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THE CHEMOTHERAPEUTIC ROLE OF p-MCA IN AVERTING NDEA INDUCED HEPATIC CARCINOGENESIS IN EXPERIMENTAL WISTAR RATS

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ABSTRACT **OBJECTIVES:** Hepatocellular carcinoma (HCC) is the primary liver cancer and second leading cause of cancer related deaths worldwide. The aim of this present study was to evaluate the biochemical, histopathological and chemotherapeutic efficacy of p-methoxycinnamic acid (p-MCA) against N- nitrosodiethylamine (NDEA) in a rat model of hepatocellular carcinoma. **MATERIALS AND METHODS:** Approximately thirty male wistar rats weighing 150-200 g were designated for this study. The rats were arbitrarily separated into five groups and each group comprised of six rats. Group 1 served as control; Group 2 rats received p-MCA at the dose of 80 mg/kg b.w. Group 3, 4 and 5 rats were induced HCC using NDEA. 2-acetylaminofluorene (AAF) was used as a promotor. Group 4 and 5 rats received p-MCA at the doses of 40 and 80 mg/kg b.w. throughout the 12 week experimental period. At the end of the experimental period, liver tissues from all the rats were collected and liver specific enzymes, lipid peroxidation, markers, xenobiotic metabolizing enzymes, antioxidant status and fibrotic markers were evaluated.**RESULTS:** NDEA administration induced hepatocyte damage, oxidative stress, cell proliferation, inflammation and fibrosis. The liver sections from NDEA induced group 3 rats showed loss of lobular architecture, morphological changes in the nuclei and DNA damage. Administration of p-MCA to NDEA treated rats restored the hepatic architecture, enzyme activities, cell proliferation, inflammation and fibrosis.**CONCLUSION:** We conclude that oral administration of p-MCA for 12 weeks exerts a significant therapeutic effect against HCC by regulating the concentration of specific hepatic and xenobiotic enzymes, suppressing oxidative stress, inhibiting cell proliferation and reducing the inflammatory response.

KEYWORDS : p-methoxycinnamic acid; N-nitrosodiethylamine; 2-Acetylaminofluorene; hepatocellular carcinoma; histopathology; liver specific enzymes.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the world's most common cause of cancer death. It is the primary form of liver cancer and is the third most common cancer death worldwide. [1] 85% of HCC is primary liver cancer widely distributed in the United States, East Asia and Sub-Saharan Africa. The risk factors of HCC include hepatitis C and non-alcoholic steatohepatitis (NASH), consumption of alcohol, tobacco, aflatoxin, environmental and industrial toxins. HCC presents with advance stage of fibrosis predominantly liver cirrhosis and DNA damage. [2]

N- nitroso diethylamine (NDEA), also known as diethyl nitrosamine (DEN), is a well-known hepatocarcinogen in rodent models that mimic human liver cancer. Exposure to NDEA in tobacco, cosmetics, pharmaceutical products, and preserved meats causes adverse effects in humans. NDEA is a most powerful chemical carcinogen which causes tumors in various organs including liver, skin, gastrointestinal tract and respiratory system. [3] The metabolism of NDEA in the liver leads to the formation of reactive oxygen species (ROS), lipid peroxidation and in turn mutagenesis and DNA damage. Several experiments also report that administration of NDEA stimulates hepatocyte proliferation, leading to hepatocarcinogenesis (HCC).

2-Acetylaminofluorene (AAF), a fluorine derivative, is a mutagenic agent that acts as a promoter of carcinogenesis in the liver, kidney and bladder. AAF is a substrate for cytochrome P-450 (CYP) enzyme, resulting in the formation of hydroxyl acetylaminoflurene, a proximal carcinogen. [4]

Phytochemicals, compounds produced by plants, have a general role in the biological activity of plant growth and their defense against pathogens. They are found in fruits, cereals, grains and vegetables. They have potent chemopreventive effects due to their ability to trigger cytotoxicity in cancer cells. [5] Phytochemicals such as phenolic acids have diverse biological and pharmacological properties against various diseases. Phenolic acids are aromatic acids that contain a phenolic ring and a carboxyl functional group. The most abundant source of phenolic acids are cereals and whole grains including bread wheat, hull-less barley, hull-less oat, durum wheat and rye which promote health. Phenolic compounds are readily absorbed through the walls of the intestinal tract, which serve as antioxidants that avoid cellular damage due to free-radical oxidation reactions. It may also promote chemopreventive and anti-inflammatory activities in the human body.

p-methoxycinnamic acid (p-MCA) is an active phenolic compound present in rice bran, brown rice, turmeric, *Kaempferia galanga* and inflorescence of buckwheat. It has been reported to have hepatoprotective,[6] antihyperglycemic,[7] neuroprotective, [8] nematicidal [9] and vasorelaxant effects. [10] The present study was designed to unravel the anti-inflammatory and anticarcinogenic effects of p-MCA against NDEA-induced rat hepatocarcinogenesis.

MATERIALS AND METHODS

ETHICAL APPROVAL

According to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) the experimental study protocol was approved by the Institutional Animal Ethics Committee (IAEC) with approval number AU-IAEC/1225/1/19, Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH), Tamil Nadu, India.

ANIMALS AND DIET

Eight week old male albino Wistar rats approximately weighing 150-200 g were obtained from the Biogen, Bangalore, India. They were maintained in the Experimental Animal Care Center of the Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University. The animals were housed under conventional laboratory conditions in a room maintained at 25±2°C with relative humidity 50±10% and regular 12h light: 12h dark cycle. The animals received standard pellet diet and water throughout the experimental period.

CHEMICALS

N- nitrosodiethylamine [N0258] and p- methoxycinnamic acid [M13807] were purchased from Sigma-Aldrich Chemical Company (USA). All other chemicals and reagents were purchased from HiMedia Private Limited (Mumbai, India).

