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CHEMO-PREVENTIVE EFFECT OF ETHANOLIC EXTRACT OF *MORINDA CITRIFOLIA* ROOT ON *N*-NITROSODIETHYLAMINE INDUCED OXIDATIVE STRESS IN RAT LIVER

Ranju Pal[1]*, Thirumoorthy N[2]

[1]Ph.D. Scholar, NIMS University, Shobha Nagar, Jaipur, (Rajasthan) India.

[2]Cherraan's College of Pharmacy, Coimbatore (Tamil Nadu) India.

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***Correspondence for Author:**

* **Ranju Pal**
Ph.D. Scholar, NIMS University, Shobha Nagar, Jaipur, (Rajasthan) India.

Pal.ranju@gmail.com

ABSTRACT

The generation of free radicals is a cause of many pathological conditions like diabetes mellitus, cancer, stroke, etc. Free radicals cause damage to cellular DNA and initiate carcinogenesis. Nitrosamine compounds are known hepatic carcinogens. In the metabolism of nitrosamines, such as *N*-nitrosodiethylamine (NDEA), there is evidence of the formation of reactive oxygen species (ROS) resulting in oxidative stress, which may be one of the factors in the etiology of cancer. In the present investigation the effects of *morinda citrifolia* root extract (MCRExt) on hepatic marker enzymes [AST, ALT, ALP], total bilirubin, total protein, lipid peroxides and antioxidants [reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), vitamin-E and C] during N-nitrosodiethylamine (NDEA) – induced hepatocarcinogenesis in wistar albino male rats were studied. Rats were divided into five groups of six animals. The animals in the groups 1 and 3 were normal control and MCRExt control, respectively. Groups 2, 4 and 5 were administered with 0.01% NDEA in drinking water for 15 weeks to induce hepatocellular carcinoma (HCC). Starting 1 week prior to NDEA administration group 4 animals were treated with MCRExt in diet for 16 weeks, 5 weeks after NDEA administration group 5 animals were treated with MCRExt and continued till the end of the experimental period (16 weeks). After the experimental period the body weight, relative liver weight, number of nodules, size of nodules were assessed. In group 2 hepatocellular carcinoma induced animals there was an increase in the number of nodules, relative liver weight. The levels of lipid

peroxides were elevated with subsequent decrease in the body weight, total protein, GSH, SOD, CAT, GPx, GST, Vitamin E & C. In contrast, MCRExt +NDEA treated groups 4 and 5 animals showed a significant decrease in the number of nodules with concomitant decrease in the lipid peroxidation status. The levels of GSH, and the activities of antioxidant in liver were improved when compared with hepatocellular carcinoma induced group 2 animals. A significant increase in the activities of serum AST, ALT, ALP and total bilirubin was observed in NDEA treated rats when compared with control animals. MCRExt +NDEA treated groups 4 and 5 animals showed significant decrease in the activities of these enzymes when compared with NDEA treated animals. The pretreatment effect of MCRExt was higher compared to post-treatment.

Key Words: Diabetes mellitus, animals, *morinda citrifolia*, hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most common type of liver cancer, representing 83% of all cases. The 5-year relative survival rate is about 7% and causes more than six lakh deaths annually worldwide [1]. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and currently represents the leading cause of death amongst cirrhotic patients [2]. Due to late diagnosis and advanced underlying liver cirrhosis, only limited treatment options with marginal clinical benefits have been available in up to 70% of patients [3]. Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer [4]. Reactive oxygen species (ROS) have been implicated in both the initiation and promotion stages of carcinogenesis and scavengers of ROS have been shown to be inhibitors at both the stages [5]. It is also reported that the generation of reactive oxygen species (ROS) by DEN causes carcinogenic effects. ROS are potentially dangerous by-products of cellular metabolism that have direct effect on cell development, growth and survival. Oxidative stress caused by ROS has been reported in membrane lipid peroxidation, DNA damage and mutagenesis associated with various stages of tumor formation process. Hence the model of NDEA-induced HCC is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis [6]. NDEA is a well known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics and pharmaceutical products. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models [7]. The severity and

