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RESEARCH ARTICLE

Protective potential of Hesperidin against Diethylnitrosamine induced Hepatocarcinogenesis in rats

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ABSTRACT

The present study was designed to investigate the hepatoprotective activity of hesperidin against Diethylnitrosamine (DENA) induced Phenobarbital (PB) promoted hepatotoxicity in Wistar albino rats. Twenty four male Wistar rats were divided into 4 groups of 6 animals each and treated as follows: Group-I rats served as vehicle control and were treated with saline, Group-II rats were given hesperidin orally (100mg/kg) for 14 weeks, Group-III rats were administered with a single dose of DENA (200mg/kg) followed by PB orally (0.05% in drinking water) after seven days for 13 weeks, Group-IV rats were given hesperidin prior to the injection of DENA and oral administration continued for 14 weeks with the administration of PB. Administration of DENA and PB altered the levels of diagnostic liver markers (AST, ALT, ALP), antioxidant enzymes (SOD, CAT, GSH, GPx, GR) and serum bilirubin. The treatment with hesperidin restored the levels near to normal thereby confirming hepatoprotective activity of hesperidin. A section of liver was also subjected to Histopathological analysis. The findings explore that the treatment with hesperidin shows promising protective effect against DENA induced PB promoted hepatic damage.

Key words: Antioxidant enzymes, Diethylnitrosamine, Hepatocarcinogenesis, Hesperidin, Liver Marker Enzymes, Phenobarbital.

INTRODUCTION:

A large number of agents including natural and synthetic compounds have been reported which have some chemo preventive value. These include the flavonoids (Hesperidin, Rutin, Quercetin, Nobeletin); phenolic acids (Capsaicin, Ellagic acid, Gallic acid); triterpenoids (Ursolic acid) etc. Chemically, Flavonoids are polyphenolic compounds that are ubiquitous in nature and are grouped according to their chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities.

Flavonoids are present in fruits, vegetables, and beverages such as tea, coffee, beer, wine, and few fruit drinks and possess strong antioxidant and radical scavenging activities. This makes flavonoids to act as antiviral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor agents. Hesperidin is one among over 4000 flavonoids found in plants and is the abundant and inexpensive by-product that comes from citrus fruits thus making it a naturally occurring citrus bioflavonoid also known as a phytochemical. Hesperidin is a citrus flavonoid possessing anti hypercholesterolemic activity [1], anti-inflammatory, analgesic activity [2], anti-fungal [3], and anti-oxidant activity [4].

MATERIALS AND METHODS:

Materials used:

Diethylnitrosamine and Hesperidin was purchased from Sigma aldrich, USA. All other chemicals used were of analytical grade and were purchased locally.

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Animals:

Male albino Wistar rats (150 ± 10gms) were used for the study and were obtained from the animal house, Vel's College of Pharmacy; Pallavaram, Chennai, India. The rats were housed under conditions of controlled temperature (25 ± 2^{O}C), humidity (50 ± 5%) and were acclimatized to 12-h light-dark cycles. Experimental animals were used after obtaining prior permission and handled according to the Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment; Government of India.

The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. The animals were deprived of food for 24 hr before experimentation but allowed free access to tap water through out.

Hepatoprotective Activity:

The animals were fed with commercially available standard rat pellet feed from M/s Hindustan Lever Ltd; Bangalore, India. The feed and water were provided *ad libitum*. The experiments were performed in day light in accordance to the current guidelines for the care of laboratory animals and ethical ethical guidelines for the investigation of pain in conscious animals. Hepatocarcinogenesis was induced by single intraperitoneal injection of DENA (200mg/kg). After 1 week, the rats were given PB (0.05% in drinking water) for 13 weeks [5].

Twenty four rats were made into four groups: *Group I:* Normal control rats were supplied with sufficient amount of food and water. *Group II:* Rats were given hesperidin orally (100mg/kg/day) for 14 weeks. *Group III:* Rats were given a single intraperitoneal injection of DENA (200mg/kg). After 1 week, the rats were given PB (0.05% in drinking water) for 13 weeks. *Group IV:* Rats were given hesperidin (100mg/kg) one week prior to the injection of DENA. The oral administration was then continued throughout the experimental period (14 weeks) along with the administration of PB.

After the end of experimental period, all the animals were anaesthetized and decapitated. Liver tissues were immediately excised and rinsed in ice-cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected and serum was separated for analysis of biochemical parameters. Small pieces of liver tissues were collected in 10% formal saline for proper fixation. These tissues were processed, embedded in paraffin wax sections of 5-6 µm thick, cut and stained with hematoxylin and eosin. The sections were examined under light microscope at 400X and photomicrographs were obtained.