COMPOUND PREPARATION AND ADMINISTRATION

p-Methoxycinnamic acid was dissolved in dimethyl sulfoxide (DMSO) just prior to treatment. It was then orally administered everyday at the doses of 40 and 80 mg/kg b.w [5] throughout the entire period of the experimental study.

CARCINOGEN PREPARATION AND ADMINISTRATION

According to Dong BJ et al., [11] N- nitrosodiethylamine (NDEA) was used to induce liver cancer. NDEA was dissolved in sterile water and was injected intraperitoneally at the dose of 200 mg/kg b.w thrice at a time interval of 15 days in between starting at the second week, fourth week and sixth week of the experiment.

EXPERIMENTAL PROCEDURE

For the whole study, rats were randomly selected and divided into six groups (Group 1-5) each group included six animals. All the rats were fed standard pellet diet and water throughout the entire experimental period. Group 1 rats served as control, Group 2 rats served as p-MCA control and were administered 80 mg/kg b.w. of p-MCA throughout the experimental period. Group 3 rats served as NDEA control and were administered 200 mg/kg b.w. NDEA, and AAF (0.02 g in 100 mg diet through orally) was injected intraperitoneally thrice at a time interval of fifteen days at the second, fourth and sixth week of the experiment. Group 4 and 5 rats served as treatment groups and were supplemented with different doses of p-MCA (40 and 80 mg/kg b.w. respectively) for twelve weeks along with NDEA and AAF as in group 3. The animals were daily monitored. Amount of food and water consumed by the animals were calculated regularly and the body weight of the rats were recorded every week throughout the experimental period.

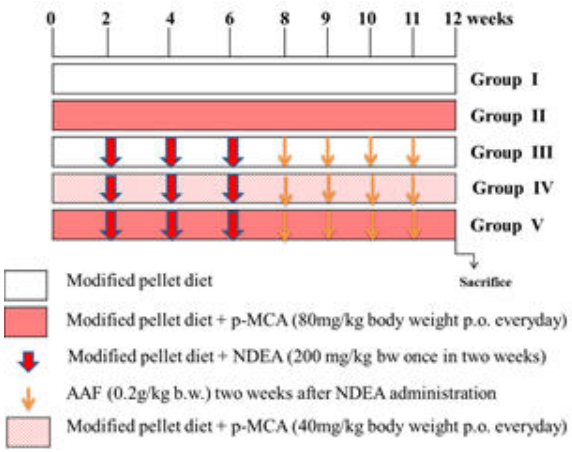


Figure1: Diagrammatic representation of the experimental design. p-MCA: p-methoxycinnamic acid, AAF: 2-acetylaminofluorene, NDEA: – nitrosodiethylamine.

ANIMAL SACRIFICE

At the end of the experimental period, animals were anesthetized by ketamine hydrochloride and sacrificed by cervical dislocation after an overnight fast and the blood was collected from the jugular vein. Liver tissue was dissected out, washed and weighed in ice cold saline.

SEPARATION OF BLOOD SAMPLES

The blood was collected from the animal's jugular vein and stored in heparinized tubes and the plasma was separated by centrifugation at 2000 × *g* for 15 min. The supernatant was used for evaluating the activities of various liver specific enzymes and antioxidants.

Preparation of cytosolic and microsomal fractions

Preparation of cytosolic and microsomal fractions was carried out by the method of Schadt et.al., 1986. Liver and kidney sample tissues were homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, centrifuged at 9000 × *g* for 20 min, and the clear cytosolic fractions were collected for assaying phase II xenobiotic enzymes. The pellets from the above centrifugation were further resuspended in ice –cold 0.15 M Tris-KCl buffer (pH 7.4) and centrifuged for 30 min at 100,000 × *g*. the pellets were resuspended in equal volumes of homogenization buffer and used for the assay of phase I xenobiotic enzymes.

Preparation of tissue homogenate

After animal sacrifice, the liver tissues were dissected and homogenized with the buffer using Potter-Elvehjam homogenizer with a Teflon pestle. The homogenate was centrifuged at 1000 rpm for 15 min at 4° C to separate cell debris. The supernatant was collected and used for further biochemical estimations.

Body weight and growth rate changes

Body weight and growth rate of both control and treated rats were assessed throughout the 12 week-experimental period. The animals were weighed before the start of the treatment, subsequently every week and finally before sacrifice.

Body weight and growth rate were calculated as follows
Body weight = Final body weight – Initial body weight

$$\text{Growth rate} = \frac{\text{Body weight}}{\text{Total number of experimental days}}$$

BIOCHEMICAL ANALYSIS

Assay of specific hepatic marker enzymes

Number of enzymes that regulate chemical reactions in the body are produced and found in the liver cells. Elevation of serum enzyme activities show damage or injury to the liver. The liver function was determined spectrophotometrically (SL 159, UV –Vis spectrophotometer, ELICO, India) by assessing the activities of hepatic-marker enzymes such as alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), alkaline phosphatase (ALP, EC.3.1.3.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) using the method of King. [12]

Assessment of lipid peroxidation

Tissue lipid peroxidation was estimated spectrophotometrically (SL 159, UV –Vis spectrophotometer, ELICO, India).The amount of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) were measured by the method of Niehaus and Samuelson [13] and Jiang et al., [14]

Assessment of the antioxidants

Activity of superoxide dismutase (SOD, EC. 1.15.1.1] was estimated by the method of Kakkar et al., [15] SOD protects cellular constituents from oxidative damage. The reaction was started by the addition of NADH. SOD activity was determined by 50% inhibition of the formation of NADH-phenazine metho sulphate-nitrobluetetrazolium (NBT) formazan. The intensity of the chromogen in butanol layer was measured at 560 nm. Catalase (CAT, EC.1.11.1.6] is an enzymatic antioxidant found in all animal tissues especially with increased activity in the liver. It protects tissues from highly reactive hydroxyl radicals by decomposing hydrogen peroxide. Thus, reduction in the activities of CAT results in several consequences due to accumulation of superoxide radicals and hydrogen peroxide. [16] Reduced glutathione (GSH) is a small molecular weight antioxidant, which protects cells from oxidative damage and the toxicity of xenobiotic electrophiles, thereby maintaining redox homeostasis. [17] The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The reaction mixture was arrested by the addition of a dichromate acetic acid reagent, and the chromic acetate formed was determined spectrophotometrically at 590 nm.

