Mangiferin a bioactive compound of mangifera indica l on oxidative damage and antioxidant status in n-diethylnitrosoamine induced hepatocellular carcinoma in animal model

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Abstract
Our study aims at elucidating the antioxidant efficiency of Mangiferin from (*Mangifera indica L.*) for its protective effect beside N-Nitrosodiethylamine induced Hepatocellular carcinoma (HCC) in rat liver carcinogenesis. Studies have shown that N-Nitrosodiethylamine induces lipid peroxidation and alters the antioxidant status in non-target organisms. An effort has been made to study the effect of N-Nitrosodiethylamine induced Hepatocellular carcinoma on biochemical parameters and ameliorating results of Mangiferin. Animals were segregated to six groups. Group A served as control, Group B induced with 0.01% DEN through water for 15 weeks to induce hepatocellular carcinoma. Group C received Mangiferin *via* intragastric intubation at a daily dose of 30 mg/kg body weight for 16 weeks every day. Groups D to F animals received 0.01% of DEN as in Group B along with Mangiferin *via* intragastric intubation at a daily dose of 10, 20 and 30 mg/kg body weight for throughout the experimental period for 4 month. The study highlights the effectiveness of Mangiferin as protective molecule against N-Nitrosodiethylamine induced carcinoma. Histological studies of liver tissue too correlated with the above biochemical findings. These results clearly suggest that Mangiferin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in DEN-induced liver carcinogenesis in rats.

Keywords: Mangifera indica L, Mangiferin, N-Nitrosodiethylamine, Antioxidants, Antioxidant enzymes.

Introduction
Carcinoma of hepatocytes is one of the most common malignant tumors in the world (EI-Serag HB et al., 2001). Accumulating evidence has suggested that several mechanisms contribute to the carcinogenesis of HCC (Thorgeirsson SS et al., 1998; Lau SH et al., 2005). Recent efforts to control the incidence of HCC have focused on developing effective new chemoprevention strategies. HCC induced by diethylnitrosamine in Wistar rats that shows similarities to human HCC is an ideal model for investigating the effect of intervention by chemopreventive agent (Thirunavukkarasu C et al., 2001). DEN, a hepatocarcinogen, is known to induce perturbations in the nuclear enzymes involved in DNA repair/replication (Bansal AK et al., 2005). Investigations have provided evidence that DEN causes a wide range of tumors in all animal species, and these compounds are considered to be effective health hazards to man. Man is exposed to DEN through diet, in certain occupational settings, and through the use of tobacco products, cosmetics, pharmaceutical products, and agricultural chemicals (Bartsch H et al., 1984). It has been reported that DEN, after its metabolic biotransformation, produces the promutagenic adducts, O_6-ethyl deoxyguanosine and O_4- and O_6-ethyl deoxythymidine that can produce DNA chain damage, depurination or binding to DNA, and often generates a miscoding gene sequence, paving a way for the initiation of liver carcinogenesis (Verna L et al., 1996). It has also been reported to produce reactive oxygen species (ROS), a potentially dangerous by-product of cellular metabolism that may directly affect cellular development, growth, and survival (Watanabe K et al., 2000). Oxidative stress caused by ROS has been reported in membrane lipid peroxidation, DNA damage, and mutation associated with the initiation of various stages of the tumor formation process (Parola M et al., 2001). Polyphenolic compounds have the most promising pharmaceutical properties and have received greater attention than any other class of natural products to counter the ill effects of oxygen radicals (Ramesh B et al., 2006).

The term oxidative stress is commonly used to describe an imbalance between the systemic manifestation of free radicals and the capability of cells to detoxify them and negate their damaging effects on proteins, lipids, and DNA (Chandra, K et al., 2015). The perceptual origin of "oxidative stress" is tracked back to the 1950s and the term began to be used frequently by scientists from 1970 as they started to unravel the effects of free radicals and ionizing radiation (Gerschman, R et al., 1954). The important relationship between oxidative stress and a wide variety of human diseases has placed this stress factor at the forefront of diseases research. Indeed, diseases such as Rheumatoid arthritis (RA), (Stamp, LK et al., 2012; Hassan, S.Z et al., 2011) Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) (Gandhi, S et al., 2012), Cardiovascular disease (CVD) (Rochette, L et al., 2013), allergies (Dozor, AJ 2010), immune system dysfunctions (Zhou, R et al., 2010), diabetes, and cancer are all related to oxidative stress. The important intracellular signaling molecules in RA are reactive oxygen species (ROS), which may damage matrix components and enhance the synovial inflammatory proliferative response in immune

system cells (Hitchon, C.A et al., 2004). Oxidative stress conditions may also make T-cells resistant to growth or death stimulators (Griffiths, HR et al., 2005). Moreover, the pathological role of mitochondrial respiratory chain dysfunction and also the roles of oxidative stress in neurodegenerative disease such as AD and PD are well known.

Reactive oxygen species (ROS) Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell, B et al., 1999). The addition of an electron to dioxygen forms the superoxide anion radical (O2•−) (Halliwell, B et al., 1999). The addition of an electron to dioxygen forms the superoxide anion radical (O_2•−), (Miller, DM et al., 1990). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly/prevalently through enzyme- or metal-catalysed processes (Valko, M et al., 2005). The production of superoxide occurs mostly within the mitochondria of a cell (Cadenas, E et al., 1998). The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Kovacic, P et al., 2005; Valko, M et al., 2004). Recently, it has been demonstrated that Complex I-dependent superoxide is exclusively released into the matrix and that no detectable levels escape from intact mitochondria (Muller, FL et al., 2004).

Materials and Methods

Chemicals

Mangiferin Standard and Mangiferin was isolated from Mango Tree Stem Part, (DEN) N-Nitrosodiethylamine was purchased from Sigma Aldrich Chemical Company, Saint Louis, MO, USA. All other chemicals used were of good quality and analytical grade.

Animal model

Male albino rats of Wistar strain (180 ±200 grams) procured from Tamil Nadu University for Veterinary and Animal Sciences, (TANUVAS) Chennai, India were used for the study. Animals were fed with standard rat pelleted feed and water was provided *ad libitum*. Animals housed in controlled temperature (25±2°C).According to the institutional animal ethical committee experiments were done.