RESULTS AND DISCUSSION:

Diethylnitrosamine chemically belonging to the N-Nitrosamine family is proved to be one of the potent carcinogens that is primarily metabolized by the cytochrome P-450 enzymes to reactive electrophiles (O6 alkyl-guanine and N7 alkyl-guanine) which are proved to be cytotoxic [6], carcinogenic [7] and mutagenic [8]. DENA through normal or futile mechanisms causes production of ROS, and continuous or excessive exposure of ROS on the cells causes DNA damage; oxidations of amino acids in proteins and polyunsaturated fatty acids in lipids [9]; as well as oxidative inactivation of the specific cellular enzymes, thereby causing oxidative stress [10].

As per the results obtained, the animals treated with DENA showed a significant incidence of liver tumour at the end of 14 weeks which was evidenced by morphological changes such as increase in rat liver weights (Fig-I) and decrease in the rats body weights. Table I shows that the final body weights of control rats after a period of 14 weeks was 274.50 ± 8.66 grams which decreased significantly after administration of DENA to 211.33 ± 13.26 grams. Treatment with hesperidin however significantly restored the body weight to 264.16 ± 15.62 grams ($p < 0.05$). The table also shows that the liver weights of control rats were 2.77 ± 0.08 which significantly increased to 6.23 ± 0.38 in case of DENA induced rats. Treatment with hesperidin however lowered the liver weights to 3.10 ± 0.12 which indicates protective effect of hesperidin on DENA induced hepatic damage.

The levels of marker enzymes in the serum provide more information about the type and extent of hepatocellular damage. The results proved that induction of DENA leads to increased levels of diagnostic liver marker enzymes (AST, ALT, and ALP) and bilirubin in serum. This is because these are cytoplasmic enzymes and are released into circulation [11]. The marked increase of serum liver markers causes damage to the structural integrity of the cell membrane [12]. Table II indicates that the AST level significantly raised from control 115.88 ± 5.16 to 365.52 ± 12.10 after DENA induction. Administration of hesperidin at a dose of 100mg/kg to intoxicated rats caused a significant reduction in AST level to 256.25 ± 10.15. ($p < 0.05$). Similarly the ALT level raised from control 42.04 ± 1.28 to 141.32 ± 5.45 after DENA administration. Treatment with Hesperidin however showed a significant

reduction in ALT level to 96.21 ± 3.10. In case of ALP also the level raised from control 58.92±2.42 to 134.48±4.24 after DENA administration. Treatment with Hesperidin caused a significant reduction in the ALT levels to 84.70±2.35.

SOD, CAT, GPx and GR are important anti per oxidative enzymes which effectively scavenge the superoxide and hydrogen peroxide levels [13]. These enzymes are responsible for the protection against oxidative damage. It has been strongly reported that the administration of DENA significantly lowered the enzymatic antioxidant defense mechanism which is attributed to the oxidative stress in liver. Table III indicates a significant decrease in the serum anti peroxidative enzymes in case of DENA induced rats. The levels of SOD, CAT, GPx and GR show a significant decrease on DENA induction. This depletion is due to consumption of these enzymes to act upon the free radicals. Treatment with hesperidin however shows that the levels of the antioxidant enzymes are significantly restores and thereby proves to be a protective agent against the DENA induced oxidative stress in rat liver which may be due to the several pharmacological activities taking place.

Table 1: Effect of HESP on DENA induced alteration in body weight, liver weight and relative liver weight in control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DENA)	Group-IV (HESP+DENA)
INITIAL BODY WEIGHT	163.16 ±.8.18	172.86±11.39^{NS}	176.83±7.38^{*a}	174.16±8.01^{*b}
FINAL BODY WEIGHT	274.50±8.66	269.50±16.4^{NS}	211.33±13.26^{*a}	264.16±15.62^{*b}
LIVER WEIGHT	7.63 ± 0.44	7.45±0.76^{NS}	13.16±0.81^{*a}	8.21±0.61^{*b}
RELATIVE LIVER WEIGHT	2.77±0.08	2.75±0.11^{NS}	6.23±0.38^{*a}	3.10±0.12^{*b}

Values are expressed as mean ± S.D. for six animals. Body weight and liver weight Unit: gm. a=Comparisons are made between Group-I with Group-III. b= Comparisons are made between Group-III with Group IV. The * represent the statistical significance at $p < 0.05$. NS represents non significance at $p < 0.05$.

Table 2: Effect of HESP on DENA induced liver marker enzymes in serum of control and experimental rats.