Assessment of xenobiotic metabolizing enzymes

Phase I xenobiotic metabolizing enzymes including cytochrome P450 [CY P450, EC.1.14.14.1], cytochrome P4502E1 [CYP2E1, EC.1.14.13.n7], cytochrome b5, NADPH-cytochrome P450 reductase [EC.1.6.2.4] and NADH-cytochrome b5 reductase [EC.1.6.2.2] were assayed by the methods of Habig et al., [18] T. Omura [19] and P. Strittmatter [20] respectively.

Phase II metabolizing enzymes in the liver including glutathione –S-transferase [GST, EC.2.5.1.18], DT-diaphorase [DTD, EC.1.6.99.2] and UDP-glucuronyltransferase [UDP-GT, EC.2.4.1.17] were assayed by the methods of Habig et al., [18] and K.J. Isselbacher. [21]

Histopathological Description

Histopathological analysis of tissues of liver and kidney were performed by the method of G.M.Akshatha et al., [22] After sacrifice, the liver and kidney tissue sections were excised, washed with ice-cold saline, allowed to dry and then weighed. The liver and kidney sections were stabilized in 10% neutral buffered formalin at room temperature. Formalin-fixed samples were embedded in paraffin wax, and sectioned using a microtome.

Hematoxylin and Eosin staining

5 µm thick paraffin embedded tissue sections were stained with Mayer's hematoxylin and shaken for 30 sec. The stained sections were rinsed with running water for 1 min and allowed to dry. Then they were stained with 1% eosin Y solution for 10-15 sec with agitation. Then specimens were dehydrated through different graded series of alcohol and extracted by adding of xylene. The tissue sections were subsequently mounted with DPX and allowed to dry. The photographs were captured at 40X by using light microscope (Carl Zeiss).

Argyrophilic nucleolar organizer regions (AgNORs) staining

Silver staining of AgNORs was performed by the method of S. P. Gulia et al., [23] The paraffin embedded blocks were deparaffinzed in xylene

and hydrated through different grades of ethanol. The tissue sections were dipped in freshly prepared silver colloidal solution (2% gelatin and 1% formic acid) and treated with 50% aqueous silver nitrate solution and incubated at room temperature for 40 min. After incubation, the sections were washed with double distilled water and dehydrated through ethanol and xylene. The sections were mounted with DPX and allowed to dry overnight. The silver dots of AgNORs stained slides were photographed under light microscope at 40X magnification (Carl Zeiss).

Mast cell staining

Toluidine blue staining for mast cells was performed according to the method of Hargrove L et al.,[24]The tissue sections were deparaffinized, hydrated and stained with toluidine blue for 3 min. Then they were washed with running distilled water and dehydrated through different concentrations of ethanol and xylene. The sections were again dried and mounted using DPX mounting solution. The dark blue granules of mast cells were captured under a light microscope at 40X magnification. (Carl Zeiss).

STATISTICALANALYSIS

The results were analyzed by one-way analysis of variance (ANOVA) and the significant differences among six groups of rats were evaluated by Duncan's multiple range test (DMRT). All the data were expressed as mean ± SD. The data was considered statistically significant at $p < 0.05$.The statistical analyses were done by using SPSS version 17.0 software package (SPSS, Tokyo, Japan).

RESULTS

Effect of p-MCA and NDEA on body weight and growth rate

The animals were carefully monitored for the entire 12-week experimental period. Amount of food and water consumption was noticed and changes in the body weight were regularly recorded at the end of every week. For the first two weeks no changes were noticed among the different groups of animals. Once treatment was initiated the body weight changes of NDEA administered rats and control rats varied gradually. The amount of food and water consumed by the NDEA treated rats were comparatively less as compared to the control and p-MCA control rats. At the end of 12 weeks, group 3 (NDEA control) rats showed drastically decreased body weight as compared to group 1 and 2 rats. Group 4 and 5 (p-MCA 40 and 80 mg/kg b.w.) rats showed a significant increase in the body weight as compare to the group 3 rats. Finally, there was no significant difference in the body weight between groups 1 and 2 control rats. Body weight of the rats was calculated as the difference between the final body weight and the initial body weight. The body weight changes in the different groups of animals are shown in figure 2.

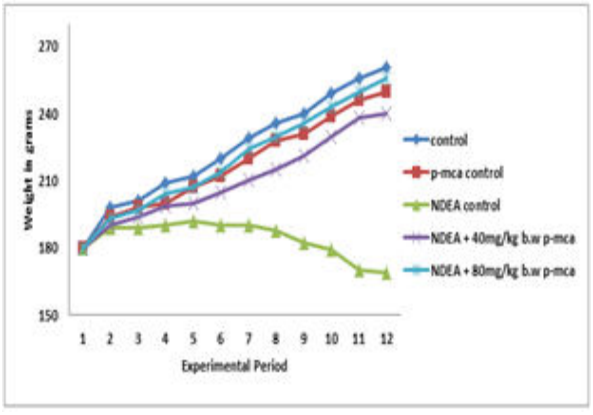


Figure 2: Body weight changes of control and experimental rats

Growth rate changes of the five groups of animals were periodically recorded. The growth rate changes of the different groups of animals was calculated by the difference between the final body weight and the initial body weight divided by the total number of days of the experiment. The growth rate of groups 4 and 5 animals were significantly increased as compared to the group 3 (NDEA control) rats. There was no any significant difference between the control and p-MCA control rats. Figure 3 shows the growth rate differences between the control and experimental rats.

