Protective Effect of α-Momorcharin on Oxidative Damage and Antioxidant Status in Diethylnitrosamine Induced Hepatocellular Carcinoma in Animal Model

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Abstract

The present investigation is part of continuing programme related to the biochemical screening of local plants used in Ancient Indian Medicine, Ayurveda, Siddha and Yunani. Our study aims at elucidating the antioxidant efficiency of α-momorcharin from (Momordica charantia L.) for its protective effect (antioxidant) against N-Nitrosodiethylamine (DEN) induced Hepatocellular carcinoma (HCC) in rat liver carcinogenesis. Studies have shown that N-Nitrosodiethylamine (DEN) induces lipid peroxidation and alters the antioxidant status in nontarget organisms. In our present study, an attempt has been made to study the effect of N-Nitrosodiethylamine (DEN) induced Hepatocellular carcinoma (HCC) on biochemical parameters and ameliorating effect of α-momorcharin. Adult male wistar rats were divided into six different groups. Rats are in group I received standard pellet diet and served as control, group II rats were induced with hepatocellular carcinoma by providing 0.01 % DEN through water for 15 weeks. Group III rats received α-momorcharin via intragastric intubation at a daily dose of 30 mg/kg body weight for 16 weeks every day. Groups IV to VI rats received 0.01% of DEN as in group II along with α-momorcharin via intragastric intubation at a daily dose of 10, 20 and 30 mg/kg body weight for throughout the experimental period of 16 weeks. N-Nitrosodiethylamine (DEN) induction leads to reduction in the levels of Enzymic and Non-Enzymic antioxidants levels. However, on treatment with α-momorcharin normalized the levels of all the biochemical parameters. These findings highlight the efficacy of α-momorcharin as protective (antioxidant) effects against N-Nitrosodiethylamine (DEN) induced oxidative stress. Histological observations of liver tissue too correlated with the above biochemical findings. These results clearly suggest that α-momorcharin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in DEN-induced liver carcinogenesis in rats.

Keywords: α-Momorcharin, N-Nitrosodiethylamine, Antioxidants, SOD, CAT, LPO, GPx.

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world.[(1)] Accumulating evidence has suggested that several mechanisms contribute to the carcinogenesis of HCC.[(2,3)] Recent efforts to control the incidence of HCC have focused on developing effective new chemoprevention strategies. HCC induced by diethylnitrosamine (DEN) in Wistar rats that shows similarities to human HCC is an ideal model for investigating the effect of intervention by chemopreventive agent.[(4)] DEN, a hepatocarcinogen, is known to induce perturbations in the nuclear enzymes involved in DNA repair/replication.[(5)] 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.[(6)] 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.[(7)] 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.[(8)] 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.[(9)] 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.[(10)]

Free Radicals

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.[(11)] The addition of an electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$).[(12)] The addition of an electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$).[(12)] 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.[(13)] The production of superoxide occurs mostly within the mitochondria of a cell.[(14)] 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.[15,16] 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.[17]

Materials and Methods

Chemicals: α-Momorcharin and β-Momorcharin was purchased from IMAM International Group Pharmaceutical Company, China. N-Nitrosodiethylamine (DEN) was purchased from Sigma Aldrich Chemical Company, Saint-Louis, MO, USA. All other chemicals used were of good quality and analytical grade.

Extraction Method for α-Momorcharin and β-Momorcharin

In our study α-momorcharin was purchased as a purified compound from IMAM International Group Pharmaceutical Company, China. According to the literature, the Company extracted, isolated and purified each compound using the following procedure. The whole fruit of bitter gourd was ground and homogenized in 2 mm sodium phosphate buffer, pH 7.5. The resulting slurry was then stirred for 3 hrs to extract the crude proteins. The insoluble component from crude proteins was removed by the filtration and centrifugation at 30,000 x g for 1 hour at 48°C. By using 2 mm sodium phosphate buffer, pH 7.5, the crude protein solution was dialysed. The dialysed protein sample was applied to DEAE Sepharose column equilibrated with 2 mm sodium phosphate buffer at pH 7.5. The unbound protein was then applied to Mono-S column which was equilibrated by 2 mm sodium phosphate buffer at pH 7.5 and eluted by 0.5 m of NaCl. The fraction corresponding to either α and β or α, β-momorcharin, which was confirmed the N-glycoside activity RNA, was concentrated and dialysed against 20 mm Tris-HCl buffer, pH 7.8. The chromatography was performed on Bio Logic Duo Flow system (Bio Rad, Hercules, CA) at 48°C. The purity of α and β or α, β momorcharin was examined by SDS-PAGE and gel filtration chromatography.The concentration of α-momorcharin was determined by spectrophotometrically using optical absorbance at A_{280} nm.