Parameters (IU L^{-1})	Group-I (Control)	Group-II (HESP)	Group-III (DENA)	Group-IV (HESP+DENA)
AST	115.88±5.16	115.70±5.16^{NS}	365.52±12.10^{*a}	256.25±10.15^{*b}
ALT	42.04±1.28	42.56±1.25^{NS}	141.32±5.45^{*a}	96.21±3.10^{*b}
ALP	58.92±2.42	60.69±2.97^{NS}	134.48±4.24^{*a}	84.70±2.35^{*b}

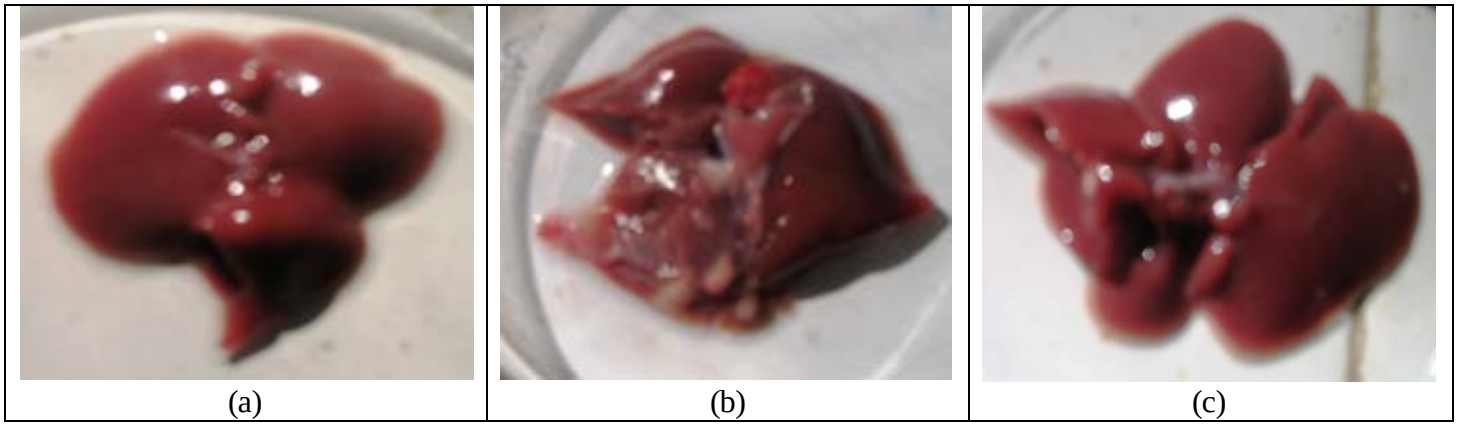
Values are expressed as mean ± S.D. for six animals. Liver marker enzymes unit: IU L^{-1} (AST, ALT and ALP). a= Comparisons are made between Group-I with Group-III. b= Comparisons are made between Group-III with Group IV. The * represent the statistical significance at $p < 0.05$. NS= represents non significance at $p < 0.05$.

Table 3: Effect of HESP on DENA induced antioxidant enzymes in liver of control and experimental rats.

Parameters	Group-I (Control)	Group-II (HESP)	Group-III (DENA)	Group-IV (HESP+DENA)
SOD (Units $min^{-1}mg^{-1}$ protein)	7.93±0.69	8.01±0.55^{NS}	4.03±0.27^{*a}	6.80±0.69^{*b}
CAT (µmole H_2O_2 consumed min^{-1} mg^{-1} protein)	69.34±4.65	68.89±3.67^{NS}	43.07±3.36^{*a}	58.37±3.48^{*b}
GPx (µmole of GSH oxidized $min^{-1}mg^{-1}$ protein);	116.85±5.28	117.73±5.74^{NS}	67.52±4.79^{*a}	89.55±7.82^{*b}
GR (µmole g^{-1} tissue)	192.55±18.23	195.32±17.66^{NS}	102.31±8.07^{*a}	151.04±7.75^{*b}