magnitude of the cancer problem makes it imperative to develop chemopreventive strategies, utilizing pharmacological or natural agents to block initiation or to arrest progression in premalignant cells [8]. Traditionally, the roots of Noni plants were used by Polynesians to produce yellow or red dye, but more importantly, they are now known to contain medicinally active components, such as anthraquinones, which, due to its antioxidant activities, possess various therapeutic properties [9]. These include anti-bacterial, anti-viral and anti-cancer activities as well as analgesic effects [10]. A group of compound in *Morinda citrifolia* root that were shown to be responsible for the plant's therapeutic properties is anthraquinones and among the different anthraquinones, damnacanthal which is present mainly in the root is of particular interest, due to its important activity in fighting against cancers [11].

MATERIAL AND METHODS

Plant material

The roots of *Morinda citrifolia* were collected from the place, Quereshi bagh nursery, Jamnagar (Gujrat) and authenticated by senior Faculty of Agriculture university, Nagpur, Maharashtra.

Extraction procedure

The roots were shade dried, broken into small pieces and powered coarsely. About 1000 gm of air dried powdered material was extracted with 99.9% of ethanol in a soxhlet extractor for 15 cycle. The extract was concentrated to dryness under reduced pressure and controlled temperature (40-50o C) using rotary evaporator. The ethanolic extract yielded an brown sticky mass weighing 32g and extractive value was found to be 8.431% w/w. the extract was used directly for total phenol and flavonoid content and also for assessment of antioxidant capacity through various chemical assays[11,12].

Acute Oral Toxicity Study

The acute oral toxicity study was conducted in compliance with the Organization for Economic Cooperation and Development (OECD) Guideline for the Testing of Chemicals No.420, adopted December 17, 2001 (OECD, 2001) with slight modification. After acclimatization, 20 rats of either sex were randomly divided into two groups of 5 each. The control group received the vehicle in a volume of 1ml/100g body weight by gavage, whereas the treatment group received the extract complex at the dose of 500mg/kg, 1000mg/kg and 2000mg/kg. The first rat of each sex in each group were dosed and observed, then a further four rats of each sex in each group were subsequently dosed when no mortality occurred

within 48h. All animals were observed individually for clinical signs of toxicity immediately after dosing and at 1, 2, 4, and 8 h after dosing. Observations were focused on changes in skin, fur, eyes, mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems as well as somatomotor activity and behavioral pattern. The number of survivors were noted after 24h and then maintained for a further14 days with a once daily observation. Animals were weighed on day 0, and on days 7 and 14. At the end of the study, all surviving animals were sacrificed with diethyl ether. Gross pathological examinations of all major internal organs such as heart, lungs livers, kidneys, spleen, pancrease, stomach and sex organs were then performed [13].

Experimental Protocol

Experimental animal

Adult male albino wistar rats (8 weeks), weighing 150 to 170 gm were used in this study. The animals were housed in clean polypropylene cages and maintained in a well-ventilated, temperature controlled animal house with a constant 12 h light/dark schedule. The animals were fed with standard rat pelleted diet (Hindustan Lever Ltd., Mumbai, India) and clean drinking water was made available *ad libitum*. All animal procedures were performed after approval from the ethical committee and in accordance with the the recommendations for the proper care and use of laboratory animals.(PBRI/IAEC/2010/PN-69).

Experimental design

Male albino wistar rats of body weight 150-170 g were divided into five groups of 10 animals each.

Group 1: Normal control rats fed with standard diet and pure drinking water.

Group 2: Rats were induced with hepatocellular carcinoma by providing 0.01% NDEA through drinking water for 15 weeks.

Group 3: Rats were administered orally with MCRExt alone (200 mg/kg body weight).

Group 4: Rats pretreated with MCRExt (200 mg/kg body weight, once a day) 1 week before the administration of 0.01% NDEA and continued till the end of the experiment.

Group 5: Rats posttreated with MCRExt (200 mg/kg body weight, once a day) from 5 weeks after the administration of 0.01% NDEA for next 10 weeks and continued till the end of experiment.