Animal Model

Male wistar rats (Rattus novergcus) (150±180g) 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-h light, 12-h 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 18 animals.

Tumor Induction: 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.

Formulation and Administration of α-Momorcharin: α-momorcharin was freshly prepared and dissolved in 10% Dimethyl sulfoxide (DMSO) at a daily dose of 10 mg/kg, 20 mg/kg, and 30 mg/kg body weight.

Experimental Design

Seggregation of Groups

Experimental animals were divided into 6 groups of 18 rats as follows.

Group I : Served as Normal control and received standard pellet diet.

Group II: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks.

Group III: Rats received α-momorcharin *via* intragastric intubation at a daily dose of 30mg/kg body weight for 16 weeks.

Group IV: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with α-momorcharin *via* intragastric intubation at a daily dose of 10mg/kg body weight for 16 weeks.

Group V: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with α-momorcharin *via* intragastric intubation at a daily dose of 20mg/kg body weight for 16 weeks.

Group VI: Rats had hepatocellular carcinoma induced by 0.01% DEN through water for 15 weeks along with α-momorcharin *via* intragastric intubation at a daily dose of 30mg/kg body weight for 16 weeks.

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

Microscopic observations of α-momorcharin in DEN-treated rat liver are given in Fig. 1, shows the histopathological examination of liver section. Control (Group I) rats revealed normal liver parenchyma cells with granulated cytoplasm, small uniform nuclei, and central vein surrounded by cords of hepatocytes. Group II DEN-treated rats showed loss of architecture and lobules of neoplastic hepatocytes with a fecal area of fatty change. Group III rats exhibited normal architecture, indicating the non-toxic nature of α-momorcharin. Groups IV and V rats along with α-momorcharin and DEN showed moderate cancerous change, fatty change, and hydropic degeneration. Group VI rats showed fewer neoplastically transformed cells and the hepatocytes maintained near-normal architecture.

Effect of α-Momorcharin on Lipid Peroxidation levels in both Serum and Liver: The levels of lipid peroxidation in Fig 1, are shown in both plasma and liver of control (Group 1I) and experimental groups. Significantly increased levels of lipid peroxidation were observed in DEN-induced liver cancer-bearing animals (Group II). Administration of α-momorcharin to DEN-induced rats (Groups IV, V, and VI) significantly decreases the lipid peroxidation level, which was brought to near-normal. There were no significant differences observed in α-momorcharin- treated (Group III) and control rats (Group I).

Effect of α-Momorcharin on the Antioxidant Defense System: The levels of plasma and liver tissue enzymatic antioxidants in Graph 1 & 2, [SOD, CAT, GPx, glutathione reductase (GR), and GST] in control and experimental rats. The control rats (Group I) had normal levels of these enzymes; whereas HCC-induced rats (Group II) showed significantly reduced levels when compared to other groups. α-momorcharin given alone (Group III) highlights the increased levels of these enzymes when compared to control rats. The administration of α-momorcharin to DEN-induced rats (Groups IV, V, and VI) restored the changes to near-normal levels due to the antioxidant efficacy of α-momorcharin.

Effect of α-Momorcharin on Hepatic Marker Enzymes (AST, ALT, ALP, & LDH): The levels of the tissue hepatic marker enzymes in Table 1, (AST, ALT, ALP, and LDH) of control and experimental rats. DEN-induced rats (Group II) exhibited a significant elevation in the activity of these marker enzymes when compared to control rats (Group I), whereas α-momorcharin treated rats (along with α-momorcharin and DEN, Groups IV, V, and VI) 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 (AST, ALT, ALP, and LDH) of control and experimental rats. DEN administered rats (Group II) showed a significant increase in the activity of these marker enzymes when compared to control rats (Group I), whereas DEN-induced rats treated with α-momorcharin (Groups IV, V, and VI) showed a drastic decline in the levels of these marker enzymes when compared with DEN induced rats (Group II).

