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Research Article

Induction of Hepatic Antioxidant Enzymes by Chrysin in *N*-Nitrosodiethylamine induced Hepatocellular carcinoma in Wistar albino rats

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ABSTRACT

The aim of this study was to examine the influence of the Chrysin against Diethylnitrosamine induced Hepatocellular carcinoma in Wistar albino rats by assessing the levels of tumour incidence, liver endogenous antioxidant enzyme, lipid peroxidation and hydro peroxides, cytosolic superoxide dismutase. Decreases in liver antioxidant status and increase tumour incidence, rapid lipid peroxidation and hydro peroxides were observed significantly in animals induced with Diethylnitrosamine. Animal treated with chrysin 50 mg/kg body weight shows significant attenuation of these changes when compare to Diethylnitrosamine induced animals. Chemopreventive effect of Chrysin further confirmed by observing the activities of cytosolic superoxide dismutase. Restoration of the liver antioxidants and reduced the levels of free radicals in tissue were observed in chrysin treated animal groups. With confining these data, the induction of antioxidant by chrysin has been well established against Diethylnitrosamine induced Hepatocellular carcinoma.

Keywords: Diethyl nitrosamine; Hepatocellular carcinoma; Catalase; Reactive oxygen species

INTRODUCTION

N-Nitroso diethyl amine (DEN) is widely known hepatocarcinogen. On repeated administration of DEN, causes perturbations in the nuclear enzymes involved in DNA repair mechanism may induce hepatic damage and producing reproducible tumours. (Slikker, 2004) The global burden of hepatocellular carcinoma is third leading cause of cancer worldwide. Several studies has been extensively explained that DEN induces free radical production, leads to increased lipid peroxidation and causing depletion of endogenous antioxidants, cytotoxicity and carcinogenesis.(McKillop, 2009) DEN also shows low carcinogenic effect on other organs and many study evidences that the free radicals play a key role in DEN induced hepatic carcinogenesis. (Thirunavukkarasu, 2003) DEN exposure leads to rapid metabolism results to the formation of major promutagenic ethyl adduct, which can lead to mutations further causes cancer in liver. (Swenberg, 1985)

Though the oxygen free radicals are natural physiological products, it is also responsible for the production of reactive oxygen species (ROS). Many studies proved that ROS causes enormous cellular damage by modifying the bio molecules and its cellular functions. (Khandrika, 2009) Under normal conditions deleterious effects of oxidative stress caused by ROS will overcome by the action of enzymatic and non enzymatic antioxidants, which leads to maintain the redox balance and prevent the DNA damage and other ROS mediated effects. (Kelkel, 2010) Increased free radicals cause damage to system through interaction with proteins, carbohydrates, DNA and membranes, resulting in cellular damage and mutations.

The modern recent approach of chemoprevention serves as a potential alteration and biologic modifiers to retard the proliferation of cancer cells. It is important to find specific scavenger to reduce free radical induced oxidative stress, and balances antioxidant defence system. (Crohns, 2009) The compounds which contribute antioxidant property have become more interested to protect the cells and tissues against free radicals and negative effects of reactive oxygen species.

Chrysin (5, 7 dihydroxy flavone) derived from a species like Passiflora, pelargonium and Pinaceae. It is naturally present in honey, plant extracts, propolis and pine wood. (Gambelunghe, 2003) Chrysin exhibits a strong complexing activity for clinical and therapeutic applications in various disease strategies. It has essential physiological activities like anti inflammation, anticancer and antioxidant. (Lin, 2010) Recent investigations reports that Chrysin exist potential effect on human thyroid, papillary cancer cell lines and also induces apoptosis in leukaemia cells. (Harris, 2006) In-vivo studies have indicated that Chrysin offers protection against oxidative stress mediated ethanol-induced liver injury and also suggests the chemo preventive effects on breast and colon cancers. (Rodrigo, 2010) (Khan,

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2010)Present study was designed to carry out a systemic investigation of the protective role of Chrysin on DEN induced Hepatocellular carcinoma by estimating enzymatic antioxidant levels and total free radical levels.