Induction of Tumor

Hepatocellular carcinoma (HCC) was induced in Male Wistar rats by administering N-Nitrosodiethylamine (DEN) at 200 mg/kg body weight in drinking water for 16 weeks. Male Wistar Rats (Rattus novergcus) (150±180 g) procured from Tamil Nadu University for Veterinary and Animal Sciences, (TANUVAS) Chennai, India were used for the study. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water was provided ad libitum. The rats were housed under conditions of controlled temperature (30±2°C) and acclimatized to 12-hours light, 12-hours dark cycle. The cages containing a hygienic bed of husk in a specific-pathogen free animal room under controlled conditions. They were provided with standard food pellets (diet composition: wheat broken moisture 9.0%, crude protein 11.5%, crude fat 1.9%, crude fiber 4%, ash 0.2%, and nitrogen-free extract 73.4%) supplied by Hindustan Lever Ltd, Mumbai, India, and tap water ad libitum. Animal experiments were conducted according to the guidelines of institutional animal ethical committee. Animals were randomly divided into 6 groups containing a total of 16 animals (Group A to Group F).

Formulation and administration of mangiferin

Mangiferin was freshly prepared dissolved in 10% Dimethyl sulfoxide (DMSO) at a daily dose of 10 mg/kg, 20 mg/kg, and 30 mg/kg body weight.

Seggregation of groups

Experimental animals were divided into 6 groups of 16 rats as follows.

Group A: Served as Normal control and received standard pellet diet.
Group B: Rats had hepatocellular carcinoma induced by 0.01% DEN through normal water for 15 weeks.
Group C: Rats received Mangiferin *via* intragastric intubation at a daily dose of 30 mg/kg body weight for 4 months.
Group D: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with Mangiferin *via* intragastric intubation at a daily dose of 10 mg/kg body weight for 4 months.
Group E: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with Mangiferin *via* intragastric intubation at a daily dose of 20 mg/kg body weight for 4 months.
Group F: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with Mangiferin *via* intragastric incubation at a daily dose of 30 mg/kg body weight for 4 months.

At the end of the experimental period, the rats were sacrificed by cervical dislocation and blood samples and liver tissue from the animals were taken for analysis.

Preparation of tissue homogenate and histopathological changes

After sacrifice, the liver tissue was macroscopically examined for the presence of tumors or other pathological lesions. Tissues with abnormal morphology were fixed in

10% buffered formalin and embedded in paraffin blocks. Histological sections stained with hematoxylin and eosin was used to confirm the presence and type of tumors by histopathological examination, which was performed by a pathologist unaware of the experimental codes. Liver tissue was removed immediately and washed with ice-cold saline and homogenized in the appropriate buffer in a tissue homogenizer.

Results and Discussions

Control Group A rats revealed normal liver parenchyma cells with granulated cytoplasm, small uniform nuclei, and central vein surrounded by cords of hepatocytes. Group B DEN-treated rats showed loss of architecture and lobules of neoplastic hepatocytes with a fecal area of fatty change. Group C rats exhibited normal architecture, indicating the non-toxic nature of α-momorcharin. Groups D and E rats along with Mangiferin and DEN showed moderate cancerous change, fatty change, and hydropic degeneration. Group F rats showed fewer neoplastically transformed cells and the hepatocytes maintained near-normal architecture.

Effect of mangiferin on lipid peroxidation levels in both serum and liver

Significantly increased levels of lipid peroxidation were observed in DEN-induced liver cancer-bearing animals in Group B. Administration of Mangiferin to DEN-induced rats in Groups D, E, and F, significantly decreases the lipid peroxidation level, which was brought to near-normal. There were no significant differences observed in Mangiferin- treated in Group C and control rats in Group A.

Effect of mangiferin on the antioxidant defense system

The levels of plasma and liver tissue enzymatic antioxidants in Graph 1 and 2 in control and experimental rats. The control rats Group A had normal levels of these enzymes whereas HCC-induced rats in Group B showed significantly reduced levels when compared to other groups. Mangiferin given alone in Group C highlights the increased levels of these enzymes when compared to control rats. The administration of Mangiferin to DEN-induced rats in Groups D, E, and F restored the changes to near-normal levels due to the antioxidant efficacy of Mangiferin.

Effect of mangiferin on hepatic marker enzymes - AST, ALT, ALP, and LDH

The levels of the tissue hepatic marker enzymes in Table 1 of control and experimental rats. DEN-induced rats in Group B exhibited a significant elevation in the activity of these marker enzymes when compared to control rats in Group A, whereas Mangiferin treated rats along with Mangiferin and DEN, Groups D, E, and F showed a significant decrease in the levels of these marker enzymes when compared with DEN-induced rats. Table 2 gives the levels of the serum marker enzymes of control and experimental rats. DEN administered rats in Group B showed a significant increase in the activity of these marker enzymes when compared to control rats in Group A, whereas DEN-induced rats treated with Mangiferin in Groups D, E, and F, showed a drastic decline in the levels of these marker enzymes when compared with DEN induced rats in Group B.

Mangiferin effect on non-enzymatic antioxidant status in control and experimental rats

The levels of hepatic tissue non-enzymatic antioxidants in Table 3 in control and experimental rats. The enzyme levels of control rats in Group A, were normal whereas the levels in DEN HCC-induced rats in Group B were significantly reduced when compared to other groups. Mangiferin alone in Group B showed increased levels of these enzymes when compared to control rats. In the rats along with Mangiferin and DEN in Groups D, E, and F, antioxidant levels were restored to near-normal by the antioxidant efficacy of Mangiferin.

Statistical Analysis

Data were statistically evaluated using one way ANOVA and expressed as Mean±SD. Kruskall Wallis test and Mann Whitney U test using 11.0 version of SPSS Software were used when applicable. $p \leq 0.001$ was considered to be significant.

Mangiferin administration to DEN-treated rats at 3 different doses (10 mg/kg, 20 mg/kg, and 30 mg/kg body weight) reverses the changes in liver-specific enzyme levels both in the serum and tissue. Mangiferin treatment may significantly attenuate the increased activities of these enzymes, which may be due to the ability of Mangiferin to protect the cells from membrane damage and maintain membrane integrity, thereby decreasing enzyme leakages.

Naturally-occurring antioxidants induce a variety of biological activities, including the induction of drug-metabolizing enzymes, inhibiting carcinogen-induced mutagenesis, and scavenging of free radicals (Hirose M *et al*., 1994). The development of life threatening diseases like cancer is linked to the availability of these antioxidants (Gutteridge JM *et al*., 1994). Chemical induction of hepatic carcinoma is associated with changes in oxygen radical metabolism. This change was demonstrated by a measurement of the antioxidant enzymes. Tumor cells have abnormal antioxidant enzyme activities (Halliwell B *et al*., 1989; Oberley LW *et al*., 1986). In our study, the cancer bearing rats showed decreased activities of enzymic antioxidants and non-enzymic antioxidants in both plasma and liver tissue.