Effect of α-Momorcharin on Non-Enzymatic Antioxidant status in control and Experimental Rats: The levels of hepatic tissue non-enzymatic antioxidants in Table 3, (vitamin C, vitamin E, and GSH) in control and experimental rats. The enzyme levels of control rats (Group I), were normal whereas the levels in DEN (HCC)-induced rats (Group II) were significantly reduced when compared to other groups. α-momorcharin alone (Group II) showed increased levels of these enzymes when compared to control rats. In the rats along with α-momorcharin and DEN (Groups IV, V, and VI), antioxidant levels were restored to near-normal by the antioxidant efficacy of α-momorcharin.

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.

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.[18] 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.[19] Lipid peroxidation is one of the major mechanisms of cellular injury caused by free radicals,[20] 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.[21] 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,[22] α-momorcharin 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 α-momorcharin and is probably mediated by α-momorcharin's ability to inhibit free radical generation. The strong inhibitory effect of α-momorcharin at a dose of 30 mg/kg body weight/day was noticed.

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.[23] The development of life threatening diseases like cancer is linked to the availability of these antioxidants.[24] 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.[25,26] In our study, the cancer bearing rats showed decreased activities of enzymic antioxidants (SOD, CAT, GPx, GST, and GR) and non-enzymic antioxidants (GSH, vitamin E, and vitamin C) in both plasma and liver tissue.

Daily α-momorcharin 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 α-momorcharin 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. α-momorcharin has one hydroxyl and one acetoxy group in the benzoid ring, which may be responsible for its antioxidant and radical scavenging properties. It is therefore suggested that α-momorcharin treatment could protect normal cell or tissues against the cytotoxic effects of carcinogens. The strong inhibitory effect of α-momorcharin 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 hepato 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 (AST and ALT) are reliable, first marker enzymes of the liver, and are used in diagnostic enzymology,[27] ALP is another important key marker enzyme located in the bile canalicular lipid membrane, so any interference with the bile flow (whether extra- or intrahepatic) 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.

α-momorcharin 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. α-momorcharin treatment may significantly attenuate the increased activities of these enzymes, which may be due to the ability of α-momorcharin to protect the cells from membrane damage and maintain membrane integrity, thereby decreasing enzyme leakages.

Conclusion

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

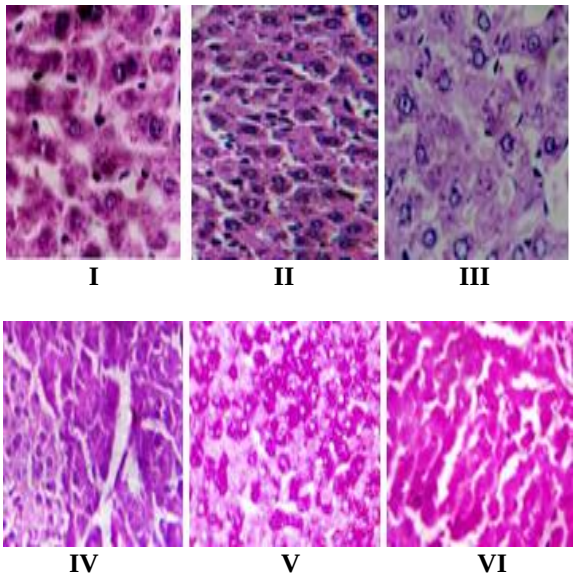
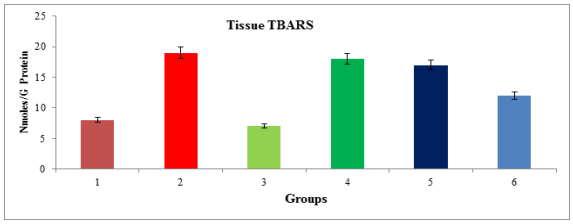
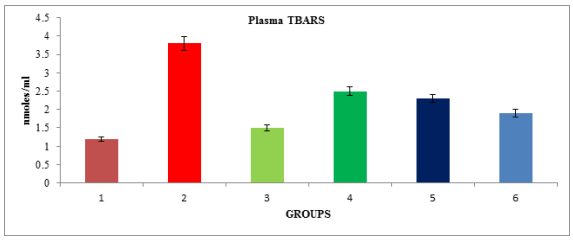
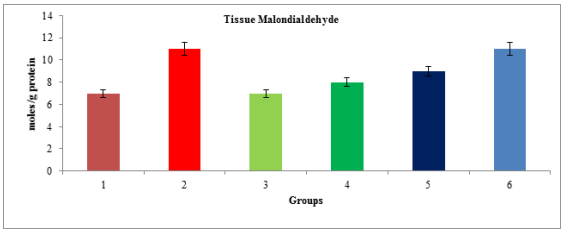