MATERIALS AND METHODS

Animals

Healthy male Wistar rats, weighing 160-180 g were obtained from Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India and maintained in a diurnal light and dark cycle of 12 h each. Rats were fed commercial rat feed (M/S Hindustan foods Ltd, Bangalore India) and given access to water ad libitum. The experimental designs were approved by the Institutional Ethical Committee of the University of Madras (360/01/a CPCSEA).

Source of Chemicals

N-nitrosodiethylamine (DEN) and Chrysin were purchased from Sigma Chemicals Co (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and obtained from SRL Chemicals, Mumbai, India**.**

Experimental protocols

Experimental animals were divided into four groups and each group consisting of six animals.

Group I: Rats received Dimethyl sulphoxide (DMSO) as a vehicle ip for 16 weeks and referred as positive control rats.

Group II: Rats were administered with 0.01% DEN through drinking water for 15 weeks and referred as Hepatocellular carcinoma induced rats.

Group III: Rats were treated with Chrysin (50 mg/kg body weight dissolved in DMSO) intraperitoneally twice in a week for 16 weeks to assess the cytotoxicity if any induced by Chrysin and rats were referred as drug control.

Group IV: Rats were received 0.01% DEN (as in Group II) along with chrysin (50 mg/kg body weight dissolved in DMSO) intraperitoneally. Chrysin treatment was started 1 week prior the first dose of 0.01% DEN administration and rats were referred as treated rats.

At the end of the experimental period the rats were fasted overnight, body weight were observed and then anesthetized followed by cervical decapitation. The blood samples were collected and plasma was separated. The buffy coat, enriched in white cells, was removed and the remaining erythrocytes were washed three times with physiological saline. A known volume of erythrocyte was lysed with hypotonic phosphate buffer at pH 7.4. The hemolysate was separated by centrifugation at 2500 rpm for 10 min and the supernatant was used for the estimation of enzymic antioxidants. Liver was rapidly removed and washed in ice cold saline weighed and blotted dry. Portion of the liver was then homogenized in 0.1M Tris buffer. Liver homogenates were used for biochemical studies such as Lipid peroxidation and, lipid hydroperoxides and enzymic antioxidants. Native-polyacrylamide gel electrophoresis (Native-PAGE) analysis carried out for activities Superoxide dismutase.

Biochemical Investigations

Both serum and liver homogenates were analyzed for total protein content by using the method of (Lowry, 1951). Activities of liver and erythrocytes Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx) were assayed followed by published papers (Sinha, 1972) (Kakkar, 1984) (Rotruck, 1973). Activities of lipid peroxidation in liver were assessed by measuring the levels of lipid peroxidation and lipid hydro peroxides in the liver following the procedures of (Jiang, 1992) (Niehaus, 1968).

Native-PAGE analysis for cytosolic SOD

The method of Native PAGE analysis were followed by (Sivaramakrishnan, 2008) Before loading the sample to the lanes of a 12% native gel, a constant current was applied to the gel in the presence of Tris (187.5 mM) and EDTA (1 mM) to remove oxidants may alter activities of enzymes (e.g., ammonium persulfate). The tissue proteins were then separated by electrophoresis in the presence of Tris (50 mM), glycine (300 mM), and EDTA (1.8 mM) at constant current (50 mA) for 3–5 h. Briefly, following electrophoresis, the gel was placed in the dark and soaked in a SOD staining solution containing riboflavin (0.028 mM), nitroblue tetrazolium (0.25 mM), EDTA (1 mM) and TEMED (28 mM), in 50 mM phosphate buffer. After 30 min incubation in a dark room, the staining solutions were removed and replaced with 50 mM phosphate buffer. The gel was then exposed to light for a fewminutes. Areas of SOD activity appeared as white bands in a blue background.