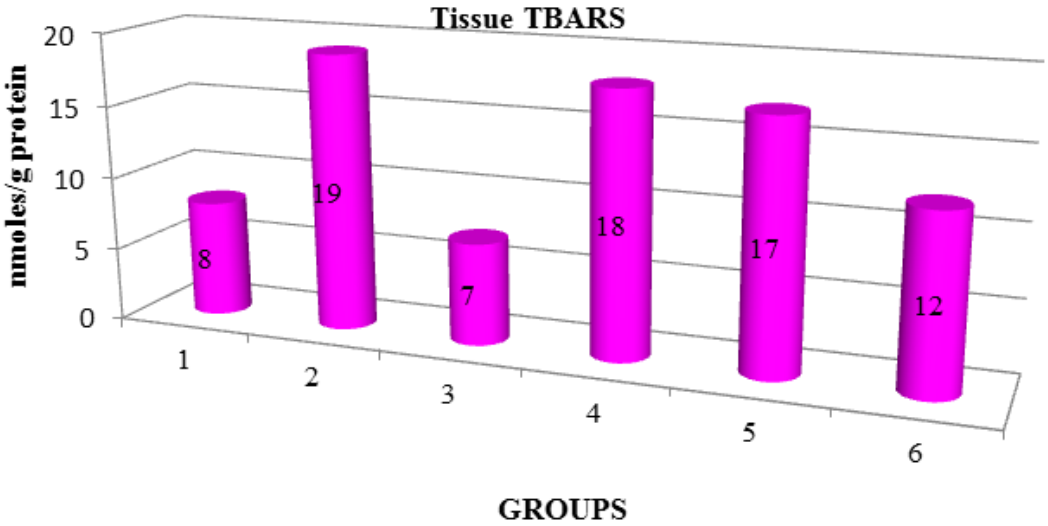
In recent years, there has been a growing interest in dietary/food substances obtained from natural products having chemoprotective properties against chemical carcinogens. HCC is a common cancer and is the 3rd leading cause of death worldwide (Bosch FX *et al*., 1999). DEN is known to induce the reproducible and complete carcinogenic biochemical changes involved in the progression of HCC. ROS are potentially dangerous byproducts of cellular metabolism that have directly affected cellular growth, development, and survival (Watanabe K *et al*., 2000). Lipid peroxidation is one of the

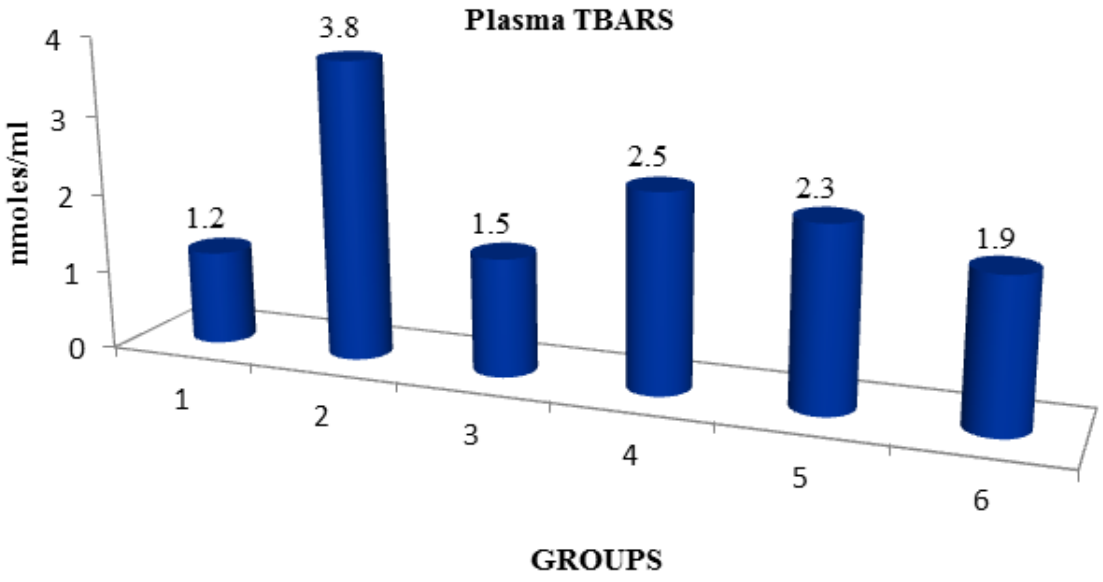
major mechanisms of cellular injury caused by free radicals (Esterbauer H *et al*., 1990), and acts as an important causative factor in carcinogenesis. DEN intoxication has been reported to generate lipid peroxidation byproducts that may interact with various biomolecules that lead to oxidative stress (Hietanen E *et al*., 1987). This may be due to the uncontrolled generation of free radicals that overwhelms the antioxidant defense system. DEN-induced rats showed increased lipid peroxidation levels (thiobarbituric acid reactive substances, malondialdehyde and conjugated dienes) in both plasma and liver tissue (Klaunig JE *et al*., 2004), Mangiferin administration to DEN-treated rats at three different doses 10 mg/kg, 20 mg/kg, and 30 mg/kg body weight every day led to significantly deceased levels of lipid peroxidation both in the plasma and liver when compared with animals induced with DEN alone. This shows the anti-lipid peroxidative role of Mangiferin and is probably mediated by Mangiferin ability to inhibit free radical generation. The strong inhibitory effect of Mangiferin at a dose of 30 mg/kg body weight/day was noticed.

