,000 people are diagnosed with this cancer each year throughout the world.

Liver cancer is also a prominent cause of cancer deaths worldwide, accounting for more than 700,000 deaths each year. The incidence rate of HCC in India for men ranges from 0.7 to 7.5 and for women 0.2 to 2.2 per 100,000 populations per year. The incidence of HCC in cirrhotics in India is 1.6% per year. The male: female ratio for HCC in India is 4:1. The age of presentation varies from 40 to 70 years. The age-standardized mortality rate for HCC in India for men is 6.8/100,000, and for women is 5.1/100,000. In the case of liver cancer treatment, surgical removal of livers is significant surgery, especially since the liver is rich in a blood vessel. There are side effects include infections, blood clots, pneumonia.

The drugs sorafenib, ramucirumab and cabo-zantinib are in use for liver cancers that can't be removed surgically and they act in part by hindering new blood vessel growth. There are other managements available for HCC but has limitation like in early-stage, resection is possible, but chances of recurrence is high. In liver transplantation, the scarcity of donor and acceptor is more so this problem is associated with transplantation.

Though the number of medications available for the treatment of liver ailments and hepatotoxicity is limited, there is the number of plant-origin medicaments which offer hepatoprotective effect. However, scientific proof of their impact is not validated. Hence, it will be worthwhile to screen such compounds, which will lead to their use in the prevention of liver cancer similar to silymarin <sup>6</sup>. Diosgenin is a bioactive steroidal sapogenin that belongs to the triterpene group and is of great benefit to the pharmaceutical industry <sup>7</sup>. Diosgenin, a well-known steroidal sapogenin obtained by the hydrolysis of the saponindioscin, from plants, namely, from Dioscorea, Trigonella, Costus <sup>8</sup> and Smilax species <sup>9</sup>. It has a role as an apoptosis inducer, an antiviral agent, an antineoplastic agent and a metabolite. Diethyl nitrosoamine (DEN), also known as n-nitrosodiethylamine, is widely used as a carcinogen in experimental animal models.

Diethyl nitrosamine (DEN) can be given by oral route with drinking water or intraperitoneal route to the animals, which produces reactive oxygen species and increases oxidative stress which leads to damage of critical cellular biomolecules such as lipids, proteins and deoxyribonucleic acid (DNA) and thereby causes hepatocellular carcinoma <sup>10</sup>. Diosgenin is reported to have anti-cancer, anti-inflammatory, and antioxidant properties. Based on the above perspective, the present study was designed to study its effect in DEN induced hepatotoxicity.

#### **MATERIALS AND METHODS:**

**Animals:** 48 male Wistar rats (weighing 150-180 gm) were obtained from the National Institute of Biosciences, Pune (India). The animals were housed in solid bottom polypropylene cages. They were maintained at  $24 \pm 1$  °C, with relative humidity 45-55% and 12:12 h dark/light cycle.

The animals had free access to standard pellet chow (Prashant enterprises, Pune, India) and open access to water throughout the experiment protocol. The experimentation was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (Registration number 1703/PO/Re/S/01/CPCSEA dated 17/06/2016) constituted under Committee for Control and Supervision of Experiments on Animals (CPCSEA). The approval number is CPCSEA/PCP/PCL12/2018-19.

**Chemicals and Their Sources:** DEN and diosgenin were procured from Sigma Aldrich, USA. Standard drug sorafenib was obtained from Lupin Limited, Pune, and Maharashtra. All other chemicals purchased from Pune, Maharashtra.

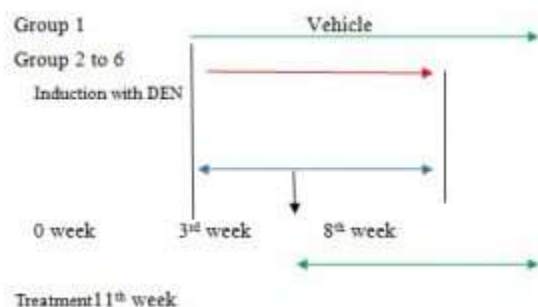
**EXPERIMENTAL DESIGN:** The experimental animals were divided into six groups, each group comprising of eight animals.

**Group 1:** Vehicle control group: Animals were not treated with the drug; the only vehicle was given to the animals for eight weeks.

**Group 2:** DEN control group: 0.01% DEN in drinking water was given to the animals for eight weeks <sup>11</sup>.

**Group 3:** Standard treated group: 0.01% DEN in drinking water was given to the animals for eight weeks and treated with sorafenib (5 mg/kg) from 3 to 11 weeks of induction.

