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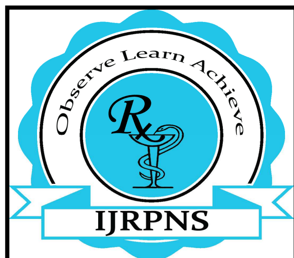

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ANTI-FIBROTIC EFFECT OF DIOSMIN AGAINST DMN-INDUCED LIVER FIBROSIS IN RATS: A BIOCHEMICAL ANALYSIS

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ABSTRACT

Diosmin is a flavone glycoside, having anti-inflammatory and anti-cancer properties. The protective effect of diosmin during hepatic fibrosis remains elusive. The main cause of liver fibrosis is the activation of hepatic stellate cells (HSC) mainly via oxidative damage. Activated HSCs was known to create imbalance in levels of liver marker enzymes like AST, ALT, ALP and triggers lipid peroxidation, and enzymatic antioxidants and alters the hepatic function. Due to the accumulation of extracellular matrix the levels of hydroxyproline were also measured to observe the collagen formation which is the principle marker of hepatic fibrosis. Our aim of the present study was to evaluate the modulatory effect of diosmin on above mentioned factors during experimentally DMN induced liver fibrosis. Group I rats served as control. Group II rats were induced with Dimethylnitrosamine (DMN) (10mg/kg b.w) for three consecuetive days in a week for 4 weeks by intraperitoneal injection. Group III rats exposed to DMN and treated with diosmin (20mg/kg b.w) intragastrically. Group IV rats were administered with diosmin alone for 4 weeks which serve as a drug control. In the present study it was observed that treatment with diosmin significantly ($p < 0.005$) attenuates the levels of LPO, SOD, CAT, GSH, GPx, GR and repair the hepatocytes thereby reduce the serum markers ALT, AST & ALP which were observed to be significantly higher in DMN induced rats. Further treatment with diosmin attenuated collagen accumulation, as demonstrated via hydroxyproline content and restored the histological alterations. Findings from the present study show that diosmin alleviates DMN-induced liver fibrosis in rats.

KEYWORDS

HSC, Diosmin, Liver fibrosis, Lipid peroxidation and DMN.

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INTRODUCTION

Hepatic fibrosis and its associated liver diseases increase the risk of morbidity and mortality if left untreated. Factors like alcohol consumption, autoimmune disorders, drugs, helminthic infection, iron or copper overload and biliary obstruction seems to cause morbidity and mortality of liver fibrosis worldwide[1]. Accumulation of extracellular matrix (ECM) in liver parenchyma is the

characteristic feature of liver fibrosis. Since hepatic fibrosis is the wound healing process, advanced fibrosis, results in an imbalance between the production and dissolution of ECM, and further develop to cirrhosis. Excessive deposition of collagen disrupts the normal architecture of the liver and alters liver homeostasis[2]. To-date scientific approach to regulate or control liver fibrosis remains a major challenge and search for an ideal agent to regulate the same is in great demand and this leads to urge for the discovery of potential agents[3].

During hepatic fibrosis, hepatic stellate cell (HSC) formally called the Ito cell responsible for the synthesis of collagen formation gets activated. The activated HSC undergo trans-differentiation from a quiescent vitamin A storing cell into a my fibroblast like cells which enable the appearance of alpha smooth muscle action (α SMA)[4]. Liver fibrosis is a chronic liver disease and hence the treatment should be more safe, tolerable and specific towards the damaged hepatocytes. Till now no anti-fibrotic agent has been approved in clinical practice[5]. Hence, search for agents that could alleviate liver fibrosis with less side effects are warranted greatly.

Diosmin, is a naturally occurring flavone glycoside, found mainly in the citrus pericarp[6]. Diosmin was reported to possess biological functions like antioxidant[7], anti-inflammatory activity[8], anti-diabetic activity and anti-proliferative and anti-cancer activities[9]. Diosmin is also known to be a vascular protecting agent, it increase the venous tone, lymphatic drainage and reduces the capillary hyper permeability, and reduces the release of inflammatory mediators[10]. The present study was an attempt to analyze the protective efficacy of diosmin against experimentally induced liver fibrosis. Here we observed that status of biochemical markers like antioxidant levels, LPO levels, collagen accumulation and histological abnormalities were modulated and there by demonstrated to possess protective efficacy of diosmin against liver fibrosis.