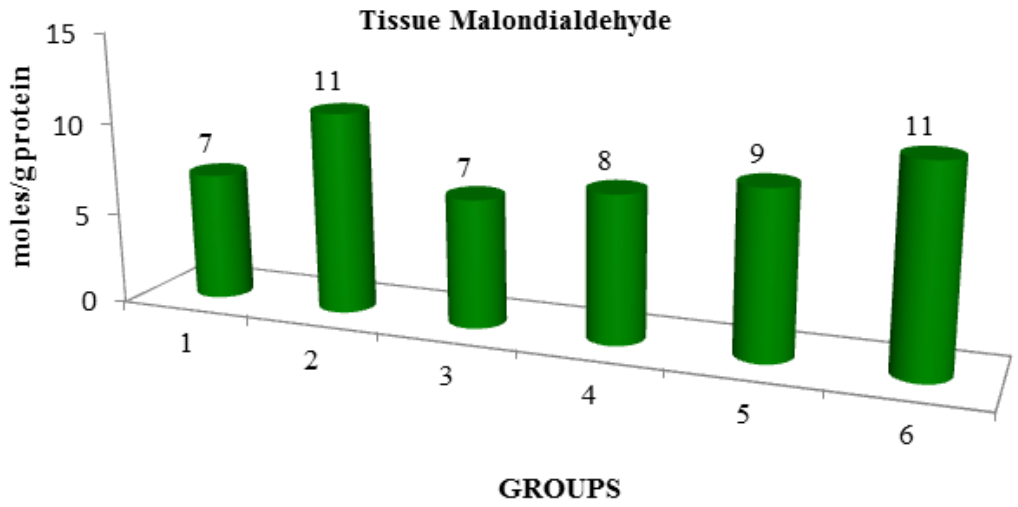
Mangiferin supplementation given to DEN-treated rats at 3 different doses (10 mg/kg, 20 mg/kg, and 30 mg/kg body weight) significantly increased all of the above antioxidants, which may be due to the ability of Mangiferin to interact with radicals, thereby subsequently scavenging them, This is because it donates electrons to unstable oxidized molecules, in turn reducing the free radicals. It also converts inactive antioxidant enzymes into active ones, thereby increasing the concentrations of antioxidant enzymes in the tissues. It is therefore suggested that Mangiferin treatment could protect normal cell or tissues against the cytotoxic effects of carcinogens. The strong inhibitory effect of Mangiferin at a dose of 30 mg/kg body weight was noticed.

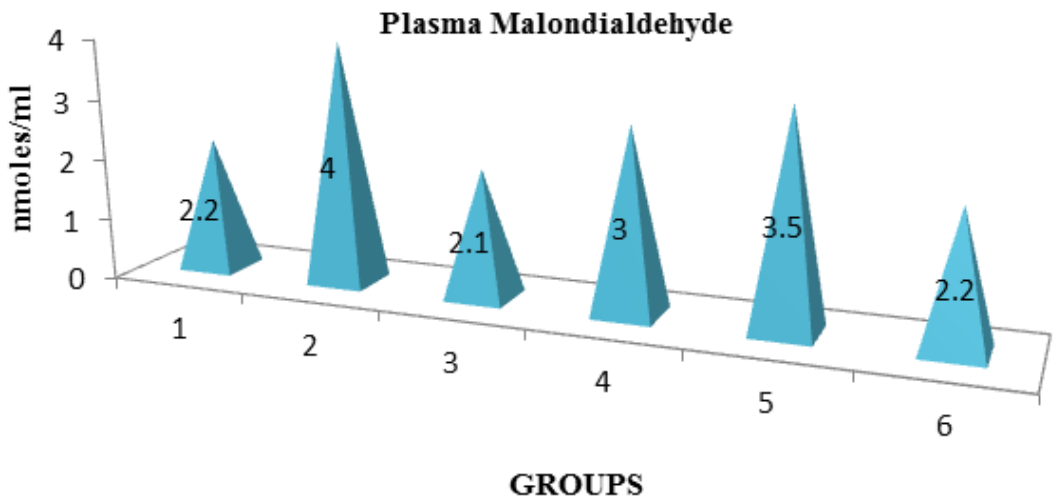
Liver damage caused by DEN generally reflects the instability of liver cell metabolism, which leads to distinctive changes in liver specific enzymes such as transaminases, phosphates, and LDH, and these enzymes leak from the damaged tissues into the body fluids due to their tissue specificity and catalytic activity. These enzymes are representative of liver function, so they are considered to be sensitive and dramatic indicators of hepatic injury and loss of functional integrity of the membrane. Transaminases are reliable, first marker enzymes of the liver, and are used in diagnostic enzymology (Lipport M *et al*., 1981), ALP is another important key marker enzyme located in the bile canalicular lipid membrane, so any interference with the bile flow leads to an alteration in these enzymes. LDH is a fairly sensitive marker of solid neoplasm. DEN-induced hepatic damage is usually accompanied by a rise of AST, ALT, ALP, and LDH due to the overproduction of these enzymes in tumor cells, which may cause increased permeability of the cell membrane, resulting in DEN intoxication.

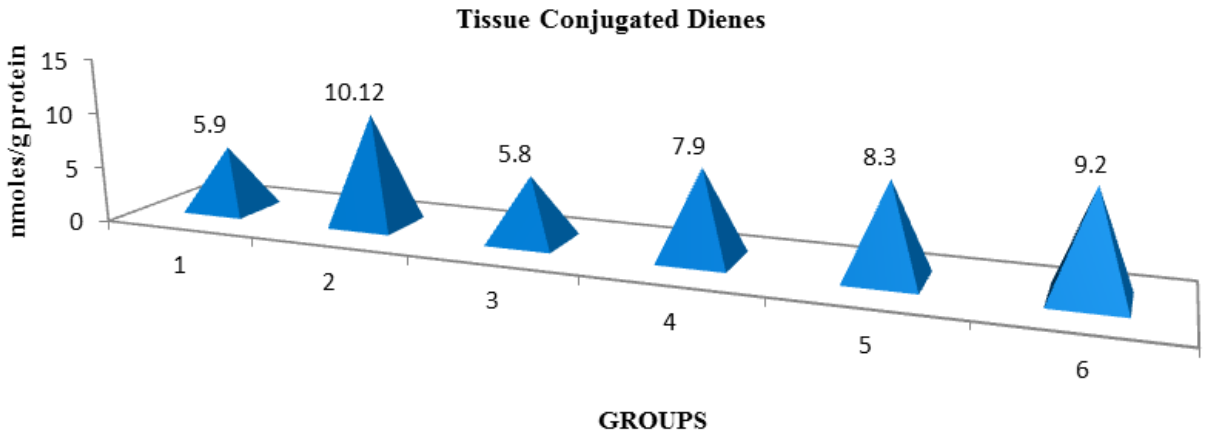
In conclusion, our study clearly indicates that the administration of Mangiferin at a dose of 30 mg/kg body weight appreciably attenuates the reversible alterations in lipid peroxidation and overall enzymatic antioxidant status and that Mangiferin reduces liver-specific enzyme leakage from the tissue of DEN-induced rat models. Hence, further studies are required to elucidate the molecular mechanism of Mangiferin.



