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A long term study to unravel the chemopreventive efficacy of p-Methoxycinnamic acid in comparison to sorafenib, a standard drug against NDEA induced hepatocarcinogenesis

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Abstract:
p- Methoxycinnamic acid (p-MCA), an active phenolic compound derived from the rice bran, turmeric and *Kaempferia galangal*, is known to exhibit numerous pharmacological properties. We investigated that chemopreventive effect of p-MCA against N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in male wistar rats. Rats were subjectively divided into five groups. Group 1 served as control and was fed modified pellet diet and water. Group 2 received the reference drug (Sorafenib [11.4 mg/kg b.w]). Group 3, 4 and 5 rats received the hepatocarcinogen (NDEA) intraperitoneally in addition to 2-acetylaminofluorene (AAF) as promotor. In addition group 4 rats received p-MCA at the dose of 80 mg/kg b.w. and group 5 rats received sorafenib at the dose of 11.4 mg/kg b.w throughout the experimental period. Our results established that supplementation with p-MCA and the reference drug (sorafenib) to NDEA induced rats significantly decreased the incidence of cancer in the liver, besides altering the phase I and phase II xenobiotic enzymes. Furthermore the increased expressions of collagen, lipid and glycogen accumulation observed in NDEA alone-induced rats were comparable with those of the control rats, p-MCA and sorafenib alone treated rats. Our results proved that p-MCA was equally effective that of the reference drug sorafenib (which is used currently for the treatment of hepatocellular carcinoma against NDEA induced hepatocarcinogenesis.

Keywords: p-methoxycinnamic acid, N-nitrosodiethylamine, 2-acetylaminofluorene, sorafenib, hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of death all over the world. It is the primary form of liver cancer and it is three times more common in men than women [1]. HCC arises due to several causative agents like alcohol consumption, food, hepatitis B virus, hepatitis C virus, obesity, tobacco smoking, diabetes, genetic factors and environmental factors [2]. Besides, in addition to known factors, exposure to genotoxic and cytotoxic chemicals such as nitroso compounds and azo dyes present in the

surroundings are also hidden factors causing liver cancer. Thus exposure to environmental carcinogens is an important factor leading to the formation of HCC.

N-nitroso diethylamine (NDEA) is one of the most important environmental carcinogens which occurs commonly in nature such as in cheese, processed meats, soybean, tobacco products, alcoholic beverages, agricultural chemicals and cosmetics [3]. It is considered as one of the most powerful carcinogens which causes tumors in various organs including liver, skin, gastrointestinal tract and respiratory system [4].

2-Acetylaminofluorene (AAF) is a fluorine derivative which induces tumors in liver, kidney and bladder. 2-AAF acts as a substrate for cytochrome P-450 (CYP) enzyme which is found in all organisms. It leads to the formation of proximal carcinogen called hydroxyl acetylaminofluorene and it is more powerful than the parent molecule [5].

Natural products with a wide range of pharmacological properties are excellent and effective sources for the development of anticancer drugs [6]. 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. They are abundant in 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, and serve as antioxidants and avoid free radical induced cell damage. They are also known to possess chemopreventive and anti-inflammatory properties [7].

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 [8], antihyperglycemic [9], neuroprotective [10], nematicidal [11] and vasorelaxant effects [12].

Our previous study revealed that p-MCA has inhibitory effects on N-nitrosodiethylamine induced hepatic carcinogenesis in a dose dependent manner. Now our aim was to explore the effects of p-MCA in comparison with the reference drug sorafenib against NDEA induced hepatic carcinogenesis.

2. Materials and methods

2.1. 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/1215/4/18, Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH), Tamil Nadu, India.

2.2. Animals and diet

For this experimental study, approximately male wistar rats weighing 200 g were obtained from Biogen, Bangalore, India. They were maintained in the Experimental Animal Care Center of 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.

2.3. 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 Limted (Mumbai, India).

2.4. 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 [13] respectively throughout the entire period of the experimental study.

2.5. Carcinogen preparation and administration

According to Khan et al., [1] N- nitrosodiethylamine (NDEA) was used to induce liver cancer. NDEA was dissolved in sterile water and injected intraperitoneally at the dose of 200 mg/kg b.w. on the second week, and subsequently fourth week and sixth week of the experiment. After carcinogen administration, acetylaminofluorene (AAF) was incorporated at the dose of 0.02 g in 100g of diet during the remaining part of the experimental period.