MATERIAL AND METHODS

Experimental animals

Adult male albino rats of Wistar strain, weighing 150–180g were used. Animals were purchased from King Institute for preventive medicine, Guindy, Chennai. All the experiments were carried out in accordance with the institutional animal ethics committee (IAEC No.06/02/2011). Prior to the experiments, the animals had free access to food and water *ad libitum* and maintained under constant conditions of temperature (23±1°C), humidity 60 %, with 12-h light/12-h dark schedule (lights on 09:00–21:00). Animals were acclimatized in polypropylene cages with a wire mesh top and a hygienic bed of husk.

Chemicals and their sources

Diosmin, Dimethyl nitrosamine, Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) were purchased from sigma chemical company, St Louis, MO, USA. All the other chemicals and reagents used were of analytical grade and were procured from SRL Chemicals Pvt. Ltd (Mumbai, India).

Experimental Design

Group I: Normal control rats fed with standard diet and water.

Group II: Hepatic fibrosis was induced by intraperitoneal injections of 10mg/kg dimethylnitrosamine (DMN dissolved in saline) for 3 consecutive days each week over a period of 4 week. (28 days).

Group III: Rats administered with DMN (as in group II) and treated along with Diosmin orally (20mg/kg in 0.1% DMSO). Diosmin treatment was started one week prior to the first dose of DMN administration and continued for 4 weeks.

Group IV: Diosmin was administered (as in group III) to assess the cytotoxicity (if any) induced by diosmin.

At the end of the experimental period, (29th day) rats were sacrificed under zylazine and ketamine anesthesia. Liver specimens were fixed in 10% neutral buffered formalin (NBF) for histochemical studies. The remaining liver tissues were homogenized with 10% homogenizing buffer and immediately utilized for biochemical assays.

Analysis of Serum markers

Aspartate transaminase (AST), alanine transaminase (ALT), and Alkaline Phosphatase (ALP) activities were estimated using a standard protocol[11, 12].

Analysis of liver fibrotic markers:

Hydroxyproline

Hydroxyproline was measured according to the method of Woessner[13]. Approximately 100 mg of liver tissue was hydrolyzed in 2 ml of 6 M HCl in a sealed tube at 110°C for 24 h. The hydrolysates were dried in a water bath set at 60°C. The residues were dissolved in 10 ml of distilled water and filtered through 0.45 mm Millipore filter. For the determination of hydroxyproline content, 1 ml of the clear filtrate was mixed with 1 ml of freshly prepared 1.4 % chloramine-T solution and allowed to stand at room temperature (RT) for 20 min. Then, it was mixed with 1 ml of 19 % perchloric acid and incubated at RT for 5 min. Finally, 1 ml of freshly prepared 20 % p-dimethylaminobenzaldehyde in CMC (critical micellar concentration) was added, mixed well and placed in a water bath at 60°C for 20 min. After cooling, absorbance was measured at 560 nm in a UV-VIS spectrophotometer.

Assay of lipid peroxidation and enzymatic antioxidants

The liver homogenates were used for the measurement of reduced glutathione (GR), superoxide dismutase(SOD)[14], catalase (CAT)[15], glutathione-S-transferase(GST) and Glutathione reductase[16], glutathione peroxidase (GPx)[17] measurement of lipid peroxidation (LPO) in terms of thiobarbituric acid reactive substance (TBARS)[18], were observed to know the efficacy of diosmin in chronic liver disease.

Histopathological analysis

For histological examination, liver tissues were collected from all groups, then washed in ice-cold normal saline, cut into small pieces and fixed in 10 % formalin for 24 h and processed further. 10 x 5 x 3 mm sized tissue blocks of the organs were processed for paraffin embedding. Thin sections of 4–5 micrometer (mm) thickness were cut with rotary microtome and stained with hematoxylin and eosin (H&E) and masson tri chrome stain. The sections were observed under light microscope and photographs were taken at different magnifications.