**Group 4:** Diosgenin (10 mg/kg) treated: 0.01% DEN in drinking water was given to the animals for eight weeks and treated with diosgenin (10 mg/kg) from 3 to 11 weeks of induction.



**FIG. 1: SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL PROTOCOL**

**Group 5:** Diosgenin (20 mg/kg) treated: 0.01% DEN in drinking water was given to the animals for eight weeks and then treated with diosgenin (20 mg/kg) from 3 to 11 weeks of induction.

**Group 6:** Diosgenin (40 mg/kg) treated: 0.01% DEN in drinking water was given to animals for eight weeks and then treated with diosgenin (40 mg/kg) from 3 to 11 weeks of induction.

All groups except the vehicle control group of animals were induced by 0.01% N-nitrosodiethylamine through drinking water for eight weeks. Groups 3 was treated with sorafenib (5 mg/kg), while 4, 5, and six were treated with diosgenin in 10, 20, 40 mg/kg p. o. Every day for eight weeks. After three weeks of DEN induction, the respective treatment was given to group 3 to 6 for eight weeks once daily orally. Then after eight weeks of treatment, the blood sample was collected for evaluation of parameters. The animals were sacrificed, and liver tissues were collected for antioxidant and histopathology parameters. Blood serum was separated by centrifugation (2500 rpm × 10 min) and stored at -80 °C until further analyzed, and part of the liver tissue was fixed in 10% neutral buffered formalin for 24 h for histopathological examination.

**Biochemical Parameters:** For biochemical estimation, blood samples were allowed to clot at room temperature and centrifuged at 1500 rpm for 10 min to separate the serum. The biochemical parameters, such as AST, ALT, ALP, and LDH, were estimated using a UV-Visible spectro-photometer (JASCO Japan). For lipid peroxidation studies and enzymatic antioxidant 10% liver tissue homogenate (0.2 M phosphate buffer, pH 7.4) was prepared, the homogenate was then centrifuged at 2500 r/min for 15 min at 4 °C in a refrigerated centrifuge. The resultant supernatants were maintained in an ice bath. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and GSH were estimated by the methods of respectively. Levels of lipid peroxidation (LPO) products were estimated in liver tissue homogenate <sup>12, 13</sup>.

**Histological Examination:** On the last day of study, animals were sacrificed by using thiopentone sodium (100 mg/kg), dissected, and liver were collected for histopathology. The liver was fixed in the neutral buffered formalin solution; Formalin-fixed liver tissues were dehydrated using gradient concentrations of ethanol, were hed in xylene, and embedded in paraffin wax. Tissue blocks were sectioned at 5-6 μM thickness, deparaffinized, and stained with hematoxylin and eosin and analyzed under a microscope.

**Statistical Analysis:** Results were expressed as mean  $\pm$  sem and analyzed by One way ANOVA followed by Dunnett's test.

## RESULTS:

**Effect of Diosgenin on Bodyweight and Liver Weight in DEN Induced Hepatotoxicity in Rats:** The bodyweight of the DEN control group was significantly decreased as compared to the vehicle control group. In the diosgenin treated group (20 and 40 mg/kg), the body weight was significantly increased as compared to the DEN control group. The body weight was significantly increased in sorafenib treated groups as compared to the DEN control group. However, the bodyweight of diosgenin (10 mg/kg) treated group was not altered when compared to DEN control **Table 1**. The body weight changes suggest that the diosgenin at the dose (20 and 40 mg/kg) could reverse DEN induced cachexia. The liver weight was significantly increased in the DEN control group as compared to the vehicle control group. On the treatment with diosgenin, the liver weight was significantly decreased in (20 and 40 mg/kg) treated groups as compared to the DEN control group. Treatment with sorafenib significantly decreased liver weights. However, the observation confirms and that diosgenin could attenuate the liver weight increase due to DEN

Values are expressed as mean  $\pm$  sem n = 8 and analyzed by one way ANOVA followed by Dunnet's test, ### p<0.001 when compared to vehicle control and \*p<0.05, \*\*\*p<0.001 when compared to DEN control

**Effect of Diosgenin on Biochemical Parameters in Den Induced Hepatotoxicity in Rats:** Oral administration of DEN in DEN control group resulted in a significant increase (p< 0.001) in the aspartate aminotransferase (AST) level as compared to vehicle control rats (49.83%). Treatment with diosgenin (10, 20, and 40 mg/kg, p. o.) for eight weeks showed a significant decrease in the level of AST (7%, 18.58%, 32%), respectively, as compared to DEN control group. Animals treated with sorafenib (5 mg/kg p. o.) for eight weeks, showed a significant decrease in the level of AST (36.44%) compared to DEN control group **Table 2**.