RESULTS

Table 1 shows the effect of DEN and chrysin on number of nodules tumour incidence in control and experimental group of animals. In group II DEN induced liver cancer bearing animals there is Significant increase ($P < 0.05$) were observed in number of nodules and tumour incidence when compare to other experimental group of animals. The chrysin co-treated (Groups IV) showed a significant decrease in the number of nodules and tumour incidence when compared with HCC animals in (Group II). However no significant difference was observed between the chrysin alone treated (Group III) and control (Group I) animals.

Table 2 and 3 depicts the effect of activities of SOD, CAT and GPx in erythrocyte and liver in control and experimental groups of animals. The activities of these enzymatic antioxidants were significantly decreased in DEN Treated rats (Group-II) compare to control animals (Group-I), On chrysin co- treated clearly shows the

Table 1: Effect of Chrysin on Number of nodules and Tumour incidence in control and experimental group of animals

Particulars	Group-I	Group-II	Group-III	Group-IV
Number of Rats examined	6	6	6	6
Total number of Nodules	0	126[a]	0	58[b]
Tumor incidence in (%)	0	79.3	0	21.17

Values are expressed as mean±S.D. (n =6). Statistical significance $p < 0.05$. a compared with group I, b compared with group II. *NS* non significant compared to group I

Table 2: Effect of Chrysin on activities of SOD, CAT, GPX in the liver in control and experimental group of animals

Particulars	Group-I	Group-II	Group-III	Group-IV
SOD	7.30±1.13	4.50±0.94[a]	6.98±1.19[NS]	5.84±1.09[b]
CAT	76.15±1.42	42.24±1.49[a]	74.61±1.36[NS]	57.41±1.67
GPX	102.3±1.32	54.2±0.59[a]	104.1±1.30[NS]	88.7±0.79[b]

Values are expressed as mean ± S.D. (n =6). Statistical significance $p < 0.05$. a compared with group I, b compared with group II. *NS* non significant compared to group I. Units for SOD, superoxide dismutase in Units/mg protein; CAT, catalase in µmol of H_2O_2 utilised/min/mg protein and GPx, glutathione peroxidase in µmol GSH utilized/min/mg protein

Table 3: Effect of Chrysin on activities of SOD, CAT, GPX in the Erythrocytes in control and experimental group of animals

Particulars	Group-I	Group-II	Group-III	Group-IV
SOD	8.30±1.13	4.12±0.94[a]	7.38±1.19[NS]	5.94±1.09[b]
CAT	168.15±1.42	132.24±1.49[a]	170.61±1.36[NS]	157.41±1.67[b]
GPX	156.7±0.22	73.3±0.91[a]	148.3±0.28[NS]	98.8±0.52[b]

Values are expressed as mean ± S.D. (n =6). Statistical significance $p < 0.05$. a compared with group I, b compared with group II. *NS* non significant compared to group I. Units for SOD, superoxide dismutase in Units/mg protein; CAT, catalase in µmol of H_2O_2 utilised/min/mg protein and GPx, glutathione peroxidase in µmol GSH utilized/min/mg protein

normalization and increase significantly ($p < 0.05$) and restored these enzymatic-antioxidants in erythrocyte and tissues when compare with DEN induced tumour bearing animals (Group-II). No significant differences observed between (Group-III) chrysin alone treated animals and control (Group-I).

Figure -1(a and b) represents the levels of lipid peroxidation markers (Lipid per oxidation and hydro peroxide) in liver of control and experimental group of animals. Significantly ($p < 0.05$) increased level of lipid per oxidation and hydro peroxide were observed in Group-II DEN induced liver cancer bearing animals. Administration of Chrysin significantly decreases the lipid peroxidation and hydro peroxide level which was brought to near normal. There is no significant differences were observed among chrysin alone treated (Group III) and control animal groups (Group I).

Figure -2 Native PAGE for cytosolic SOD in the liver. In DEN induced HCC animals (Group 11) shows significant decreased activities of endogenous enzymic antioxidants when compared with (group 1) normal control animals. On chrysin treatment significantly increased the activities of cystolic SOD (Group IV). However no significant differences were observed among chrysin alone treated (Group III) and control animal groups (Group I).