Statistical analysis

All the data were expressed as mean ± standard deviation (SD) of number of six animals in each group of experiments (n = 6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS Version 15. Values were considered statistically significant at ($P < 0.05$).

RESULTS

Effect of Diosmin on body weight and liver weight changes

Table No.1 shows the effect of DMN and diosmin on body weight, liver weight in control and experimental group of animals. There was a significant increase ($P < 0.05$) in liver and body weight gain were observed in DMN induced animals. Diosmin treatment markedly ($P<0.05$) reduces body weight and liver weight gain. Control and drug control animals do not exhibit any significant alterations.

Effect of Disomin on status of Serum marker enzymes

Table No.2 shows the activities of serum marker enzymes in control and experimental animals. Serum marker enzyme AST, ALT and ALP were found to be markedly ($P<0.05$) elevated in DMN administered rats. Treatment with diosmin, significantly reduced ($P<0.05$) the activities of these enzymes ($P<0.05$) as compared to DMN administered rats. Control and drug control animals does not exhibit any significant alterations.

Effect of Diosmin on the levels of hydroxyproline content

Table No.3 shows the evaluation of liver fibrosis by measuring the hepatic hydroxyproline. Levels of hydroxyproline will represents the amount of collagen present. Administration of DMN shows increased level of hydroxyproline content compared to that of control group, whereas diosmin treatment attenuated the DMN-induced rise in hepatic hydroxyproline content compared to that of group 2 rats. This result shows a representation percentage of collagen accumulation in control and experimental groups. No significant change observed in group 1 and group 4 rats.

Effect of Disomin on the status of Lipid peroxidation and enzymatic antioxidants

Table No.4 shows the status of lipid peroxidation and enzymatic antioxidants in control and experimental group of rats. Malondialdehyde (MDA) is a product of LPO and serves as an oxidative damage index. Due to increase in oxidative stress and LPO the antioxidative levels (SOD, CAT, GPx, GST, GSH, GR) are also decreased in DMN treated groups. There was a significant increase in the levels of MDA in DMN administered rats ($P<0.05$) when compared to normal control rats. Diosmin treatment significantly reduced the levels of MDA ($P<0.05$) and increases the levels enzymatic antioxidants when compared to DMN induced animals. Control and drug control animals doesn't exhibit any significant alterations.

Effect of Disomin on histological changes

Figure No.1 and 2 shows the effect of diosmin on DMN-induced hepatic fibrosis as evaluated by histopathological examination of the liver with H and E staining and accumulation of collagen by massontrichrome stain. In the H and E stain the DMN induced animals exhibited significantly disrupted tissue architecture and caused centrilobular necrosis in the hepatic sinusoid. However, these centrilobular necrosis in the hepatic sinusoid, mainly the necrotic area, these were reduced in the liver sections of the diosmin animals. Control and drug control animals exhibited normal liver architecture. In Figure No.2 shows the accumulation of collagen in the hepatocytes in DMN induced rats, these comparatively gets reduced upon diosmin treatment groups.

DISCUSSION

Hepatic fibrosis (HF) arises as a result of imbalance between ECM production and degradation. The components of hepatic ECM comprises of structural and supporting molecules, amongst HSCs are the major connective tissue-producing cell type in liver[19]. Free radical generation, mitochondrial dysfunction and depletion of antioxidants lead to the progression of fibrosis and cirrhosis[20]. In the present study administration of DMN to rats caused visual and quantifiable responses that were demonstrated from alterations in the body and liver weight gain, distinct stained patterns in collagen staining and histological abnormalities. All these observations were indications of hepatic fibrosis and it supports the documented reports that DMN induced hepatic fibrosis[21].