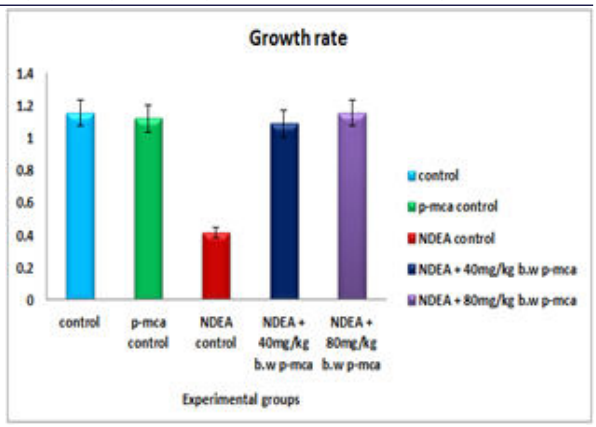


Figure 3: Growth rate changes of control and experimental rats. All the data are expressed as mean ± SD. The data was considered statistically significant at $p < 0.05$.

Changes in the liver morphology of the control and experimental rats

Figure 4 shows the morphological differences between the control and treatment groups of animals. NDEA treated rats (Group 3) rats showed increased nodule formation in the hepatic tissue (fig 4C). Administration of p-MCA with NDEA (Group 4) at the dose of 40mg/kg b.w. showed less number of nodule formation (fig 4D). 80mg/kg b.w. of p-MCA + NDEA treated rats (Group 5) showed no changes in the liver morphology. There were no changes in the liver morphology of the control and p-MCA control rats (fig 4A and 4B).

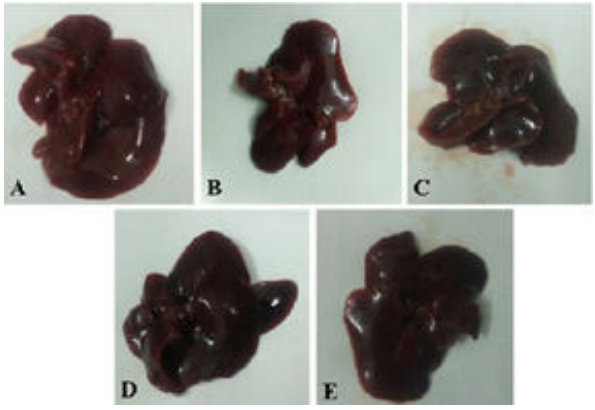


Figure 4: Morphological changes of the liver of control and experimental rats. **A and B** – Liver tissue of control and p-MCA treated rats showed normal morphology. **C-**Liver tissue of NDEA treated rats showed nodule formation in their morphology. **D and E-**Liver of NDEA + p-MCA administered rats showed reduced number of nodule formation.

Changes in the kidney morphology of the control and experimental rats

Kidney morphology of control and treatment groups of animals is shown in figure 5. Kidney of control and p-MCA treated control rats showed normal morphology of tubules, glomeruli and renal parenchymal cells. The kidneys of NDEA treated rats showed enlarged kidney morphology, and nodule formation and edema exudates. However, NDEA treated rats on supplementation with p-MCA showed reduced number of nodules and normal appearance of the kidney.

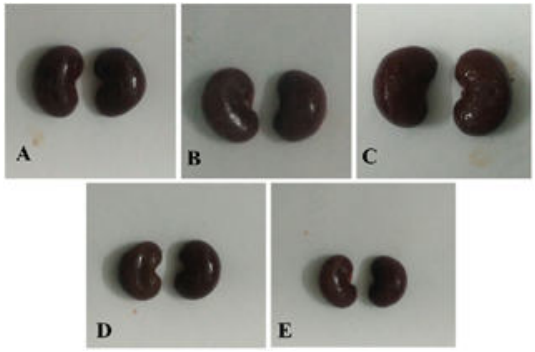


Figure 5: Morphological changes in the kidney of control and experimental rats. **A and B** – Kidney of control and p-MCA treated rats showed normal morphology. **C**-Kidney tissue of NDEA treated rats showed enlarged morphology. **D and E**-Kidney of NDEA + p-MCA administered rats showed reduced number of nodule formation.

Effect of p-MCA and NDEA on hepatic marker enzymes

Table 1: Effect of p-MCA on liver specific marker enzymes in the serum of control and experimental rats

Parameters (IU/L)	Control	p-MCA control	NDEA alone	NDEA + p-MCA (40 mg/kg b.w)	NDEA + p-MCA (80 mg/kg)
AST	71.53± 5.45[a]	74.73± 5.72[a]	102.42± 7.80[b]	96.29± 7.37[c]	73.36± 5.59[a]
ALT	54.53± 4.15[a]	53.26± 4.08[a]	88.20± 6.72[b]	69.19 ± 5.30[c]	53.65 ±4.09[a]
ALP	82.94 ± 6.32[a]	81.16 ± 6.21[a]	128.63 ± 9.79[b]	107.41 ± 8.22c	80.17± 6.10[a]
LDH	92.38 ± 7.03[a]	94.09 ± 7.20[a]	131.98 ± 10.05[b]	120.26 ± 9.21[c]	91.55 ± 6.97[a]

Values are expressed as the mean + SD of six rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $p<0.05$ (DMRT).

AST-aspartate transaminase, ALT-alanine transaminase, ALP-a alkaline phosphatase, LDH-lactate dehydrogenase

Table 1 shows the effect of p-MCA and NDEA on liver marker enzymes such as ALT, AST, ALP and LDH. The activities of enzymes such as ALT, AST, ALP and LDH were significantly increased in NDEA supplemented rats (group 3) as compared to the control rats (group 1) indicating tumour formation and liver damage. Administration of p-MCA to NDEA treated rats showed a significant decrease in the activities of hepatic marker enzymes such as ALT, AST, ALP and LDH as compared to the NDEA treated rats (group 3).Especially 80 mg/kg b.w p-MCA treatment to NDEA treated rats caused prominent decrease in the enzyme activities. There were no significant differences between the enzyme activities of control and p-MCA control rats.