Sample collection

At the end of the experimental period (16 weeks), total body weights were recorded and animals were sacrificed by cervical decapitation without anesthesia to avoid animal stress, then livers were immediately removed and weighed, in order to obtain the organ weight ratio. The relative liver weight of organs (%) was calculated as g/100g body weight. Blood samples were collected in EDTA tubes and centrifuged at 2200×g for 15 min. at 4^0C. Plasma samples were stored at -20^0C for biochemical analysis of proteins, total bilirubin and enzymes (ALT, AST and ALP).

At the end of experimental period effect of MCRExt on number of hepatocellular nodules were estimated by measuring the parameters like total number of nodules, average number of nodules/ nodule bearing liver and relative size of nodule.

Preparation of tissue homogenate

The tissues were weighed and 10% tissue homogenate was prepared with 0.025 M Tris-HCl buffer, pH 7.5. After centrifugation at 10,000 rpm for 10 min, the clear supernatant was used to measure thiobarbituric acid reactive substances (TBARS). For the determinations of vitamin E level the liver tissues was weighed and lipids was extracted from tissues by the method of Folch et. al.(1957) using chloroform-methanol mixture ($CHCl_3$: MeOH)(2:1 v/v). The extract used for the estimation of vitamin E.

For the estimation of non-enzymic and enzymic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 min and the resulting supernatant was used for enzyme assays [14].

Estimation of Hepatic Biomarker

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) , alkaline phosphatase (ALP) enzyme activities and serum bilirubin were determined using reagent kits in a computerized Semi-Autoanalyser.

Estimation of Lipid Peroxidation/Thiobarbituric Acid Reactive Substances (TBARS)

TBARS were assayed by the method of Niehaus and Samuelsson[21]. To 1 mL of tissue homogenate, 2 mL of TCA-TBA-HCl reagent was added, mixed thoroughly and kept in boiling water bath for 15 minutes. The precipitate was removed by centrifugation. A series of standards was processed similarly and the absorbance was read at 535 nm [15].

Enzymatic Antioxidant Activity

Estimation of catalase activity

The reaction mixture (1.5 ml, vol) contained 1.0 ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was read at 620 nm; CAT activity was expressed as µM of H_2O_2 consumed/min/mg protein [16].

Estimation of superoxide dismutase (SOD) activity

0.5 ml of tissue homogenate was diluted with 1 ml of water. In this mixture, 2.5 ml of ethanol and 1.5 ml of chloroform (all reagents chilled) were added and shaken for 1 min at 4 °C then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 µM PMS, 0.3ml of 30 µM NBT, 0.2 ml of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 s the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control. One unit of the enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute under the assay conditions [17].

Reduced glutathione (GSH)

GSH was estimated by the method of Ellman. 0.5 mL of tissue homogenate was precipitated with 2 mL of 5% TCA. After centrifugation, 1 mL of supernatant was taken and added 0.5 mL of Ellman's reagent (19.8 mg of 5,5' dithio(*bis*)nitrobenzoic acid in 100 mL of 1% sodium citrate) and 3 mL of phosphate buffer. Standards were treated in a similar way and the colour developed was read at 412 nm.

Glutathione peroxidase (GPx)

GPx activity was measured by the method of Rotruck *et. al.* To 0.2 mL of buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide and 0.5 mL of tissue homogenate were added. To that mixture, 0.2 mL of glutathione solution and 0.1 mL of hydrogen peroxide were added. The

contents were mixed well and incubated at 37°C for 10 minutes along with the control tubes containing all the reagents but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 mL of 10% TCA. 0.2 mL of tissue homogenate was added to the control tubes. The tubes were centrifuged and supernatant was assayed for glutathione content by adding Ellman's reagent [18].

Glutathione-S-transferase (GST)

GST activity was measured by the method of Habig *et al*. The reaction mixture containing 1 mL of buffer, 0.1 mL of 1-chloro-2, 4-dinitrobenzene (CDNB), 0.1 mL of homogenate and 1.7 mL of distilled water was incubated at 37°C for 5 minutes. The reaction was then started by the addition of 1 mL of glutathione. The increase in absorbance was followed for 3 minutes at 340 nm. The reaction mixture without the enzyme was used as blank [19].