DMN contributes to fibrotic progression through multiple mechanisms like oxidation of metabolic products, oxidative stress, compromised antioxidant defenses and augmented lipid peroxidation. In the present study DMN induced rats lacks body weight gain as compared with controls Table No.1. Factors like decreased nutrient absorption, increased energy utilization and metabolic efficiency were presumed to be responsible for the decreased body weight gain during DMN administration. Disomin treatment leads to increased body weight gain in DMN administered rats. This observed efficacy is attributed to its ability to alter nutrient absorption and restores liver homeostasis and this highlights the protective efficacy of disomin.

Oxidative stress is the main cause of chronic liver injury. Upon chronic injury the liver gets oxygen in demand and generates free radicals. This upon decrease the antioxidant level and increase the LPO. Further the status of antioxidant capacity in vivo was reduced in DMN induced rats when compared to control rats, whereas diosmin treatment in turn elevated the levels to near normalcy. On the other hand, DMN induced rats exhibited increased values of cellular lipid peroxidation which measured through the TBARs levels when compared with control rats. Levels of SOD, CAT, GPx, GST, GSH in the liver homogenates of DMN administered rats were significantly lower when compared with control animals. Treatment with disomin reduced the extent of LPO and restored the antioxidant levels. This observation substantiate the fact that diosmin scavenges the reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion O_2^-, and hydroxyl radical, thereby enhances antioxidant defense system. This observation is in agreement

with previous report denotes that diosmin possess antioxidant activity and it inhibits LPO *in vivo*[22].

In the DMN administered rats we determined the levels of serum markers ALT, AST, ALP in Table No.2 as index for liver function. DMN administered rats showed significantly increased levels of these markers when compared to control animals. This could to be attributed to the mitochondrial damage and necrotic hepatocytes and a possible cholestasis that was caused by DMN which is further supported by loss of histoarchetecture of hepatocytes[23].

The main target of diosmin in hepatic fibrosis is targeting the non-parenchymal hepatic stellate cells which will attenuate the deposition of extracellular matrix. This was confirmed by measuring the hydroxyproline, responsible for the synthesis of collagen. Further DMN caused damage to erythrocyte membrane thereby leading to increase serum bilirubin concentrations. The end product of hemoglobin, the bilirubin serves as an index of hepatic injury and this supports the necrotic condition of hepatocytes[24]. This denotes that disomin by its ability to protect erythrocyte integrity, maintains homeostasis in carbohydrate, protein and lipid metabolism exerts anti-fibrotic activity. All these biochemical properties were finally proved in Histopathological stain and masson trichrome stain which exhibit there changes in hepatocyte architechture.

Table No.1: Effect of disomin on the status of body weight and liver weight in control and DMN induced liver fibrosis rats

S.No	Serum marker	Group 1 Control	Group 2 Induced	Group 3 Treated	Group 4 Drug Control
1	AST U/L	45±1.8	101±9.90^{a}	48±2.88^{b}	42±2.1^{ns}
2	ALT U/L	49±2.45	120 ±9.6^{a}	40±2.4^{b}	50±1.5^{ns}
3	Total Protein mg/dl	6.1±0.12	5.5±0.27^{a}	6.8±0.27^{b}	6.4±0.12^{ns}
4	ALP U/L	220±8.8	380±26.6^{a}	245±14.7^{b}	238±11.9^{ns}

Table No.1: Rats administered with DMN by intraperitoneal (i.p) injection three times per week for 4 weeks showed significant difference ($P < 0.05$) in body and liver weight again when compared with normal control rats. DS administered intragastrically (i.g) six times per week for 4 weeks along with DMN showed significant changes ($P < 0.05$) when compared with group 2 rats. Values are given as mean ± SD from six rats in each group.

Table No.2: Effect of diosmin on the levels of serum marker enzymes in control and DMN induced liver fibrosis rats

S.No	Groups	Group 1 Control	Group 2 DMN induced	Group 3 DS + DMN	Group 4 DS alone
1	Initial body weight(g)	250±12.5	245±19.6[a]	248±14.88[b]	240±12[ns]
2	Final body weight (g)	255±12.7	260±20.8[a]	258±15.48[b]	246±12.3[ns]
3	Liver weight (g)	8.07±0.4	9.60±0.86[a]	9.1±0.63[b]	8.0±0.48[ns]

Table No.2: Shows the status of serum markers AST, ALT, ALP and total protein during DMN induced liver fibrosis. Levels show a significant change ($p < 0.05$) in group 2 and group 3 when compared with group 1 and group 2, respectively. Values are expressed as mean ± SD from six rats in each group.