Oral administration of DEN resulted in a significant increase (p<0.001) in the alanine aminotransferase (ALT) level of the DEN control group compared to vehicle control rats (56.2%). Treatment with diosgenin (10, 20, and 40 mg/kg, p. o.) for eight weeks showed a significant decrease in the level of ALT (11.4%, 22.2%, 40%) as compared to DEN control group. Animals treated with sorafenib (5mg/kg p. o.) for eight weeks showed a significant decrease in the level of AST (46.86%) compared to DEN control group **Table 2**.

Oral administration of DEN resulted in a significant increase (p< 0.001) in the alkaline phosphatase (ALP) level of the DEN control group compared to vehicle control rats (48.36%). Treatment with Diosgenin (10, 20, and 40 mg/kg, p. o.) for eight weeks showed a significant decrease in the level of AST (11.64%, 23.68%, 24.39%) as compared to DEN control group. Animals treated with sorafenib (5 mg/kg p.o.) for eight weeks showed a significant decrease in the level of ALP (33.08%) compared to DEN control group **Table 2**.

Oral administration of DEN resulted in a significant increase (p< 0.05) in the lactate dehydrogenase LDH level of the DEN control group compared to vehicle control rats (52.26%). Treatment with diosgenin (10, 20, and 40 mg/kg, p. o.) for eight weeks, showed a significant decrease in the level of AST (18.83%, 23.93%, 29.30%) as compared to DEN control group. Animals treated with sorafenib (5 mg/kg p. o.) for eight weeks, showed a significant decrease in the level of LDH (42.15%) compared to DEN control group **Table 2**.

Values are expressed as mean  $\pm$  sem n = 8 and analyzed by One way ANOVA followed by Dunnett's test, ### p<0.001 when compared with vehicle control and \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 when compared to DEN control

**Effect of Diosgenin on Antioxidant Parameters in DEN Induced Hepatotoxicity in Rats:** Oral administration of DEN resulted in a significant decrease (p<0.001) in the GSH level of DEN control group compared to vehicle control rats (49.91%).

Treatment with diosgenin (10, 20, 40 mg/kg, p. o.) for 8 weeks, showed significant increase in level of GSH (9.75%, 23.1%, 37.08%) as compared to DEN control. Animals treated with sorafenib (5 mg/kg p. o.) for 8 weeks, showed significant increase in level of GSH (36.40%) compared to DEN control group **Table 3**.

Oral administration of DEN resulted in a significant increase (p<0.001) in the MDA level of the DEN control group compared to vehicle control rats (74.28%). Treatment with diosgenin (10, 20, 40 mg/kg, p. o.) for eight weeks, showed a significant decrease in the level of MDA (24.77%, 39.13%, 49.10%) as compared to DEN control group. Animals treated with sorafenib (5 mg/kg p. o.) for eight weeks, showed a significant decrease in the level of MDA (62.23%) compared to DEN control group **Table 3**.

Oral administration of DEN resulted in a significant increase (p< 0.05) in the SOD level of the DEN control group compared to vehicle control rats (68.44%). Rats treated with diosgenin (10, 20, 40 mg/kg, p. o.) for eight weeks showed a significant decrease in the level of SOD (3.8%, 8.8%, 13.7%) as compared to DEN control group. Rats treated with sorafenib (5 mg/kg p. o.) for eight weeks showed a significant decrease in the level of SOD (62.59%) compared to the DEN control group. However, the SOD level of diosgenin (10 mg/kg) treated group was not changed when compared to DEN control **Table 3**.

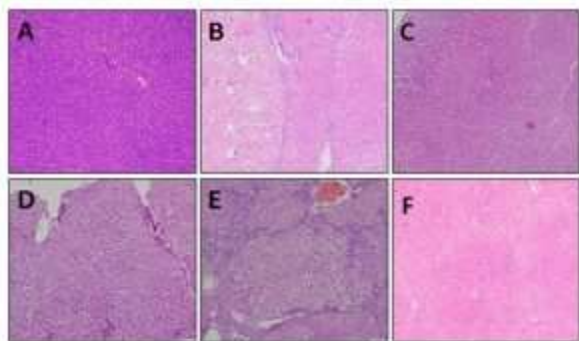
Oral administration of DEN resulted in a significant increase (p<0.001) in the catalase level in the DEN control group compared to vehicle control rats (53.26%). Treatment with diosgenin (10, 20, 40 mg/kg, p. o.) for eight weeks showed a significant decrease in the level of catalase (12.9%, 40%, 67.7%) as compared to DEN control group. Animals treated with sorafenib (5 mg/kg p. o.) for eight weeks, showed a significant decrease in the level of catalase (41.18%) compared to DEN control group **Table 3**.