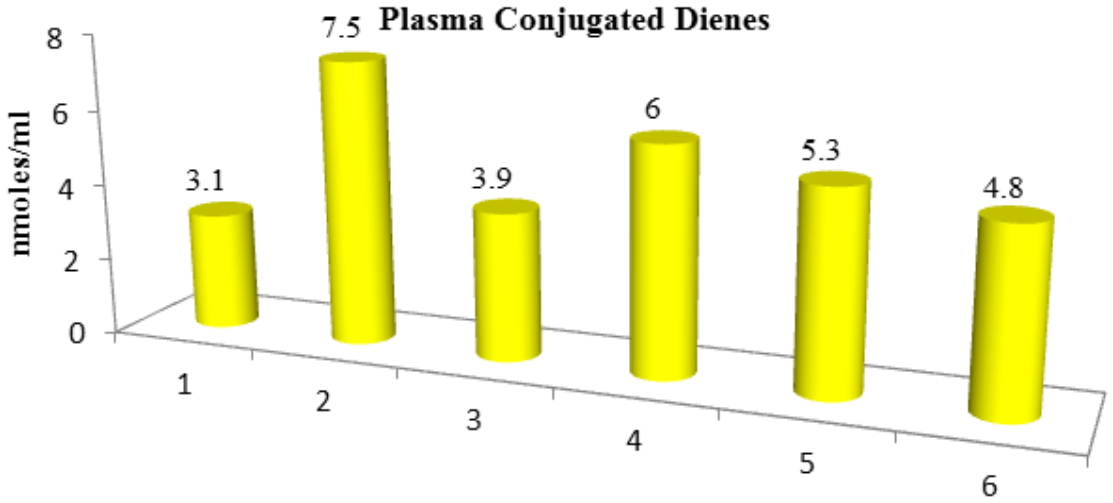

Plasma TBARS
nmoles/ml
4
3
2
1
0
1.2
3.8
1.5
2.5
2.3
1.9
1
2
3
4
5
6
GROUPS

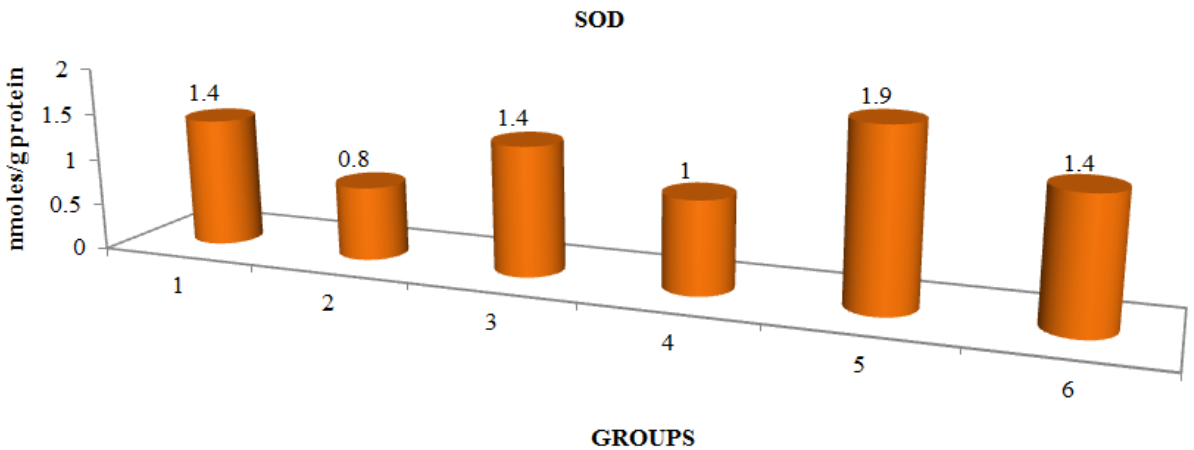

Tissue Malondialdehyde
moles/g protein
15
10
5
0
7
11
7
8
9
11
1
2
3
4
5
6
GROUPS

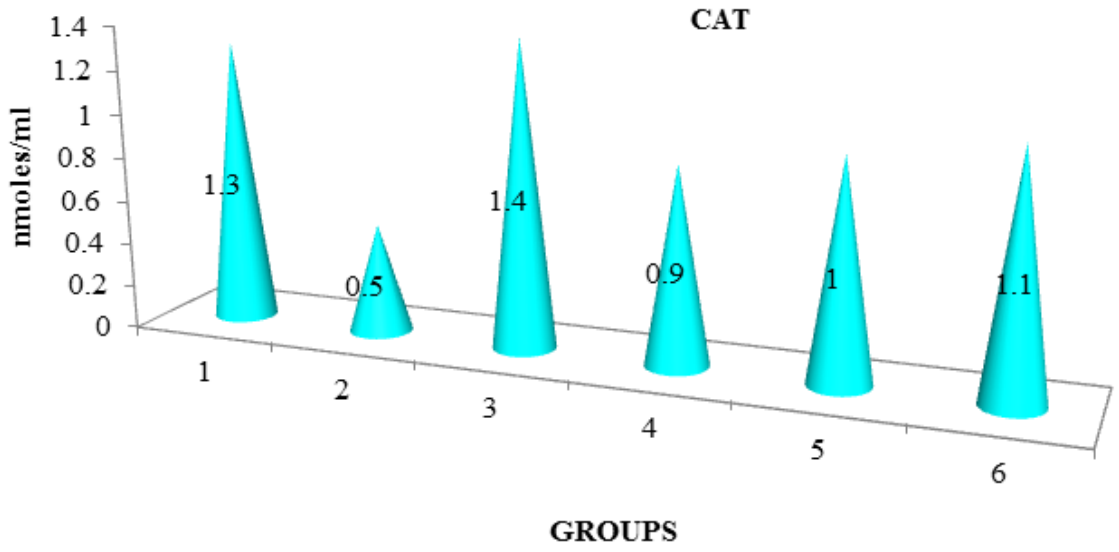

Plasma Malondialdehyde
nmoles/ml
4
3
2
1
0
2.2
4
2.1
3
3.5
2.2
1
2
3
4
5
6
GROUPS