Table No.3: Effect of Disomin on the status of Hydroxyproline in control and experimental groups of animals

S.No	Groups	Hydroxyproline mg/g of protein
1	Group 1 (Control)	5± 0.15
2	Group 2 (DMN)	11.5 ± 0.69[a]
3	Group 3 (DS+DMN)	7.2 ± 0.43[b]
4	Group 4 (DS)	6 ±0.3 [ns]

Table No.3: Shows the status of Hydroxyproline in liver tissues of control and experimental groups of animals. HP level is significantly increased in DMN administered animals as compared to normal rats. Treatment with disomin significantly reduced the HP levels as compared to DMN administered rats. Values are expressed as mean ± SD from six rats in each group. (a) control vs. DMN group, (b) DMN vs. DS+DMN group, (ns) non-significant.

Table No.4: Effects of diosmin on hepatic antioxidant enzyme activities in control and DMN induced rats

S.No	Enzymic antioxidants	Group 1 Control	Group 2 DMN	Group 3 DS+DMN	Group 4 DS
1	SOD Units/min/mg ptn	8.8±0.08	3.5±0.1[a]	5.5±0.11[b]	8±0.08[ns]
2	CAT µmol H_2O_2 consumed/ min/mg ptn	60±1.2	41.1±2.05[a]	48.55±1.94[b]	59±1.18[ns]
3	GPx µmol of GSH consumed /min/mg ptn	91.5±1.83	52.1±3.1[a]	63±2.52[b]	89.04±1.78[ns]
4	GST nmol of CDNB conjugated/min/mg ptn	3.89±0.07	1.22±0.061[a]	2.9±0.08[b]	3.9± 0.07[ns]
5	GR µmol of NADPH oxidized/min/mg ptn	140±1.4	76.64±3.8[a]	95.1±4.7[b]	146.8±4.4[ns]
6	GSH (µM/mg of tissue)	1.9±0.03	1.2±0.084[a]	1.6±0.06[b]	2.09±0.04[ns]
7	Malondialdehyde (nmol/mg protein)	0.195±0.0097	0.255±.02[a]	218±6.54[b]	0.199±0.003[ns]

Table No.4: Shows the status of LPO and enzymatic antioxidants in liver tissues of control and experimental groups of rats. Group 2 animals show a significant change ($p < 0.05$) during DMN induced intraperitoneally when compared with the normal standard diet. Group 3 rats showed significant increase in the level of antioxidant enzymes when compared to group 2 animals. Control and drug control animals does not showed any changes in the levels of antioxidant enzyme activity. Values are expressed as mean ± SD from six rats in each group. (a) control vs. DMN group, (b) DMN vs. DS+DMN group, (ns) non-significant. SOD: superoxide dismutase. CAT: catalase. GPx: glutathione peroxidase. GST: glutathione S transferase. GR: glutathione reductase.