Values are expressed as mean  $\pm$  sem n = 8 and analyzed by One way ANOVA followed by Dunnett's test, ### p<0.001 when compared with vehicle control and \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 when compared to DEN control

**Histological Examination:** The liver section of the vehicle control (A) group showed the vehicle architecture of hepatocyte and intact cell membranes. DEN control group (B) showed hepatocyte morphology with diffused vacuolization and bright cell foci (white cell space) and also raised border of cells and condition of dysplasia (pre-hepatoma phase).

The standard group (C) showed a vehicle liver pattern with loss of vacuolization and radially arranged hepatocytes similar to the vehicle control group. The treatment group with low dose (D) indicates significantly decrease vacuolization as compared to the DEN control group (B). The mid-dose (E) indicates the rearrangement of the cells. The treatment group with a high dose (F) significantly alters the morphology of cells. The high dose group showed mild vacuolization of cells similar to the vehicle group (A).

**Histopathological Representation of Liver of Following groups, H and E stain 10X:** Image A: vehicle control, image B: DEN control, image C: standard (Sorafenib 5 mg/kg), image D: diosgenin 10 mg/kg, image E: diosgenin 20 mg/kg, image F: diosgenin 40 mg/kg.



**FIG. 2: EFFECT OF DIOSGENIN IN DEN INDUCED HEPATOTOXICITY ON HISTOPATHOLOGICAL ALTERATION IN LIVER OF RATS:**

**DISCUSSION:** Liver cancer is a prominent cause of cancer deaths worldwide, accounting for more than 700,000 deaths each year. Approximately 7.5 Lakhs of new cases of HCC per year occur worldwide, which makes HCC as the 5th common cause of cancer. The incidence rate of HCC in India for men ranges from 0.7 to 7.5 and for women 0.2 to 2.2 per 100,000 populations per year. The incidence of HCC in cirrhotics in India is 1.6% per year <sup>14</sup>.

Hepatic injury exaggerated by DEN causes uncertainty of liver metabolism and changes the serum enzyme activities. N-nitroso compounds are a massive group of diet and environment-borne carcinogens and are related to the development of many types of cancer, including HCC. DEN is one of the N-nitroso chemicals used to induce liver cancer in experimental animal models. DEN is a reactive carcinogen which is metabolized *in-vivo* by cytochrome P450 enzymes for its biological activity and produces toxicity <sup>15</sup>.

The first bioactivation step of DEN is hydroxylation of the  $\alpha$ -carbon of an alkyl group to form  $\alpha$ -hydroxyl nitrosamine. Thus formed metabolites ( $\alpha$ -hydroxynitrosamines) decompose instinctively to alkanediazohydroxides and then to alkyl diazonium ions, which can alkylate DNA bases. The subsequent pro mutagenic adducts, O6-ethyl deoxyguanosine, and O4- and O6-ethyl deoxythymidine are believed to be mostly responsible for DEN-induced carcinogenesis <sup>16</sup>.

DEN is a potent hepatocarcinogenic agent that causes disturbances in the nucleic acid repair mechanism and also produces reactive oxygen species (ROS), leading to oxidative stress. Oxidative stress is an imbalance between ROS and antioxidant cell capability for detoxifying the reactive intermediates.

This disturbance is caused by ROS accretion, further depletion of antioxidants, or both. Additionally, oxidative stress has been verified in membrane lipid peroxidation, tissue injury, DNA damage, and mutagenesis associated with various stages of the tumor formation process. The mechanism of DEN-induced oxidative stress may further cause severe hepato-carcinogenesis.

The bodyweight of animals was observed on a weekly basis in all groups. Throughout the study, body weight was found to be significantly decreased in the DEN control group as compared to the vehicle group due to cachexia. In the diosgenin treated group, body weight was found to be significantly increased as compared to the DEN control group, which is in accordance with the earlier reports <sup>17</sup>. The liver is the major detoxification organ that deactivates and removes toxic chemicals to be excreted in the form of urine. The oxidative stress-mediated toxicity of chemicals involves destruction primarily to liver tissue (hepatotoxicity), which could lead to cancer. Liver damage caused by DEN could lead to the escape of enzymes (such as ALT and AST) from the liver tissue into the bloodstream. The serum levels of these enzymes are illustrative of the liver function, and increases are a valuable indicator of liver diseases. In this study, there was a



significant increase in ALT, AST, ALP, and LDH in DEN-exposed rats, which is in accordance with earlier reports <sup>18</sup>.