Non Enzymatic Antioxidant Activity

Estimation of ascorbic acid (vitamin C)

0.5 ml of tissue homogenate was mixed thoroughly with 1.5 ml of 6% TCA and centrifuged for 10 min at 3500 g. After centrifugation, 0.5 ml of the supernatant was mixed with 0.5 ml of DNPH reagent and allowed to stand at room temperature for an additional 3 h then added 2.5 ml of 85% sulphuric acid and allowed to stand for 30 min. Then the absorbance was read at 530 nm. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly along with a blank. Ascorbic acid values were expressed as µg/mg tissue [20].

Estimation of vitamin E

0.1 ml of lipid extract, 1.5 ml of ethanol and 2 ml of petroleum ether were added, mixed and centrifuged for 3000 rpm for 10 min. The supernatant was evaporated to dryness at 80°C then 0.2 ml of 2, 2-1-dipyridyl solution and 0.2 ml of ferric chloride solution was added and mixed well. This was kept in dark for 5 min and added 2 ml of butanol. Then the absorbance was read at 520 nm. Standards of α-tocopherol in the range of 10-100 µg were taken and treated similarly along with blank containing only the reagent. The values were expressed as µg/mg-tissue [21].

RESULTS

Table 1. Body weight and liver weight of control and experimental group of animals.

Groups initial body	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Relative liver weight (g)
Group 1 (control)	167± 2.96	248± 7.96	6.78± 0.29	2.7±0.20
Group 2 (NDEA)	164 ± 4.84 a*	210 ± 4.95 a*	9.5± 0.52 a*	4.21±0.79 a*
Group 3 (MCRExt alone)	168 ± 7.72 b^{ns}	268 ± 7.47 b^{ns}	7.8 ±0.22 b^{ns}	2.57±0.07 b^{ns}
Group 4 (NDEA + MCRExt pretreatment)	168 ± 2.07 ce*	235 ± 4.89ce*	8.1± 0.40 ce*	3.27±0.51 ce*
Group 5 (NDEA + MCRExt post-treatment)	167 ± 2.42 d*	215 ± 9.56 d*	8.6 ± 0.26ns	3.8±0.67ns

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

Statistical comparision

a : Group I - Group II b : Group I – Group III c : Group II – Group IV

d : Group II – Group V e : Group IV – Group V

Symbols : * p < 0.001 * * p < 0.0001 p >0.05- ns - non significant

Table 2. Effect of MCRExt on number and size of hepatocellular nodules during NDEA induced hepatocarcinogenesis.

Groups	Total no. of nodules	Average number of nodules/nodule bearing liver	Relative size (% of total number)		
			<1mm	>1mm<3mm	>3mm
Group 2 (NDEA)	101	16.83 ± 2.31	56 (55%)	32 (32%)	13 (13%)
Group 4 (NDEA + MCRExt pretreatment)	41 a*	7 ± 1.54	15 (36%)	18 (43%)	9 (21%)
Group 5 (NDEA + MCRExt post-treatment)	80 b*	13.5 ± 1.64	40 (50%)	24 (30%)	16 (20%)

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

Statistical comparision:

a : Group II - Group IV b : Group II – Group V

Symbols : * p < 0.001 * * p < 0.0001 p>0.05- ns - non significant

Table 3. Effect of MCRExt on the hepatic enzyme status in liver of control and experimental group of animals.