DISCUSSION

Liver is the major site of metabolism of ingested components; it is highly susceptible to carcinogenic effect. (Stelzner, 2006) Hepatocellular carcinoma (HCC) is one of the fifth most occurring common human cancer. DEN widely used for studying the mechanism of Hepatocellular carcinoma in animal experimental model. (Ramakrishnan, 2006) Lipid peroxidation plays a key role initiation and progression of carcinogenesis. It is initiated by the reaction of a free radical on a fatty acid (or) fatty acyl side chain.(Pope, 2007) Lipid peroxidation may lead to the formation of several toxic by products such as malondialdehyde and 4-hydroxynonenal which can attack cellular targets including DNA, inducing mutagenicity. (Halliwell, 1984)

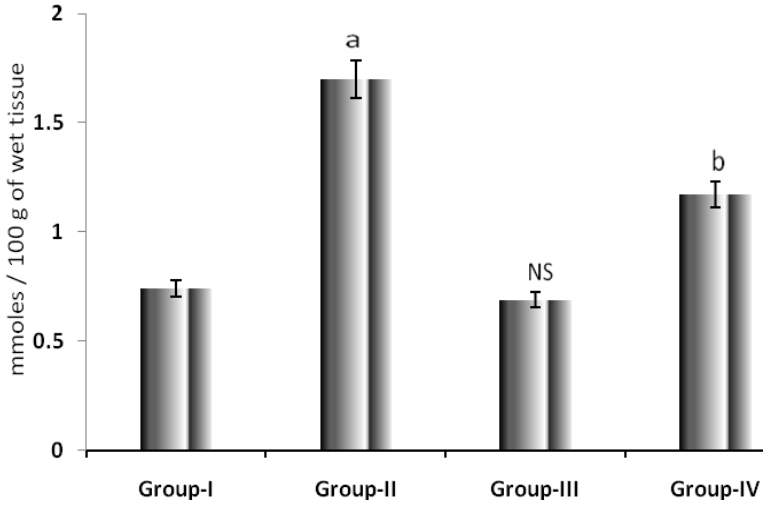

Figure 1: (a) Effect of Chrysin on the level of lipid peroxidation in the liver of control and experimental group of animals. Results were expressed as mean ± SD. Significance at P<0.05. a) Compared with group I. b) Compared with group II. NS non-significant compared to group I

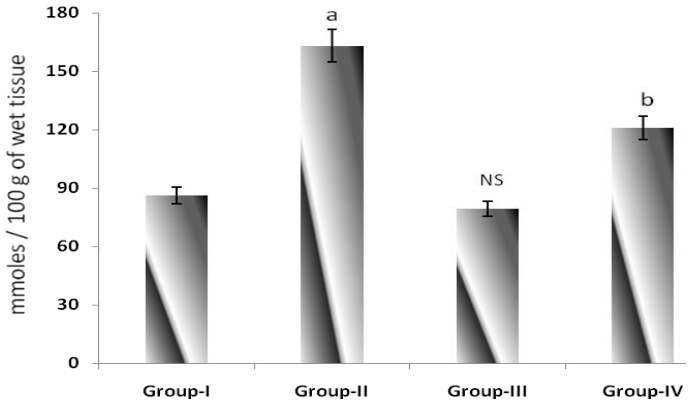

Figure 1: (b) Effect of Chrysin on the level of lipid hydro peroxides in the liver of control and experimental group of animals. Results were expressed as mean ± SD. Significance at P<0.05. a) Compared with group I. b) Compared with group II. NS non-significant compared to group I