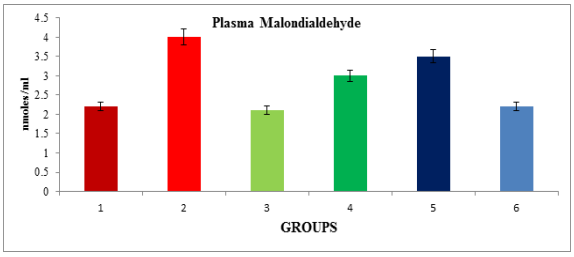
Fig. 1: Representative examples of histopathological changes in the liver of control andexperimental rats as observed macroscopically and under a light microscope

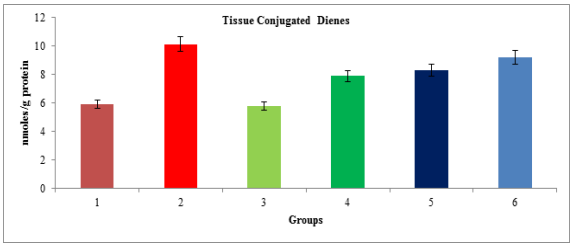
(Group I): Control rat liver showed normal architecture of hepatocytic cells arranged in a trabecular pattern.
(Group II): Remarkable cancerous change with hepatocellular carcinoma.
(Group III): Normal hepatocyte architecture, as in control rats.
(Group IV): Moderate cancerous change, fatty change and hydropic degeneration.
(Group V): Moderate cancerous change, fatty change and hydropic degeneration.
(Group VI): Mild cancerous change, fatty change and hydropic degeneration.