Groups	AST	ALT	ALP	BIL
Group 1 (Control)	93.25 ± 3.39	83.06 ± 4.08	76.5 ± 2.07	0.65 ± 0.12
Group 2 (NDEA)	133 ± 5.22 a*	123.25 ±3.74 a*	122 ± 2.71 a*	1.84 ± 0.22 a*
Group 3 (MCRExt alone)	92.01 ± 2.07 b^{ns}	85.21 ± 2.96 b^{ns}	120. ± 3.52 b^{ns}	0.62± 0.07 b^{ns}
Group 4 (NDEA + MCRExt pretreatment)	109.5 ±4.96 ce*	98.6 ±6.28 ce*	93.2 ±3.04ce*	0.91 ±0.03 ce*
Group 5 (NDEA + MCRExt post-treatment)	114.1 ± 3.72 d*	116.9 ± 5.49 d*	112.28 ±5.04 d*	1.26 ± 0.19 d*

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

Statistical comparision

a : Group I - Group II b : Group I – Group III c : Group II – Group IV

d : Group II – Group V e : Group IV – Group V

Symbols : * p < 0.001 * * p < 0.0001 p >0.05- ns - non significant

Table 4. Effect of MCRExt on lipid peroxidation in liver of control and experimental group of animals.

Groups	Group 1 (control)	Group 2 (NDEA)	Group 3 (MCRExt alone)	Group 4 (NDEA + MCRExt pretreatment)	Group 5 (NDEA + MCRExt posttreatment)
Lipid peroxidation	3.37± 0.24	5.58± 0.39 a*	3.41 ±0.13 b^{ns}	4.09± 0.62 ce*	4.86± 0.71 d*

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

Units – µmoles of MDA released/mg protein

Statistical comparision:

a : Group I - Group II b : Group I – Group III c : Group II – Group IV

d : Group II – Group V e : Group IV – Group V

Symbols : * p < 0.001 * * p < 0.0001 p >0.05- ns - non significant

Table 5. Effect of MCRExt on the antioxidant status in liver of control and experimental group of animals.

Groups	CAT	SOD	GPx	GST
Group 1 (Control)	81.01± 2.07	7.75±0.44	179± 6.16	8.31±0.14
Group 2 (NDEA)	36.83 ± 3.13a*	3.84 ± 0.59 a*	87.3± 4.32 a*	4.36±0.06 a*
Group 3 (MCRExt alone)	79.81 ± 2.07 b*	8.13 ± 0.66 b*	181.8 ±6.04 b*	8.05±0.09 b*
Group 4 (NDEA + MCRExt pretreatment)	62.3 ±3.26 ce*	5.79 ± 0.66 ce*	134.3± 0.04 ce*	7.73±0.38 ce*
Group 5 (NDEA + MCRExt post-treatment)	55.5 ± 3.72 d*	4.61 ± 0.26 d*	121 ± 1.83 d*	6.24±0.53 d*

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

SOD – Units/mg protein, CAT – µmoles of H_2O_2 utilized/min/mg protein, GPX – mmoles of GSH oxidised/min/mg protein, GST- Unit/ mg of protein.

Statistical comparision

a : Group I - Group II b : Group I – Group III c : Group II – Group IV

d : Group II – Group V e : Group IV – Group V

Symbols : * p < 0.001 * * p < 0.0001 p >0.05- ns - non significant

Table 6. Effect of MCRExt on the non- enzymatic antioxidant status in liver of control and experimental group of animals.

Groups	Ascorbic acid	Vit-E	GSH
Group 1 (Control)	1.92 ± 0.06	16.88 ± 0.34	41.81± 2.20
Group 2 (NDEA)	0.68 ± 0.13 a*	6.71 ± 0.45 a*	11.6± 1.14 a*
Group 3 (MCRExt alone)	1.89 ± 0.08 b^{ns}	17.37 ± 0.91 b^{ns}	42.92 ±1.22 b^{ns}
Group 4 (NDEA + MCRExt pretreatment)	1.43 ± 0.15 ce*	13.28 ± 0.76 ce*	37.5± 4.30 ce*
Group 5 (NDEA + MCRExt post-treatment)	1.10 ± 0.19 d*	9.06 ± 1.09 d*	29.44 ± 3.40 d*

Data are expressed as mean ± SD; n=6 animals in each group. Values are statistically significant at *P<0.001.