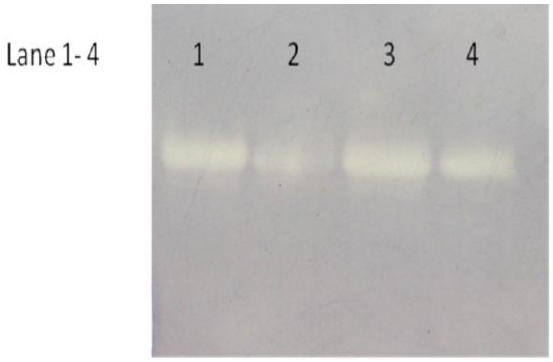

Figure 2: Native-poly acryl amide gel electrophoresis (Native-PAGE) activities staining of superoxide dismutase in liver of control and experimental animals. Lane 1- Control, Lane 2- DEN alone Lane 3- Chrysin alone, Lane 4- DEN + Chrysin

Many experimental observational studies reveals nodules are the precursor of liver cancer and also severity of hepatic cancer may correlates with size and number of nodules. (Bull, 2000) Nodule growth inhibition and reduction of tumour incidence by chrysin were observed in this present study establish important property of chrysin in cancer prevention. Chrysin significantly reduces the tumour incidence, and also delay tumour onset which was confirm by reduced morphological changes has its minimum toxicity establish the evidence for cancer chemo preventive efficacy against DEN induced liver cancer. Oxidative damage to cells

and macro molecules is an important event in the development of variety of pathological conditions including chronic liver damage, cirrhosis and Hepatocellular carcinoma. (Farinati, 1999) The hepatotoxic effect of DEN is attributed to oxidative stress. This study demonstrates that the chrysin effectively prevented DEN induced hepatocarcinogenesis. Since excessive or chronic oxidative stress is considered an important factor in the etiology of many diseases.

Chrysis involves in the enhancement of endogenous antioxidant enzymes and also decreases the total lipid peroxides. (Pushpavalli, 2010) These results provide the scientific basis of the hepatoprotective nature of chrysin against DEN induced HCC. Chrysin is being widely studied for its possible antioxidant, anti-inflammatory and also inhibit cancer. (Khoo, 2010) In a wide range of malignancies; the elevated lipid per oxidation is associated with reduced activity of antioxidants. (Murakami, 2006) Continuous damage to biomolecules and DNA leads to further mutagenic actions. Finally the activities of these antioxidant enzymes are decreased in cancerous conditions though the exact mechanism not understood yet, depleted antioxidant levels leads to oxidative stress. (Limon-Pacheco, 2009) Such studies substantiate with our findings as there was a significant depletion in the activities of enzymic antioxidants in liver of animals treated with DEN when compared to normal animals.

Antioxidants protect cells by either directly or indirectly against adverse effects of xenobiotics, carcinogens and toxic radical reactions. The negative effects of oxidants progression to neoplastic condition are intentionally by primary antioxidants such as SOD, CAT and GPx. (Janani, 2010) Superoxide dismutase is the primary step of defense mechanism against the oxidative stress. Mechanism of SOD includes conversion of superoxide radical to peroxide and molecular oxygen, which will be effectively neutralized by Catalse. (Devasagayam, 2004) SOD levels were decreased in DEN induced HCC animals. Also the activity SOD enzyme from native page results further confirms the decreased activity in hepatoma rats, activity was regained in chrysin induced animal groups.

Catalase thought to be the first line of defence against oxidative damage caused by hydrogen peroxide and other radicals induced by carcinogen decomposes hydrogen peroxide and protects the cell from highly reactive hydroxyl radicals. (Valko, 2007) The decreased level of CAT activities in Group II DEN induced cancer bearing animals may be due to the utilisation of this enzyme in the removal of highly produced hydrogen peroxide radicals caused by DEN administration. (Muthukumar, 2008) In the present study activity of GPx in Group II- HCC bearing animals was reduced significantly decreased in HCC-bearing animals. (Sivalokanathan, 2006) Reduction in SOD and GPx in hepatoma conditions would be expected to have dire consequences. The greater relative importance of GPx over SOD can be attributed by the capability of GPx to detoxify hydrogen peroxide in compare to SOD. But reduction in GPx is found to be more deleterious than SOD. Based on the above findings it is concluded that the Chrysin protects against DEN induced Hepatocelluar carcinoma by effectively reducing the increased levels of lipid peroxidation and stabilizes the antioxidant defence system.

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