Effect of p-MCA and NDEA on lipid peroxidation

Table 2: Effect of p-MCA on hepatic lipid peroxidation byproducts of control and experimental rats

Groups	TBARS	LOOH
Control	0.91 ± 0.07[a]	49.83 ± 3.79[a]
p-MCA control	0.96 ± 0.07[a]	47.74 ± 3.65[a]
NDEA alone	4.32 ± 0.33[b]	86.39 ± 6.58[b]
NDEA + p-MCA(40 mg/kg)	2.94 ± 0.23[c]	77.06 ± 5.90[c]
NDEA + p-MCA (80 mg/kg)	0.94 ± 0.07[a]	48.99 ± 3.73[a]

All the values are expressed as the mean± SD of six rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $p < 0.05$ (DMRT)

TBARS-thiobarbituric acid reactive substances (mmol/mg tissue); LOOH-lipid hydroperoxides (mmol/mg tissue)

Lipid peroxidation is the oxidative degradation of the lipids. It is the mechanism by which free radicals steal the electrons from the lipids in the cell membrane leading to cell damage. Table 2 shows the effect of p-MCA and NDEA treatment on lipid peroxidation in the liver of control and experimental rats. NDEA treated rats (group 3) showed significant increase in the levels of TBARS and LOOH as compared to the control rats (group 1). Administration of p-MCA to the NDEA treated rats (group 4 & 5) restored the levels of TBARS and LOOH as compared to the control rats. Administration of 80 mg/kg b.wp-MCA showed a much more marked difference in reducing lipid peroxidation as compared to the other dose. There was no significant difference in the levels of the lipid peroxidation between the control and p-MCA control rats.

Effect of p-MCA and NDEA on the antioxidant status

Table 3: Effect of p-MCA on hepatic enzymic and non-enzymic antioxidants of control and experimental rats

Groups	SOD	CAT	GSH
Control	7.96 ± 0.61[a]	53.84 ± 4.10[a]	76.59 ± 5.83[a]
p-MCA control	7.74 ± 0.59[a]	52.81 ± 4.04[a]	77.96 ± 5.97[a]
NDEA Alone	4.82 ± 0.37[b]	12.28 ± 0.94[b]	36.43 ± 2.77[b]
NDEA + p-MCA (40 mg/kg)	5.04± 0.39[c]	23.64 ± 1.81[c]	42.98 ± 3.29[c]
NDEA + p-MCA (80 mg/kg)	7.93 ± 0.60[a]	51.64 ± 3.93[a]	76.97 ± 5.86[a]

All the values are expressed as the mean± SD of six rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $p < 0.05$ (DMRT)

SOD-superoxide dismutase (50% NBT reduction/min/mg protein); CAT-catalase (µmoles of H_2O_2 utilized/min/mg protein); GSH-glutathione (mmole/mg tissue protein)

The antioxidant property was evaluated by the determining the enzymic and non-enzymic antioxidants of the control and experimental rats. Table 3 shows the antioxidant status of p-MCA and NDEA treated control and experimental rats. Group 3 NDEA treated rats showed a significant decrease in the activities of the antioxidants such as SOD, CAT and GSH as compared to the control rats (group 1).Administration of p-MCA to the NDEA treated rats showed significant increase in the activities of these antioxidants as compared to the group 3 NDEA alone treated rats. Administration of 80 mg/kg b.w p-MCA showed more significance in this regard. p-MCA control rats (group 2) showed no significant difference in the antioxidant activity as compared to the control rats (group 1).

Effect of p-MCA and NDEA on the xenobiotic metabolizing enzymes

Table 4 showed the effect p-MCA and NDEA on the activities of phase I and phase II enzymes in the liver. Administration of NDEA to group 3 rats showed an increase in the activities of phase I enzymes followed by a decrease in the activities of phase II enzymes (GST, DTD and UDP-GT) as compared to the control rats (group 1).Administration of p-MCA to NDEA treated rats showed significant decrease in the enzyme activities as compared to the group 3 NDEA treated rats. Supplementation with 80 mg/kg b.w p-MCA to NDEA treated rats (group 5) was observed to suppress the activities of these enzymes as compared to the NDEA alone treated (group 3) rats. There were no significant variations in the enzyme activities between the control (group1) and p-MCA control (group 2) rats.

Table 4: Effect of p-MCA on liver phase I and phase II enzymes of the control and experimental rats

Parameters	Control	p-MCA control	NDEA alone	NDEA + p-MCA (40 mg/kg)	NDEA + p-MCA (80 mg/kg)
Cytochrome P450	2.76 ± 0.21[a]	2.40 ± 0.18[a]	7.20 ± 0.55[b]	5.86 ± 0.45[c]	2.29 ± 0.17[a]
Cytochrome P4502E1	4.82 ± 0.37[a]	4.76 ± 0.36[a]	9.53 ± 0.73[b]	7.92 ± 0.61[c]	4.69 ± 0.36[a]
Cytochrome b5	3.12 ± 0.24[a]	4.09 ± 0.31[a]	11.46 ± 0.87[b]	8.87 ± 0.68[c]	3.98 ± 0.30[a]
NADPH-cytochrome P450reductase	39.17 ± 2.98[a]	42.85 ± 3.28[a]	92.34 ± 7.03[b]	70.76 ± 5.42[c]	40.75 ± 3.10[a]
NADPH-cytochrome b5 reductase	16.42 ± 1.25[a]	16.68 ±1.28[a]	28.23 ± 2.15[b]	20.05 ± 1.53[c]	16.46 ± 1.25[a]
GST	0.36 ± 0.03[a]	0.38 ± 0.03[a]	0.21 ± 0.02[b]	0.28 ±0.02[c]	0.39 ± 0.03[a]
DTD	1.26 ± 0.10[a]	1.39 ± 0.11[a]	0.42 ± 0.03[b]	0.25 ± 0.02[c]	1.42 ± 0.11[a]
UDP-GT	3.56 ± 0.27[a]	3.72 ± 0.28[a]	1.68 ± 0.13[b]	2.28 ± 0.17[c]	3.60 ± 0.27[a]