AST, ALT, ALP, and LDH are the pathophysiological markers used to find liver and tissue damage. The liver damage such as ischemic injury, toxicity injury, hepatitis, and biliary obstruction results in the increase of ALT, AST, and ALP. The increase in these enzymes confirms the loss of hepatocyte integrity <sup>19</sup>, which is further observed in our histopathological studies. Lactate dehydrogenase is an enzyme that is increased in the early stages of acute liver failure <sup>20</sup>. The level of liver enzymes was found to be decreased in diosgenin treated groups as compared to DEN control, which confirmed the preservation of hepatocyte integrity by diosgenin, which is also observed in the histopathological studies. The decrease in LDH in the diosgenin treated group confirms the hepatoprotective effect of diosgenin.

The primary line of defense against the cell-damaging effects of oxidative stress for the liver is the antioxidant defense system that counteracts the deleterious effects of free radicals. The oxidative damage is eluded by several enzymatic mechanisms that are activated in the hepatic cells. Chemical induction of liver damage by DEN administration causes an uncompromised generation of free radicals in the liver, which was validated by assessing the activity of the antioxidant enzymes. SOD, the major antioxidative enzyme in the liver, is a manganese-containing enzyme that catalyzes the dismutation of superoxide anions into hydrogen peroxide and molecular oxygen. In turn, hydrogen peroxide is rapidly altered into the water by CAT or GSH, thereby act as protection against ROS. CAT or GSH serves as a marker of evaluation oxidative stress, and it acts as an antioxidant at both cellular and intracellular levels. Decreased activity of GSH and CAT in the level in the DEN control group was observed. Diosgenin treated group increased the GSH and CAT levels, which suggest its antioxidant and hepatoprotective property of diosgenin <sup>21</sup>.

An end product of lipid peroxidation, MDA, is also considered a perilous indicator of oxidative damage because it is linked to the increased ROS caused when cytochrome P450 enzymes mediate the metabolism of DEN <sup>22</sup>. In lipid peroxidation, oxidative stress is known to be involved in carcinogenesis <sup>23</sup>, and a higher level of their product plays an early phase of tumor growth. In the present study, DEN induced group showed increased activity of the lipid peroxidation level. The diosgenin treated group significantly altered lipid peroxidation and reversed nearly to the vehicle control level, thus indicating anti-lipid peroxidative property. Hepatic contents of MDA indirectly reflect the degree of cell damage <sup>22</sup>. Administration of diosgenin to DEN-fed rats in this study countered DEN-induced oxidative stress, as shown by the restoration of antioxidant levels in the liver and the reduction of critical markers of oxidative stress, MDA.

Histopathological examination confirmed the dysplasia and pre-hepatoma caused by DEN. This is in accordance with the observed increase in liver weight in the DEN control group of animals. Treatment with diosgenin caused normalization of the cells with mild vacuolization, which further confirms the protective effect of diosgenin.

Diosgenin a steroidal sapogenin belongs to the triterpene group, which can be obtained from plants, like *Dioscorea* and *Trigonella* species. It has been reported to be an apoptosis inducer, antineoplastic and antioxidant. Furthermore, the present study demonstrated that diosgenin administration reduced DEN-induced oxidative stress, inflammatory response, and revoked the formation of preneoplastic liver nodules. Additional research is essential to explore the exact mechanism of the hepatoprotective effect of diosgenin and assess its clinical use as an adjuvant to chemotherapy or as a dietary supplement for improving liver function. Our results conclude that diosgenin is suitable for hepatoprotective effect in oxidative stress-induced liver damage.

**CONCLUSION:** Diosgenin demonstrated significant beneficial effects against DEN induced HCC in rats. The above observations suggest that diosgenin possesses chemopreventive action. The hepatoprotective properties observed in the present study could be due to the presence of phytoconstituents such as trigoneoside, trigofenoside, asparasaponin, stigmasterol, azelaic acid, etc.

Diosgenin at high dose (40 mg/kg) suppresses the tumors and decreases the biochemical marker, which is elevated in HCC. This will open new perspectives that diosgenin is a chemopreventive compound to prevent, slow, or treat the occurrence of hepatotoxicity.

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**CONFLICTS OF INTEREST:** No conflicts of interest.

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