GSH, vitamin C and vitamin E – µg/mg protein

Statistical comparision:

a : Group I - Group II b : Group I – Group III c : Group II – Group IV

d : Group II – Group V e : Group IV – Group V

Symbols : * $p < 0.001$ * * $p < 0.0001$ $p > 0.05$- ns - non significant

DISCUSSION

The liver is a multifunctional organ that plays essential roles in metabolism, biosynthesis, excretion, secretion and detoxification. These processes require energy, making the liver a highly aerobic, oxygen-dependent tissue. These processes also cause vulnerability of the liver to anoxia, increased susceptibility to noxious insults, and create a demand for cell replacement after tissue loss. Enhanced liver cell death and impaired regeneration are indeed features of most liver disorders.

The extract at a dose of 2000mg/kg produced no treatment-related signs of toxicity or mortality in any of the animals during 14 days of the study. In addition no weight loss was detected and all internal organs examined at necropsy were free from any gross pathological changes. Since no mortality was observed at 2000 mg/kg. It was thought that 2000mg/kg was the cut off dose. Therefore $1/10^{th}$ dose (i.e. 200mg/kg) was selected for all further studies.

Table 1 shows the body weight and liver weight of control and experimental group of animals. In group 2 animals there is a significant decrease in the final body weight when compared with groups 1 and 4. The group 4 MCRExt pretreated animals showed a significant increase in the final body weight when compared with group 2 animals. In group 2 animals the relative liver weight is significantly increased when compared with group 1 animals and there is a significant decrease in the liver weight in MCRExt treated groups 4 and 5 animals when compared with group 2 animals. Table 2 shows the total number of nodules and number of nodules per nodule bearing liver and nodular sizes in millimeter in tumor bearing animals. The MCRExt treated groups 4 and 5 showed a significant decrease in the number of nodules when compared with group 2 animals.

Effect on Hepatic Biomarker

In this study, we demonstrated that the injection of NDEA to rats lead to a marked elevation in the levels of serum AST, ALT, ALP and total bilirubin which is indicative of hepatocellular damage, as previously reported. This elevation could potentially be attributed

to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage. Serum AST, ALT, ALP and total bilirubin are biomarkers in the diagnosis of hepatic damage. Our data revealed that the administration of NDEA induced a significant increase in ALT, AST, ALP activity and bilirubin level in serum as compared to control. Treatment with MCRExt showed a significant decrease in serum ALT, AST and ALP activity (Table 3,) induced by NDEA. The pretreatment effect of MCRExt was higher compared to post-treatment. Administration of MCRExt significantly reduced (Table 3) the level of total bilirubin in NDEA induced rats. Due to the ability of MCRExt to reduce free radical-induced oxidative damage in the liver, it has been shown to decrease liver enzymes in serum and prevent liver damage of rats. MCRExt prevents liver damage may be by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes.

Antioxidant Study

NDEA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis. Lipid peroxidation plays an important role in carcinogenesis and may lead to the formation of several toxic products, such as malondialdehyde (MDE) and 4-hydroxynonenal. These products can attack cellular targets including DNA, thereby inducing mutagenecity and carcinogenicity. Recently, the increase in lipid peroxidation was reported during NDEA-induced hepatocarcinogenesis. In line with this finding there was a significant increase in the level of lipid peroxidation in the liver of rats treated with NDEA. However, groups treated with MCRExt displayed a significant reduction in lipid peroxidation when compared to animals treated with NDEA alone (Table 1). Restoration in the levels of lipid peroxidation in groups treated with MCRExt could be related to its ability to scavenge the hydroxyl and peroxyl radicals.

In hepatocellular carcinoma, there is disequilibrium between oxidant and antioxidant balance, which is tilted towards the oxidant side. Reactive oxygen species (ROS) are believed to cause genetic oxidation and damage to DNA and other macromolecules. Unchecked, this oxidative damage may lead to a host of conditions including cancer. Normally, this process is held in check by elaborate endogenous or exogenous antioxidant processes. Various enzymatic and non-enzymatic systems have been developed by the cell to cope with ROS and other free radicals. Since many of the anomalies that are induced by NDEA can arise from oxidative

stress, which is also known to accompany cancer development, it was of a prime interest to evaluate oxidative stress levels under those circumstances.