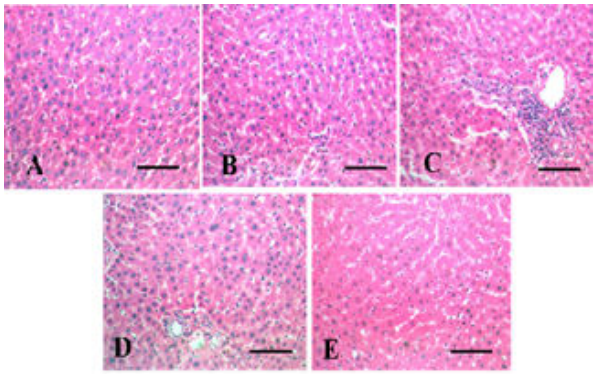
All the values are expressed as the mean± SD of six rats in each group. Values not sharing a common superscript letter (a- c) differ significantly at $p < 0.05$ (DMRT)

Cytochrome P450-µmol/mg protein; Cytochrome P4502E1-mmol of p-nitrocatechol liberated/min/mg protein; Cytochrome b5-µmol/mg protein; NADPH-cytochrome P450 reductase-one unit of enzyme activity is defined as that causing the oxidation of one mole of NADPH per minute; NADPH-cytochrome b5 reductase-one unit of enzyme activity is defined as that causing the reduction of one mole of ferricyanide per minute; GST-glutathione-S-transferase (µmol of 1-chloro-2,4,-dinitrobenzene (CDNB)-GSH conjugate

formed/min/mg protein); DTD-DT-diaphorase (µmol of 2,6,-dichlor ophenolindophenol reduced/min/mg protein); UDP-GT-UDP-glucuronyl transferase (nmol/min/mg protein)

Effect of p-MCA and NDEA on liver histology: Hematoxylin and Eosin staining

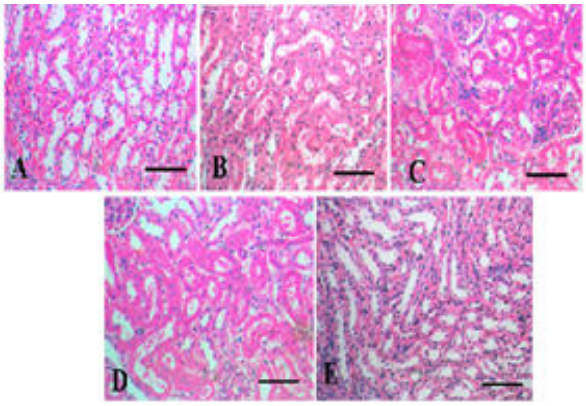
Figure 4: Histological changes in the liver of control and experimental rats (H and E staining)



A and B- Liver sections of control and p-MCA treated rats showing normal architecture. C- Liver section of NDEA-induced rat showing fibrosis with inflammation. D-Liver section of NDEA + 40 mg/kg b.w p-MCA treated rat showing inflammatory cells. E-Liver section of NDEA + 80 mg/kg b.w p-MCA treated rat showing degeneration of hepatocytes.

Figure 4 illustrates the representative photo micrographs of the liver sections of control and experimental rats stained with hematoxylin and eosin. Liver sections of groups 1 and 2 (control and p-MCA control) rats showed normal hepatic architecture with central portal vein. Administration of NDEA (group 3) revealed fibrosis with inflammatory cell infiltration and cell proliferation and degeneration. p-MCA treatment to the NDEA administered rats showed near normal appearance of hepatocytes with reduced degeneration. The hepatic section of 80 mg/kg b.w p-MCA (group 5) treated, NDEA administered rats showed normal hepatic architecture with less formation of nodules and was comparable to that of the p-MCA control (group 2) rats.

Figure 5: Histological changes in the kidneys of control and experimental rats (H and E staining)

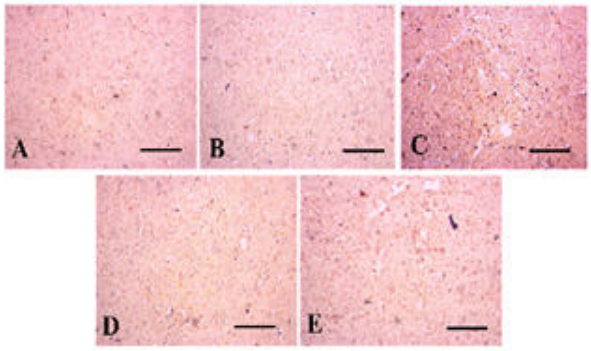


A and B- Kidney sections of control and p-MCA treated rats showing normal architecture with glomerulus, proximal convoluted tubules, and distal convoluted tubules. C- Kidney section of NDEA-induced rat showing tubular necrosis, sloughing of tubular cells. D-Liver section of NDEA + 40 mg/kg b.w p-MCA treated rat showing few inflammatory cells with glomerular atrophy. E-Liver section of NDEA + 80 mg/kg b.w p-MCA treated rat showing glomerular degeneration.

Figure 5 illustrates the histological image of the kidney sections of the control and experimental rats stained with hematoxylin and eosin. Kidney sections of NDEA treated rats showed injured architecture with damage to the tubules (proximal and convoluted), and glomeruli indicated by red fluorescence in the damaged areas. P-MCA treated NDEA administered rats showed near normal appearance of the kidney tissue with reduced degeneration of glomerulus.