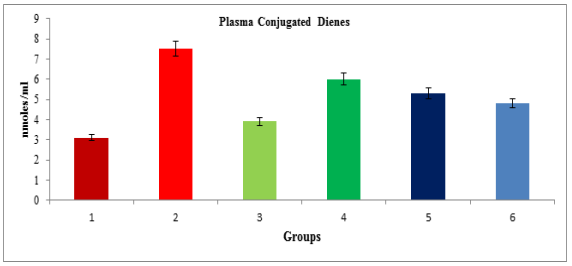




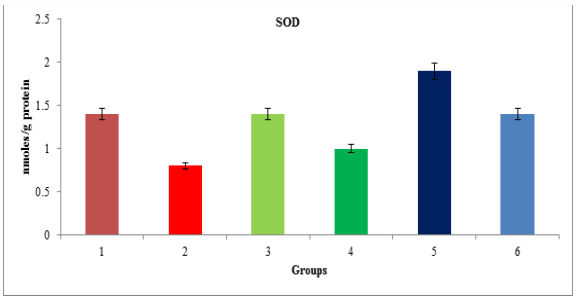


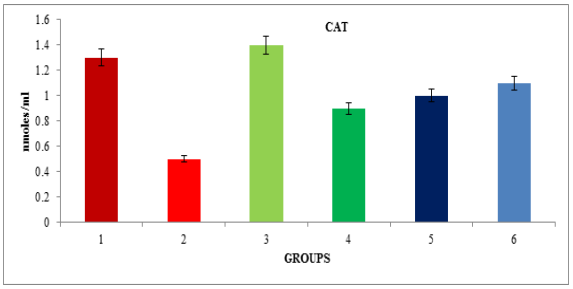


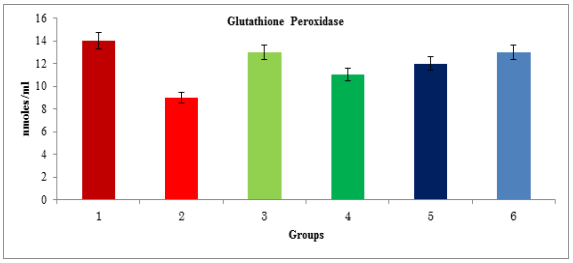


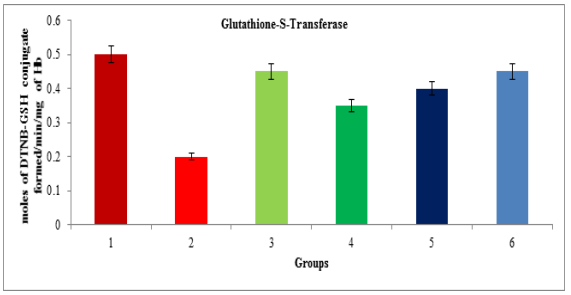


Graph 1: Effect of α-momorcharin on circulatory and liver tissue lipid peroxidation of control and experimental rats. Data are presented as the Mean±SD of each group. P<0.001 among the 6 groups (Kruskal Wallis test). P = 0.004 (Mann-Whitney test).

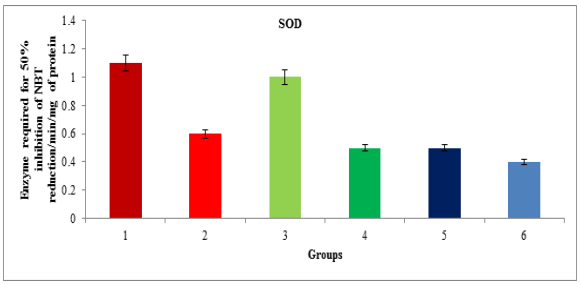


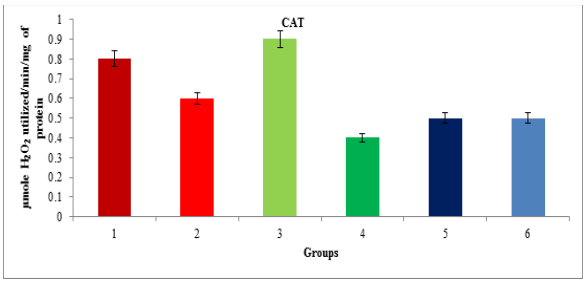


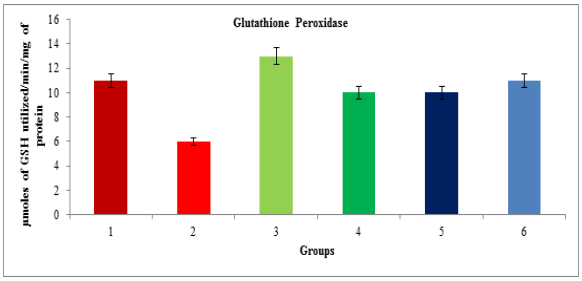


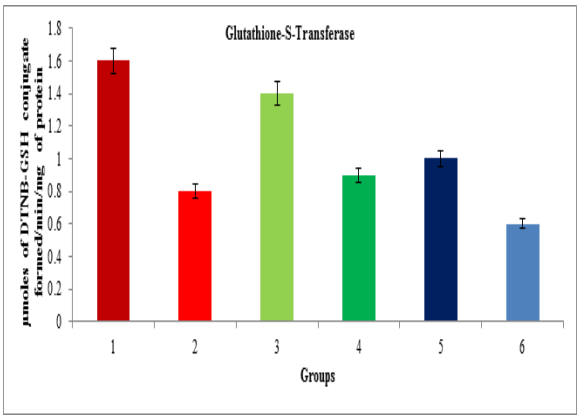


Graph 2: Effect of α-momorcharin on circulatory antioxidant enzymes in control and experimental rats. Data are presented as the Mean±SD of each group. P<0.001 among the 6 groups (Kruskal Wallis test)