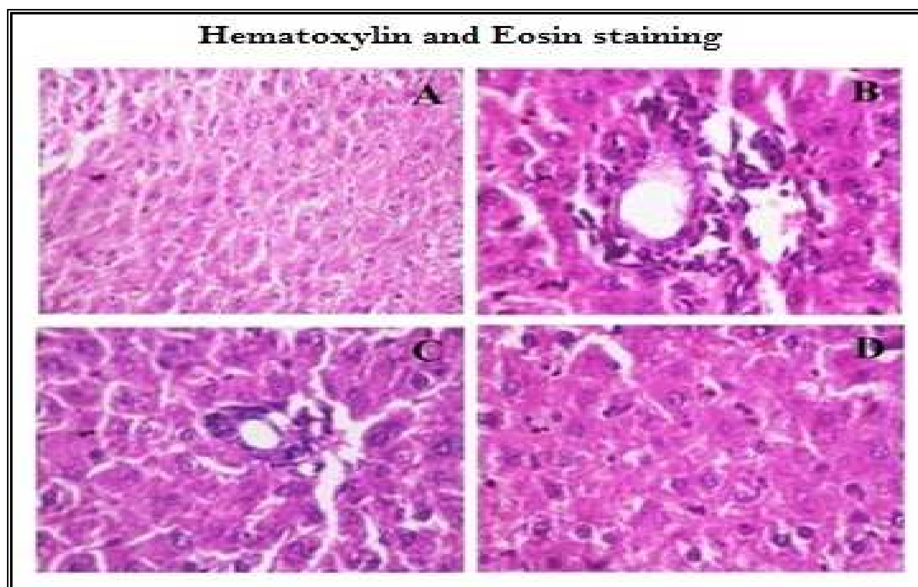

Figure No.1: Histological analysis of liver sections. The sections were stained with hematoxylin-eosin (H&E) (A) Normal. (B) DMN (10 mg/kg per day for three consecutive days of each week for 4 weeks) alone-treated group. Centrilobular necrosis and fibrosis were seen in this group. (C) DMN with diosmin (20 mg/kg)-treated group shows the recovery of hepatocyte damage cell.

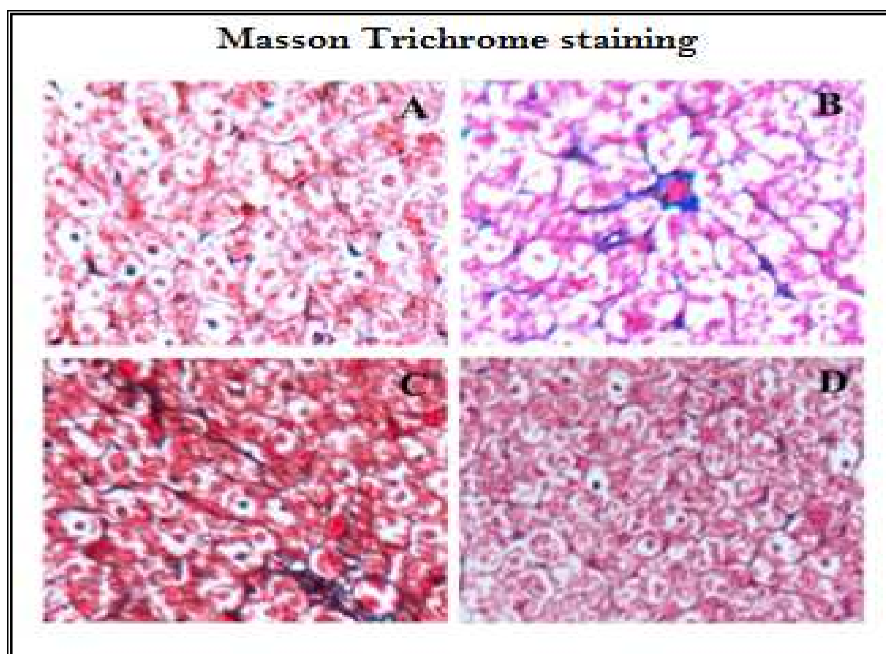

Figure No.2: Shows the presence of fibrosis marker collagen stained in blue in the HSC cells. (A) shows the normal architechture. (B) shows the relative fibrosis area which stained in blue, expressed as the percentage of the total liver area with collagen accumulation (C) the accumulation of collagen get condensed in DS treated groups. (D) Does not shows any changes. All this was determined using Masson's trichrome-stain.

CONCLUSION

All the observations analyzed in the present study provided a biochemical evidence for the hepatoprotective efficacy of diosmin against DMN induced liver fibrosis. Further the mechanistic insights and the role of diosmin in reducing pathological status like inflammation and hepatic cell death during DMN induced liver fibrosis are warranted. Further studies involving the role of Disomin in modulating signaling mechanisms are in progress.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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