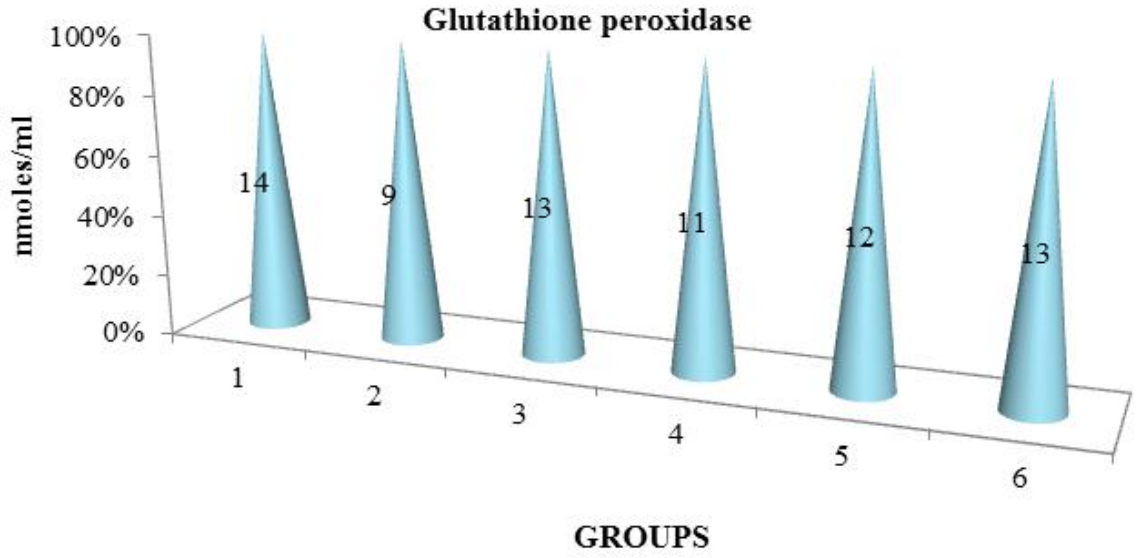


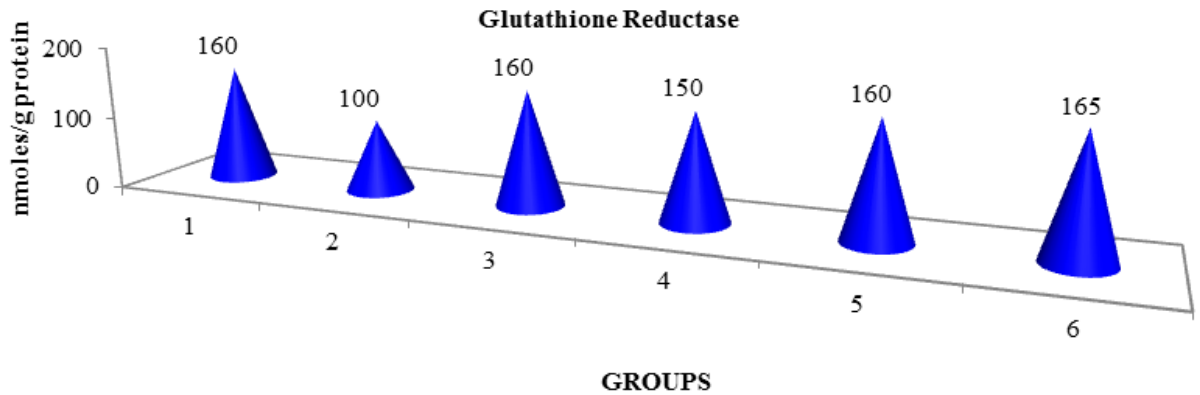
Graph 1: Effect of mangiferin on circulatory and liver tissue lipid peroxidation of control and experimental rats. Data are presented as the Mean±SD of each group.