Graph 3: Effect of α-momorcharin on liver tissue antioxidant enzymes in control and experimental rats. Data are presented as the Mean±SD of each group. P<0.001 among the 6 groups (Kruskal Wallis test)

Table 1: Effects of α-Momorcharin on hepatic tissue marker enzymes in control and experimental rats

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Control	93.83±0.16	85.53±0.28	88.70±0.22	88.72±0.21
DEN	133.47±0.25	123.66±0.24	139.81±0.08	139.66±0.23
Momorcharin 30mg/kg	92.58±0.19	87.59±0.27	90.64±0.26	87.69±0.21
DEN+Momorcharin 10mg/kg	125.66±0.23	121.63±0.27	130.67±0.26	130.78±0.20
DEN+Momorcharin 20mg/kg	119.61±0.19	111.68±0.24	111.69±0.22	111.69±0.21
DEN+Momorcharin 30mg/kg	102.94±0.05	100.97±0.02	99.79±0.19	99.71±0.19

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).

Table 2: Effects of α-Momorcharin on serum hepatic marker enzymes in control and experimental rats

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Control	122.71±0.22	42.69 ±0.20	37.58±0.31	122.64±0.22
DEN	276.59±0.17	104.73±0.25	96.77±0.14	276.59±0.18
Momorcharin 30mg/kg	125.75±0.20	43.65±0.27	38.62±0.29	121.42±0.37
DEN+Momorcharin 10mg/kg	128.77±0.24	42.81±0.16	36.95±0.04	171.53±0.25
DEN+Momorcharin 20mg/kg	129.72±0.24	44.70±0.23	36.94±0.02	157.45±0.44
DEN+Momorcharin 30mg/kg	132.68±0.21	46.12±0.61	38.66±0.29	132.56±0.25

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).

Table 3: Effects of α-Momorcharin 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	18.28±0.03	5.84±0.02	0.754±0.03	0.462±0.03
DEN	12.16±0.04	3.66±0.008	0.347±0.03	0.164±0.03
Momorcharin 30mg/kg	17.26±0.03	5.98±0.012	0.847±0.03	0.461±0.02
DEN+Momorcharin 10mg/kg	13.85±0.04	3.02±0.013	0.407±0.03	0.241±0.02
DEN+Momorcharin 20mg/kg	16.74±0.04	3.09±0.006	0.507±0.02	0.281±0.09
DEN+Momorcharin 30mg/kg	18.06±0.05	3.49±0.009	0.604±0.04	10.325±0.03

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).

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Summary & Conclusion

Hepatocellular carcinoma (HCC) represents a major source of global mortality, still rising in worldwide. The present study aims at elucidating the antioxidant efficiency of α-momorcharin in N-Nitrosodiethylamine (DEN) induced rat liver carcinogenesis. N-Nitrosodiethylamine (DEN) induction in experimental animals resulted in increased activities of liver marker enzymes and lipid peroxidation levels and decreased levels of antioxidant enzymes. α-momorcharin 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 α-momorcharin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in N-Nitrosodiethylamine (DEN) induced liver carcinogenesis in rats.

Nitrosodiethylamine (DEN) induced Hepatocellular carcinoma (HCC) in experimental animals resulted in increased activities of liver marker enzymes and lipid peroxidation levels and decreased levels of antioxidant enzymes. α-momorcharin 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 α-momorcharin treatment prevents liver damage, lipid peroxidation and protects the antioxidant defense system in Nitrosodiethylamine (DEN) induced Hepatocellular carcinoma (HCC) liver carcinogenesis in animals. In conclusion, the study clearly indicates that the management of α-momorcharin at a dose of 30 mg/kg body weight appreciably attenuates the reversible alterations in lipid peroxidation and overall enzymatic antioxidant status and that α-momorcharin reduces liver-specific enzyme leakage from the tissue of Nitrosodiethylamine (DEN) induced Hepatocellular carcinoma (HCC) induced in animals. However, further studies are required to elucidate the molecular mechanism of α-momorcharin.

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