Free radicals are regularly produced *in vivo* as a result of carcinogen treatment causing oxidative stress that leads to damage of nucleic acids, proteins and lipids resulting in chromosomal instability, mutations, genetic oxidation, damage to DNA, loss of organelle function and membrane damage which play an important mechanistic role in the development of cancer. The endogenous antioxidant system may counteract the ROS and reduce the oxidative stress with the enzymic antioxidants SOD, CAT, GPx and GST. The relatively unreactive superoxide anion free radical is converted by superoxide dismutase (SOD) into H_2O_2, which in turn takes part in the "Fenton reaction" using transition metal ions (copper or iron) as catalysts, to produce the highly reactive hydroxyl radical. The hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion controlled rates. The hydroxyl free radical is more reactive towards cellular constituents than superoxide radicals and hydrogen peroxide. SOD is said to act as the first line of defense against superoxide radical generated as a by-product of oxidative phosphorylation. Further, CAT or GPx converts H_2O_2 to H_2O. Depletion in the activities of these antioxidant enzymes can be owed to an enhanced radical production during NDEA metabolism. In this present observation an increase in MDA was presumably associated with increased free radicals, confirming the fact that these free radicals inhibited the activities of SOD, CAT and GPx. Here the superoxide radical itself is also capable to inhibit the activity of SOD and CAT. This is supported by earlier studies that showed during the NDEA induced hepato carcinogenesis. GST is a family of proteins that catalyzes the conjugation of glutathione with various electrotrophils, many of which are toxic. Also, GST plays a key role in cellular detoxification by catalyzing the reaction of glutathione with toxicants to form an S-substituted glutathione.

Our data revealed that the administration of NDEA induced a significant decrease in SOD, CAT, GPx and GST activity in liver as compared to control. In MCRExt treated groups 4 and 5 animals there is a significant increase in the activities of SOD, CAT, GPx and GST (Table 5) when compared with tumor bearing group 2 animals. The pretreatment effect of MCRExt was higher compared to post-treatment. This improvement may have resulted from changing the tissue redox system by scavenging the free radicals and improving the antioxidant status in the liver during NDEA hepatotoxicity.

Vitamin E, Vitamin C and GSH are well known non enzymic antioxidant defense system of cells. Among these vitamin E is a well recognized, important biological free radical scavenger in the cell membrane. It has been shown to provide protection against superoxides as well as H_2O_2 and it contributes to membrane stability. In hepatoma bearing animals the level of vitamin E was decreased considerably. Vitamin C is water soluble, antioxidant vitamin and can react with vitamin E radicals to regenerate vitamin E. GSH, a non protein thiol is involved in many cellular processes including the detoxification of endogenous and exogenous compounds.

Glutathione is required to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. GSH is said to be involved in many cellular processes including the detoxification of endogenous and exogenous compounds. NDEA, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecule and carcinogen interaction. Accordingly GSH might be depleted partly by the GPx mediated excess utilization of GSH. These three non-enzymic antioxidants are inter related by recycling processes. Earlier report reveals that the levels of these non-enzymic antioxidants were also decreased in hepatoma bearing animals. This observed reduction might be attributed to the utilization of these antioxidants to alleviate free radical induced oxidative stress. Our study shows the increase in the level of vitamin-E, vitamin-C and GSH in group 4 and group 5 as compared to group 2 (Table 6). The increase in the level of these antioxidants after the administration of MCRExt may be due to the direct reaction of bioflavonoids with ROS.

CONCLUSION

From this study, it is concluded that MCRExt suppressed the free radical processes by scavenging hydroxyl radicals in rats. This results underline the capacity of MCRExt to modulate the levels of LPO and significantly increase the endogenous antioxidant defense mechanisms in NDEA induced hepatocellular carcinogenesis. Our results also shows that the significant increase in the levels of serum markers and tumor markers was prevented by MCRExt treatment. Thus the present investigation highlights the tumor suppressive effect of MCREx in *N*-nitrosodiethylamine induced hepatocarcinogenesis which may involve the free radical scavenging mechanism.

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