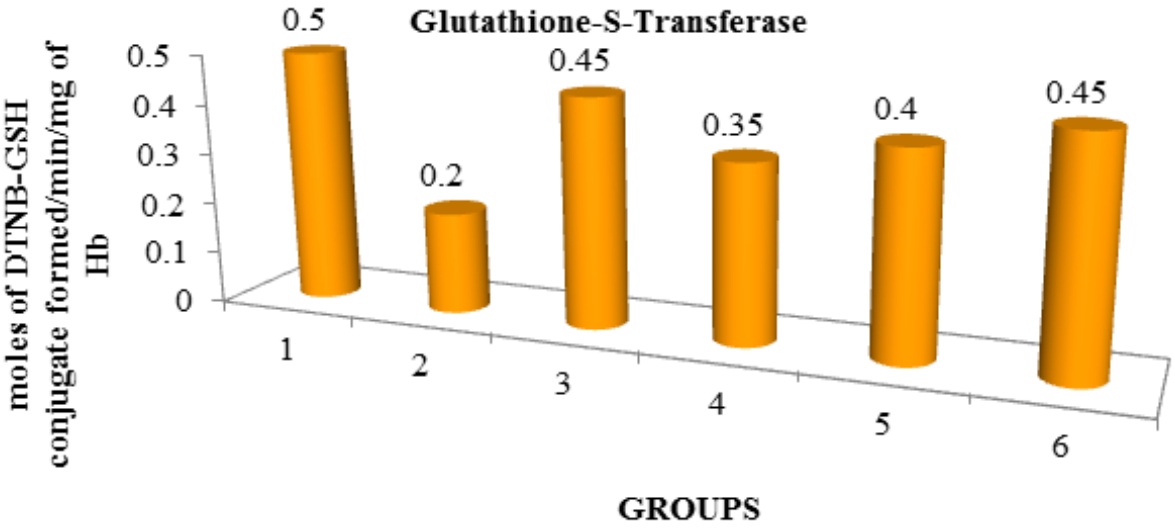


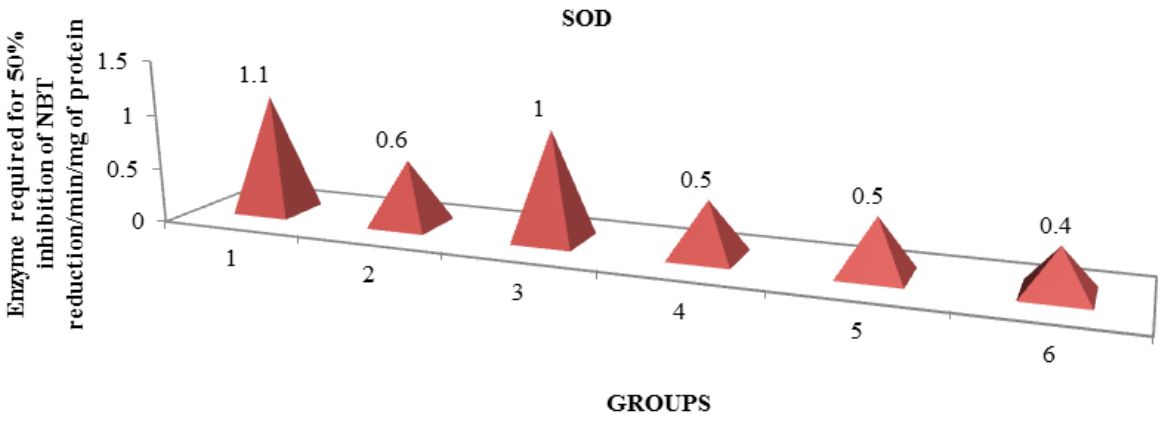

CAT
nmoles/ml
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.3
0.5
1.4
0.9
1
1.1
1
2
3
4
5
6
GROUPS

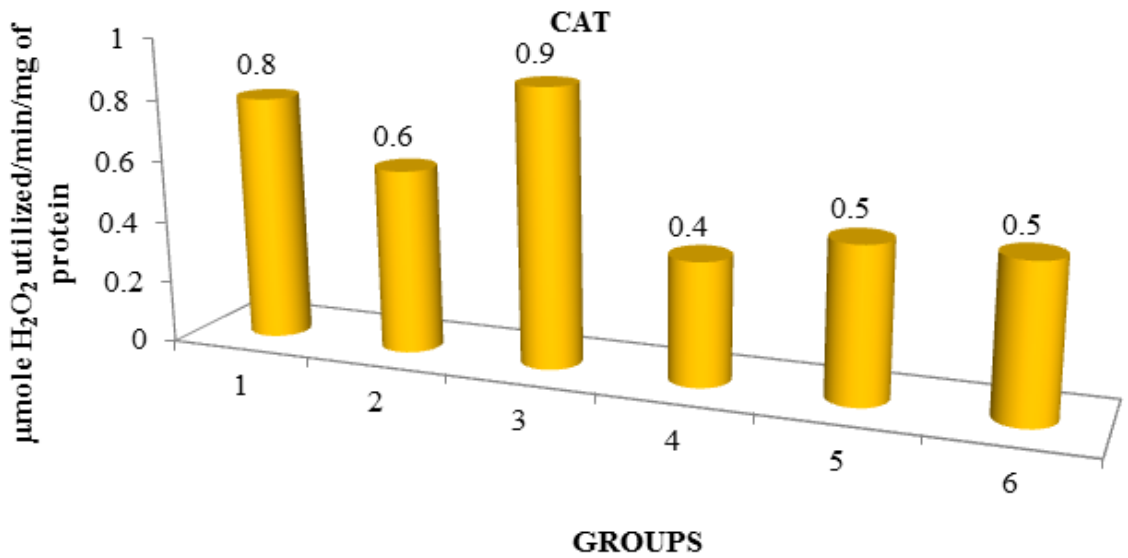

Glutathione peroxidase
nmoles/ml
100%
80%
60%
40%
20%
0%
14
9
13
11
12
13
1
2
3
4
5
6
GROUPS

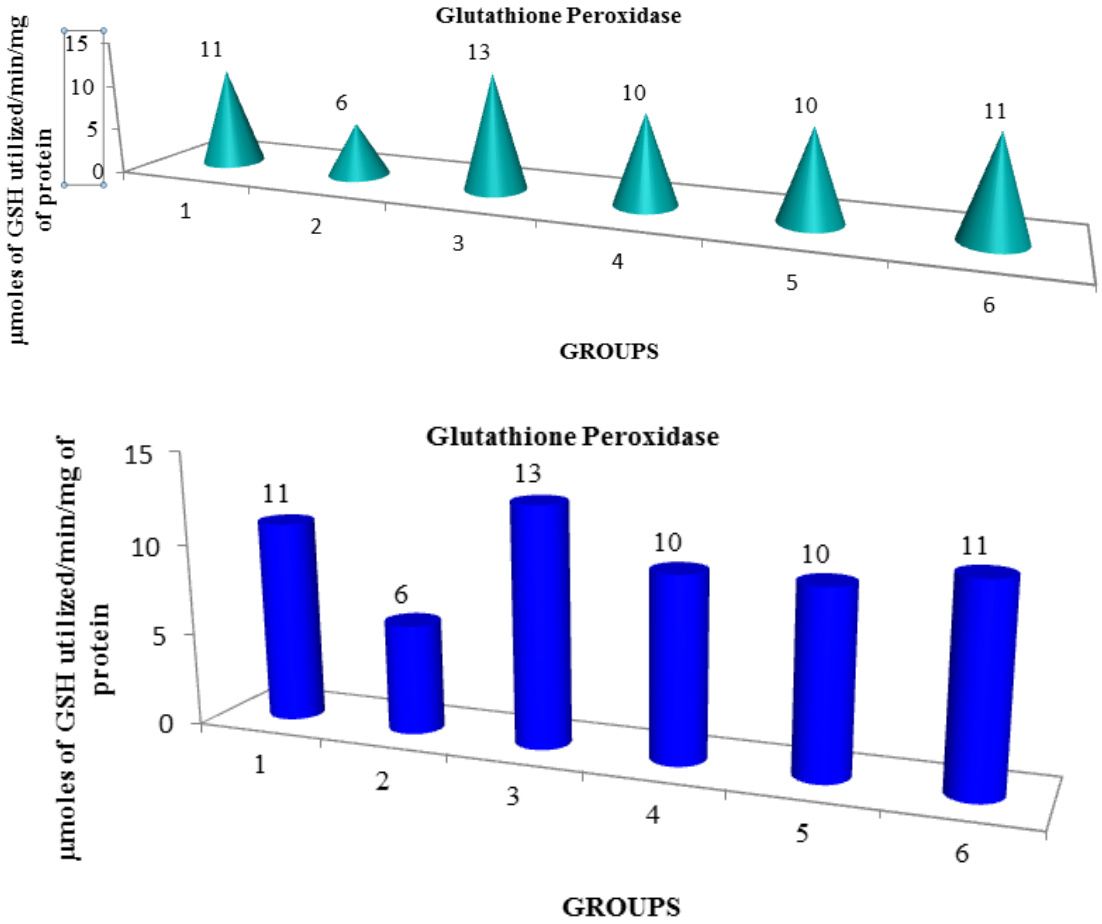

Glutathione Reductase
nmoles/g protein
200
100
0
160
100
160
150
160
165
1
2
3
4
5
6
GROUPS

Graph 2: Effect of mangiferin on circulatory antioxidant enzymes in control and experimental rats. Data are presented as the Mean±SD of each group.