Effect of p-MCA and NDEA on liver AgNORs staining

Figure 6: Histological changes in the liver of control and experimental rats (AgNORs staining)

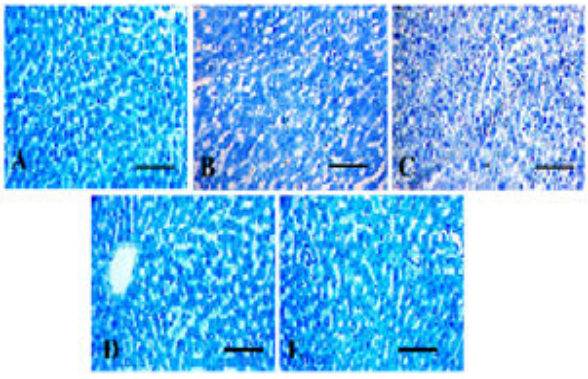


A and B- Liver sections of control and p-MCA treated rats showing less AgNORs in the nucleus. C- Liver section of NDEA-induced rat showing increased AgNORs in the nucleus (black dots in the nucleus). D and E - Liver section of NDEA + 40 mg/kg b.wp-MCA and 80 mg/kg b.w p-MCA treated rat showing decreased AgNORs in the nucleus.

The presence of argyrophilic nucleolar organizing region (AgNORs) in nucleus of the hepatocytes was determined by silver nitrate staining. Figure 6 illustrates the presence of AgNORs in the control and experimental rats stained with silver nitrate. AgNORs was identified by the presence of black dots in the nucleus of the cell. Administration of NDEA (group 3) showed increased number of AgNORs as compared to the control (group 1) and p-MCA control (group 2) rats. However, the number of AgNORs was significantly reduced in p-MCA supplemented (group 4 and 5) NDEA treated rats. Especially 80 mg/kg b.w p-MCA (group 5) supplemented NDEA treated rats showed less reduction in AgNORs and was comparable to that of the control and p-MCA control rats.

Effect of p-MCA and NDEA on liver toluidine blue staining

Figure 7: Histological changes in the liver of control and experimental rats (mast cell staining)



A and B- Liver sections of control and p-MCA treated rats showing no accumulation of mast cells. C- Liver section of NDEA-induced rat showing increased number of mast cell accumulation (blue granules). D - Liver section of NDEA + 40 mg/kg b.w p-MCA treated rat showing a slight reduction in the accumulation of mast cells. E – Liver section of NDEA + 80 mg/kg b.w p-MCA showing reduced accumulation of mast cells.

Figure 7 shows the representative photomicrographs of the liver of the control and experimental rats showed for mast cells. The results revealed excessive accumulation of mast cells in NDEA treated rats (group 3) as compared to the control (group 3) rats. p-MCA supplemented rats (group 4 and 5) showed reduced accumulation of mast cells in the liver as compared to the NDEA treated rats. The effect of p-MCA was more pronounced in group 5. There were no any significant changes in the number of mast cells in the liver sections of control rats (groups 1 and 2).

DISCUSSION

Several natural products have medicinal properties and are used to treat various diseases. [25] Similarly our study with p-MCA, a phenolic component present in rice bran, brown rice, turmeric and *Kaemperfia galanga*, demonstrated noticeable protective effects against NDEA-induced hepatocellular carcinoma (HCC) in albino wistar rats. Our study also shows that NDEA treatment provoked the development of HCC and resulted in the formation of hepatic malignancy. Further, treatment with NDEA significantly reduced the body weight gain by the animals. However, oral administration of p-MCA to HCC bearing rats showed increase in the body weight, simultaneously decreasing the occurrence of hepatic malignancy. Likewise, administration of NDEA resulted in the occurrence of preneoplastic hepatic lesions.

AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of α-ketoglutaric acid to generate oxalacetic and pyruvic acids respectively, which are important contributors to the citric acid cycle. Both enzymes require pyridoxal- 5-phosphate (vitamin B6) in order to carry out this reaction, although the effect of pyridoxal-5-phosphate deficiency is greater on ALT activity than on that of AST. In addition, LDH, an oxidoreductase enzyme is used as a diagnostic tool for many diseases including liver cancer. LDH catalysing the pH dependent interconversion of lactate into pyruvate. There are many reasons for the increase in the concentration of LDH in the blood of cancer patients. First during cancer development the cell number increases requiring more amount of glucose produced from glycolysis which increases LDH level when the condition is anaerobic. [26] Second, due to the enhanced growth of cancer cells other normal cells in the tissues are damaged thereby releasing LDH (intracellular enzyme) into the blood stream. Third, during carcinogenesis the production of LDH is increased, due to the activation of the tyrosine phosphorylation mechanism. [27] In liver disease, elevations of LDH are not as great as the increases in aspartate amino transferase (AST) and alanine aminotransferase (ALT).

Liver function was evaluated by measuring the activities of ALT, AST, ALP and LDH. Any damage to the liver cells results in increased release of the intracellular enzymes such as ALT, AST, ALP and LDH into the circulatory system. Thus administration of NDEA, a carcinogen leads to severe damage to the plasma membrane of the hepatocytes resulting in the release of the enzymes from the cytosol into the blood stream. [28]

Our present study correlates with the above findings as evidenced by the elevated of activities of hepatic enzymes such as AST, ALT, ALP and LDH indicating that NDEA induces liver damage and hepatocarcinogenesis. Supplementation with p-MCA to HCC-bearing rats significantly reduced the activities of these enzymes thereby reducing liver damage and hepatocarcinogenesis.