2.6. Experimental procedure

For the whole study, rats were randomly selected and divided into five groups (Group 1-5) with each group comprising of six animals. Group 1 rats served as control and were fed standard pellet diet and water throughout the entire experimental period. Group 2 rats served as sorafenib control and administered 11.4 mg/kg b.w. of sorafenib orally with standard pellet diet and water throughout the experimental period. Group 3 rats served as NDEA control and received AAF, (0.02g/100g of diet everyday) and NDEA (200 mg/kg b.w. ip) thrice at a time interval of fifteen days at the second, fourth, sixth, eight, tenth, eleventh and twelfth week of the experiment. Group 4 and 5 rats served as treatment groups and were treated with NDEA and AAF as in group 3. In addition group 4 rats were supplemented with p-MCA (80 mg/kg b.w.) and group 5 rats received sorafenib (11.4 mg/kg b.w.) for twenty four weeks. The animals were daily monitored, amount of food and water given to the animals were calculated regularly and the body weights of the rats were recorded every week throughout the experimental period.

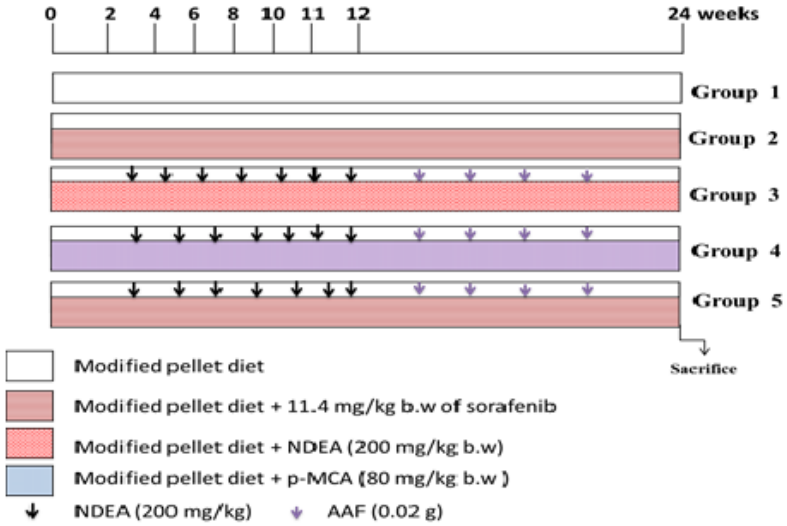

Figure1. Diagrammatic representation of the experimental design. p-MCA: p-methoxycinnamic acid, NDEA: N- nitrosodiethylamine, AAF: 2-acetylaminofluorene

2.7. 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.

2.8. 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.

2.9. 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.

2.10. Preparation of cytosolic and microsomal fractions

Preparation of cytosolic and microsomal fractions was carried out by the method of Schadt et.al.,[14]. 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.

2.11. 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 - end and finally before sacrifice.

Growth rate was calculated as follows

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

2.12. Biochemical analysis

2.12.1. Assay of specific hepatic marker enzymes

A number of enzymes that regulate chemical reactions in the body are produced and found in the liver cells. Elevation of serum enzyme activities denote damage or injury to the liver. The liver function was determined spectrophotometrically (SL 159, UV –Vis spectrophotometer, ELICO, India) by assaying the activities of hepatic-specific enzymes such as alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), and alkaline phosphatase (ALP, EC.3.1.3.1).

2.12.2. 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 [15] and Jiang et al.,[16] respectively.

2.12.3. Assessment of the antioxidants

Activity of superoxide dismutase (SOD, EC. 1.15.1.1] was estimated by the method of Kakkar et al.,[17]. The reaction was started by the addition of NADH. 50% inhibition of the formation of NADH-phenazine metho sulphate-nitroblue tetrazolium (NBT) formazan was measured by assessing the colour intensity of the chromogen in butanol layer at 560 nm. For the assay of catalase [CAT, EC.1.11.1.6], the reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. CAT activity was measured at 230 nm. Reduced glutathione (GSH) was determined by the method of Ellman [18]. GSH involves oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis (2-nitrobenzonic acid) (DTNB) to form an yellow derivative 5'-thio-2-nitrobenzonic acid (TNB), which was measured at 412 nm.

2.12.4. 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., [19], Omura T [20] and Strittmatter P [21] 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., [19], Mehmet Kanter et al., [22], and K.J. Isselbacher [23].

2.13. Histopathology

Histopathological analysis of the liver and kidney tissues were performed by the method of Akshatha G.M et al., [24]. After sacrifice, the liver and kidney tissues were excised, washed with ice-cold saline, allowed to dry and then weighed. The tissue sections were stabilized in 10% neutral buffered formalin at room temperature. Formalin-fixed samples were embedded in paraffin wax, and sectioned using a microtome.

2.13.1. 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 (AxioScope A1, Carl Zeiss, Jena, Germany).