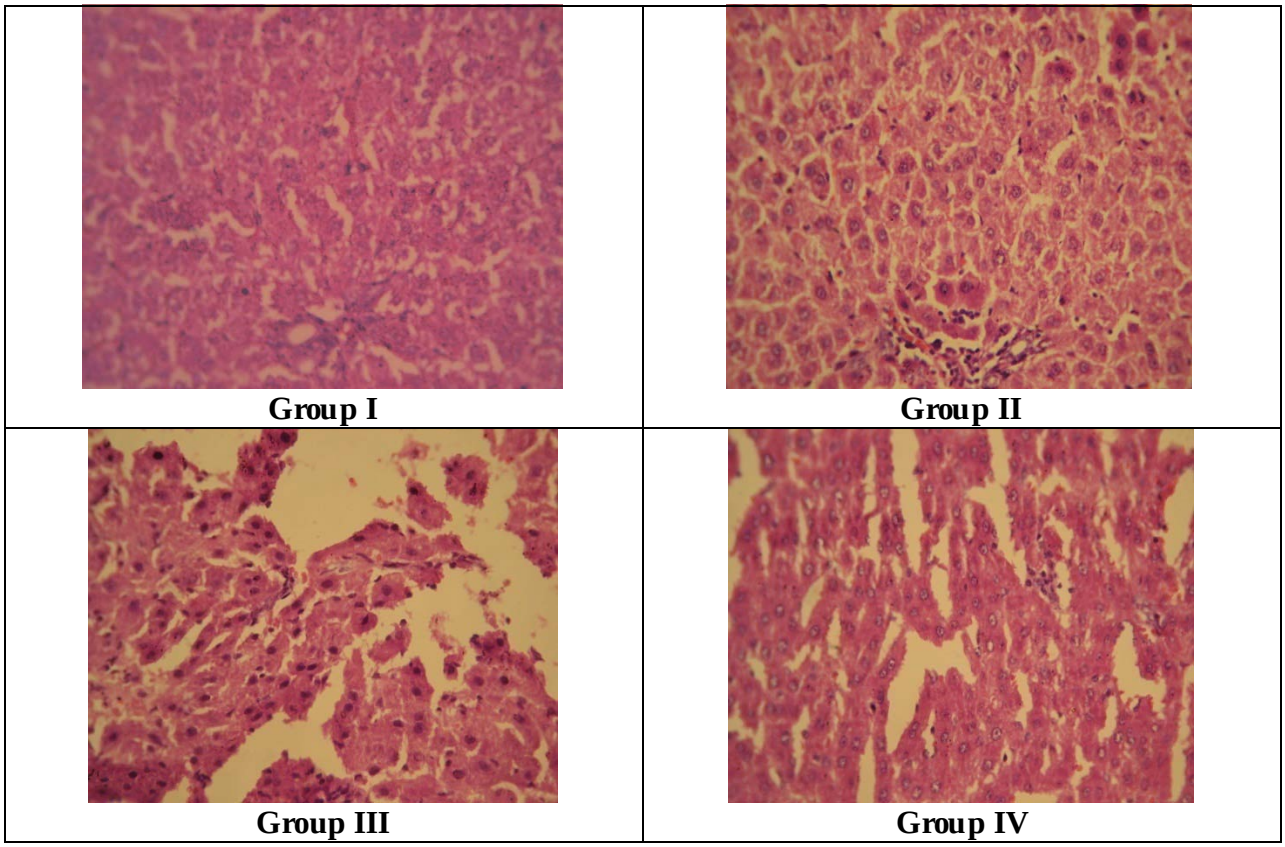
Values are expressed as mean ± S.D. for six animals. Units: SOD (Units $min^{-1}mg^{-1}$ protein); CAT (µmole H_2O_2 consumed $min^{-1}mg^{-1}$ protein); GPx (µmole of GSH oxidized $min^{-1}mg^{-1}$ protein); and GR (µmole g^{-1} tissue). a= Comparisons are made between Group-I with Group-III. b= Comparisons are made between Group-III with Group IV. The * represent the statistical significance at $p < 0.05$. NS represents non significance at $p < 0.05$.

Figure 1: Morphological changes in liver of albino rats



(a) — Normal Liver; (b) DENA Treated Liver; (c) DENA and Hesperidin Treated Liver

Figure 2: Histopathological changes in liver of albino rats



Group I and Group II indicates normal parenchymal cells with granulated cytoplasm and small uniform nuclei arranged. Group III indicates loss of normal architecture of hepatocytes with Enlarged nodules and frequently binucleated large vesicles. Group IV indicates recovery of parenchymal cells and with micro vesicular changes.

CONCLUSION:

The present study is an effort to evaluate the protective efficacy of Hesperidin by studying the morphological and biological alteration observed in DENA induced hepatocellular carcinoma in rats. Hesperidin treatment against DENA brought back the activities of marker enzymes to near normal by protecting the cell membrane integrity. Hesperidin treatment exerted a significant free radical quenching and antioxidant strengthening effects by decreasing the lipid peroxidation with consequent improvement in antioxidant status proving to be a potent antioxidant. These findings confirm that hesperidin

treatment shows promising protective effect and further studies are needed to understand the molecular mechanisms of the hesperidin. Hence it is concluded that hesperidin as potential for further development as a therapeutic agent for liver cancer.

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