Graph 3: Effect of mangiferin on liver tissue antioxidant enzymes in control and experimental rats. Data are presented as the Mean±SD of each group.

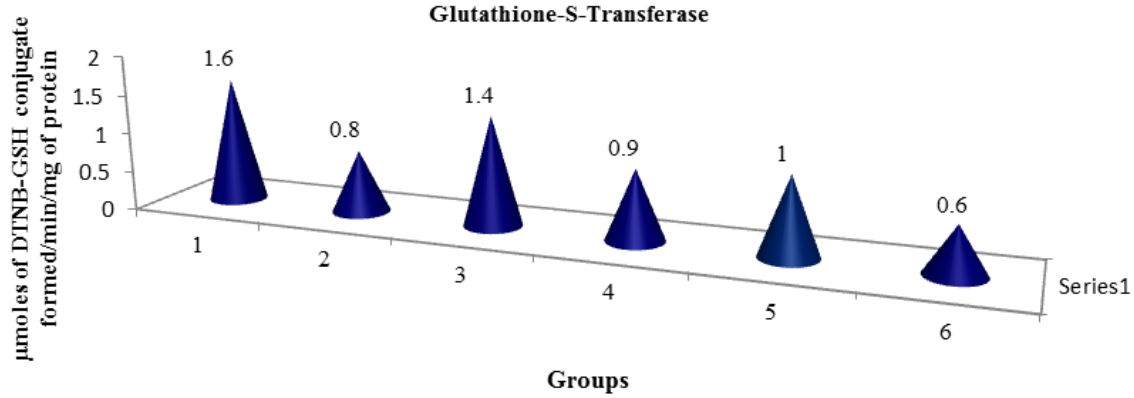


Table 1: Effects of mangiferin on hepatic tissue marker enzymes in control and experimental rats.

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Control	92.82±0.15	84.52±0.27	87.69±0.21	87.71±0.20
DEN	132.46±0.24	122.65±0.23	138.80±0.07	138.65±0.22
Mangiferin 30mg/kg	91.57±0.18	86.58±0.26	89.63±0.25	86.68±0.20
DEN+ Mangiferin 10 mg/kg	124.65±0.22	120.62±0.26	129.66±0.25	129.77±0.19
DEN+ Mangiferin 20 mg/kg	118.60±0.18	110.67±0.23	110.68±0.21	110.68±0.20
DEN+ Mangiferin 30 mg/kg	101.93±0.04	99.96±0.01	98.78±0.18	98.70±0.18

Data are presented as the Mean±SD of each group.

Table 2: Effects of mangiferin on serum hepatic marker enzymes in control and experimental rats.

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Control	121.70±0.21	41.68 ±0.19	36.57±0.30	121.63±0.21
DEN	275.58±0.16	103.72±0.24	95.76±0.13	275.58±0.17
Mangiferin 30 mg/kg	124.74±0.19	42.64±0.26	37.61±0.28	120.41±0.36
DEN+ Mangiferin 10 mg/kg	127.76±0.23	41.80±0.15	35.94±0.03	170.52±0.24
DEN+ Mangiferin 20 mg/kg	128.71±0.23	43.60±0.22	35.93±0.01	156.44±0.43
DEN+ Mangiferin 30 mg/kg	131.67±0.20	45.11±0.60	37.65±0.28	131.55±0.24

Data are presented as the Mean±SD of each group.

Table 3: Effects of mangiferin on liver tissue and plasma non-enzymatic antioxidant status of control and experimental rats.

	Tissue GSH (nm/g)	Plasma GSH (nm/g)	Vitamin C (µg/mg protein)	Vitamin E (µg/mg protein)
Control	17.27±0.02	4.83±0.01	0.753±0.02	0.260±0.02
DEN	11.15±0.03	2.65±0.007	0.246±0.02	0.063±0.02
Mangiferin 30 mg/kg	16.25±0.02	4.97±0.011	0.746±0.02	0.360±0.01
DEN+ Mangiferin 10 mg/kg	11.84±0.03	2.01±0.012	0.306±0.02	0.140±0.01
DEN+ Mangiferin 20 mg/kg	15.73±0.03	2.07±0.005	0.404±0.01	0.180±0.08
DEN+ Mangiferin 30 mg/kg	17.05±0.04	2.48±0.008	0.503±0.03	9.324±0.02

Data are presented as the Mean±SD of each group. $P<0.001$ among the 6 groups. (Kruskal Wallis test). $P = 0.004$ (Mann-Whitney test).

Conclusion

Hepatocellular carcinoma represents a major source of global mortality, still rising in worldwide. The present study aims at elucidating the antioxidant efficiency of Mangiferin in N-Nitrosodiethylamine induced rat liver carcinogenesis. N-Nitrosodiethylamine induction in experimental animals resulted in increased activities of liver marker enzymes and lipid peroxidation levels and decreased levels of antioxidant enzymes. Mangiferin treatment restored the elevated activities of liver marker enzymes and antioxidant status to near-normal with decreased lipid peroxidation levels. Histological observations of liver tissue too correlated with the above biochemical findings. These results clearly suggest that Mangiferin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in N-Nitrosodiethylamine induced liver carcinogenesis in rats.

Nitrosodiethylamine induced Hepatocellular carcinoma in experimental animals resulted in increased activities of liver marker enzymes and lipid peroxidation levels and decreased levels of antioxidant enzymes. Mangiferin administration restored the elevated activities of liver marker enzymes and antioxidant status to near-normal with decreased lipid peroxidation levels. Histological observations of liver tissue too correlated with our study. Our results clearly suggest that Mangiferin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in Nitrosodiethylamine induced Hepatocellular carcinoma liver carcinogenesis in animals. In conclusion, the study clearly indicates that the management of Mangiferin at a dose of 30 mg/kg body weight appreciably attenuates the reversible alterations in lipid peroxidation and overall enzymatic antioxidant status and that Mangiferin reduces liver-specific enzyme leakage from the tissue of Nitrosodiethylamine induced Hepatocellular carcinoma induced in animals. However, further studies are required to elucidate the molecular mechanism of Mangiferin.

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