Studies revealed that NDEA arouses lipid peroxidation and is the major factor contributing to carcninogenesis in the liver by producing free radicals and formation of by-products such as TBARS and LOOH which promotes hepatocarcinogenesis. Oxidative stress is caused by the disruption between the ROS production and the alterations in the activities of the antioxidants such as SOD, CAT and GSH. [29] In erythrocytes these antioxidants enzymes SOD and CAT are present in high concentrations. SOD is used in the dismutation of reactive superoxide anion to oxygen and to a less reactive hydrogen peroxide and CAT is used to prevent cell damage by converting H_2O_2 into molecular oxygen and water. GSH is an innate antioxidant, which has an important role in detoxification and also in the scavenging of several free radicals. In the present study, NDEA alone exposed rats had increased levels of TBARS and LOOH and a significant decrease in CAT, SOD and GSH in the liver as compared to the liver of the control rats. Supplementation with p-MCA to HCC bearing rats significantly decreased the levels of TBARS and LOOH and significantly increased the activities of the antioxidants.

Drugs, chemicals, and endogenous substrates are all metabolised by cytochrome P-450 (CYPs). Hepatic CYPs play a role in the pathogenesis of a number of liver diseases. Hepatotoxicity is caused by the conversion of drugs to toxic metabolites by the CYP enzymes. CYP2E1 has been implicated in the pathogenesis of alcoholic liver disease and non-alcoholic steatohepatitis in many studies. Augmented CYP2E1 activity is linked to lipid peroxidation and the formation of free radicals in these environments, resulting in secondary damage to cellular membranes and mitochondria. CYP2E1 has also been proposed as a cofactor for HCC due to its ability to activate carcinogens. [30]

GST, DTD, and UDP-GT are the phase II metabolizing enzymes that transfer the xenobiotic compounds into non-toxic and easily excreteable form. GST acts as a defense mechanism against toxic and reactive electrophiles that are generated by cellular oxidative reactions catalyzed by CYP450. [31] DTD, a flavoprotein catalyzes the reduction of quinones without producing ROS and protects the cells against mutagenicity. In our present study, the activities of phase I enzymes (CYP450, CYP2E1) were significantly increased and those that of the phase II enzymes (GST, DTD and UDP-GT) were significantly reduced in NDEA alone treated rats which could be due to their ability to counteract NDEA- induced toxicity or due to their reduced metabolizing capacity. Administration of p-MCA to NDEA supplemented rats significantly enhanced the activities of phase II enzymes whereas suppressed the activities of phase I enzymes.

The histopathological characteristics of the tissue sections were identified by the hemotoxylin and eosin staining. This staining displayed the cytoplasm, nucleus and extracellular matrix of the hepatocytes. Hemotoxylin has a deep-purple colour which stains nucleic acids and eosin is in pink colour which stains cytoplasm and extracellular matrix. The hepatic tissue of HCC bearing rats showed distortion in the arrangement of hepatocytes, congestion of sinusoids around central vein, granular degeneration and vacuolization. Histopathological changes observed in our present study observed were closely similar to the findings reported by G.M.Akshatha et al. [22] who reported that NDEA supplemented rat liver presented with vacuolated hepatocytes, leukocytic infiltration and hyperchromatic nuclei. p-MCA treatment to NDEA supplemented rats for 12 weeks revealed only slight changes in the hepatic architecture and was comparable to that of the control rats. In the control rats, hepatic tissue showed normal architecture with normal appearance of hepatocytes with reduced degeneration and normal central veins.

Argyophillic nucleolar organizing regions (AgNORs) are a set of nucleolar proteins used for ribosomal synthesis. The amount of AgNORs interphase is well known to be larger in neoplastic than in corresponding benign or normal tissues. The amount of interphase AgNORs is directly related to cell proliferation activity. The AgNORs value rises during the G1 phase before peaking during the S phase. The higher the interphase AgNORs activity in proliferating cells, the faster the cell proliferation. Findings suggest that a high hepatocyte proliferation rate, as measured by high AgNOR numbers in the hepatocyte nuclei of patients with chronic liver disease, is associated with a higher incidence of HCC. [32] It can be demonstrated by silver staining which appears as black dots. In our study p-MCA supplementation to NDEA exposed rats showed less number of AgNORs in the hepatic tissue sections as compared to the control group.

Toluidine blue (also called tolonium chloride) is an acidophilic metachromatic dye that stains acidic tissue components specifically (sulfates, carboxylates, and phosphate radicals). Toluidine blue (TB) has a strong affinity for nucleic acids, it stabilizes the nuclear material in tissues with a lot of DNA and RNA. Chromosomes in tissues were identified by the toluidine blue staining. It stains nucleic acids and polysaccharides purple. Mast cells are mainly made up of granules that contain mediators such as histamine, serotonin, tryptase, and heparin. Mast cells can also release freshly developed lipid mediators such as prostaglandin D2 and platelet-activating factor, as well as cytokines and chemokines. Small amounts of histamine are secreted by cholangiocytes (which house the machinery for histamine synthesis), causing an autocrine regulation of hyperplastic and neoplastic development. In the present study, large number of mast cells were found in the liver sections of NDEA treated rats indicating the inflammatory process, whereas treatment with p-MCA reduced the mast cell accumulation in the hepatocytes exhibiting its anti-inflammatory efficacy against HCC.

CONCLUSION

The present study concludes that the oral administration of the lowest dose of 40 mg/kg b.w p-MCA was not effective against NDEA induced hepatic carcinogenesis, which may due to its insufficient concentration to protect against the carcinogen induced toxicity. 80 mg/kg b.w of p-MCA showed significant anticancer effects against hepatocarcinogenesis. So we conclude that 80 mg/kg b.w p-MCA as the effective optimum dose against NDEA induced HCC. Further studies are being carried out to study the mechanism of action of p-MCA against NDEA induced hepatocarcinogenesis in detail.

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Conflicts of interest

The authors declare that there are no conflicts of interest

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