2.13.2. Picrosirius red staining

Picrosirius red staining was performed by the method of Benjamin V 2015. The liver sections were deparaffinized and hydrated with distilled water. The sections were incubated in hematoxylin for 3 min, and rinsed in distilled water. Then it was stained with sirius red solution for 10 min and rinsed in distilled water. Later the dried sections were mounted with DPX and allowed to dry. Accumulation of collagen in the tissues was observed under a light microscope (AxioScope A1, Carl Zeiss, Jena, Germany).

2.13.3. Milligan's trichrome stain

The liver sections were deparaffinized and hydrated with distilled water. The slides were preheated at 58° C with Bouin's solution for 15 min and rinsed with distilled water. Then the sections were incubated in hematoxylin for 5 min kept at dark place and rinsed with distilled water for 3-4 times. The sections were placed in acid-alcohol solution for 3-5 sec and rinsed with distilled water for 3-4 times. Then the slides were incubated in Biebrich Scarlet-Acid Fuchsin solution for 5 min and rinsed with distilled water for 3 times. After the slides dried, they were kept in 1% phosphomolybdic acid solution for 2 min and aniline blue solution for 5 min and washed with distilled water. Then the slides were placed in acetic acid solution for 30 sec and washed with distilled water 2 times, and allowed to dry. Slides were further processed using xylene and mounted with DPX. The images were photographed under light microscope (AxioScope A1, Carl Zeiss, Jena, Germany).

2.13.4. Staining for reticulum fibers

The slides were deparaffinized and oxidized in potassium permanganate for 5 min and rinsed in running water, subsequently dipped in 2% oxalic acid for 2 min and again rinsed in running water. The slides were fixed in 4% iron alum for 5 min, rinsed in running water, sensitized in ferric ammonium sulphate and rinsed again in running water. Next, the slides were impregnated in ammonical silver solution for 1 min, followed by quick rinse in running water. Subsequently, slides were dipped in 20% formalin for 3 min and rinsed in distilled water. Later, the slides were fixed with 5% sodium thiosulphate for 2 min and counterstained with nuclear fast red for 2 min and rinsed with distilled water briefly. Then the slides were dehydrated with ethanol, cleared with xylene and mounted using DPX. The images were photographed under light microscope (AxioScope A1, Carl Zeiss, Jena, Germany).

2.13.5. Sudan Black B staining

The slides were deparaffinized, rehydrated with 70% alcohol, placed in propylene glycol for 5 min and rinsed with deionized water. The slides were then placed in Sudan black B for 3 h. After incubation, the slides were then rinsed with 70% isopropyl alcohol and subsequently with deionized water. The sections were counterstained with nuclear fast red solution for 3 min and rinsed in 2 changes of distilled water. The slides were then mounted with DPX and the images were photographed using light microscope (AxioScope A1, Carl Zeiss, Jena, Germany).

2.13.6. Best's Carmine staining

The slides were embedded in paraffin and cut into 4µm thick section. The sections were pre incubated in an oven at 60° C for 30 min. The slides were deparaffinzed, hydrated with distilled water, kept in sudan black solution for 7 min and rinsed with three changes of distilled water. The tissue sections were then counterstained with hematoxylin solution for 2 min and rinsed with distilled water. The slides were rehydrated with ethanol, cleared with xylene and mounted using DPX.

2.14. Immunohistochemistry

Immunohistochemistry was done with super sensitive polymer HRP detection system kit, obtained from Biogenex, USA. The paraffin embedded tissues were sliced into 4- 5 µm thickness, deparaffinized with xylene and rehydrated using ethanol. The slides were subjected to antigen retrieval and blocked with a proteinaceous blocking solution and then incubated with diluted primary antibodies (AFP, VEGF and AKT) overnight. The slides were then treated with super enhancer solution for 30 min, and super sensitive Poly-HRP solution for 30 min. The colour formation was visualized by treating 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin for 30 sec. The sections were observed under a light microscope and photographed at 40x magnification (AxioScope A1, Carl Zeiss, Jena, Germany).

2.15. Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) and the significant differences among the 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).

3. Results

3.1. Effect of NDEA, p-MCA and sorafenib on body weight and growth rate changes

The animals were carefully monitored for the entire 24-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 there were no changes 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 24 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 80 mg/kg b.w. and sorafenib 11.4 mg/kg b.w) rats showed a significant increase in the body weight as compared 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 2A.

Growth rate changes of the five groups of animals were periodically recorded. The growth rate changes of the different groups of animals were calculated by the difference between the final body weight and the initial body weight divided as 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 significant difference between the control and sorfenib control rats. Figure 2B shows the growth rate differences between the control and experimental rats.

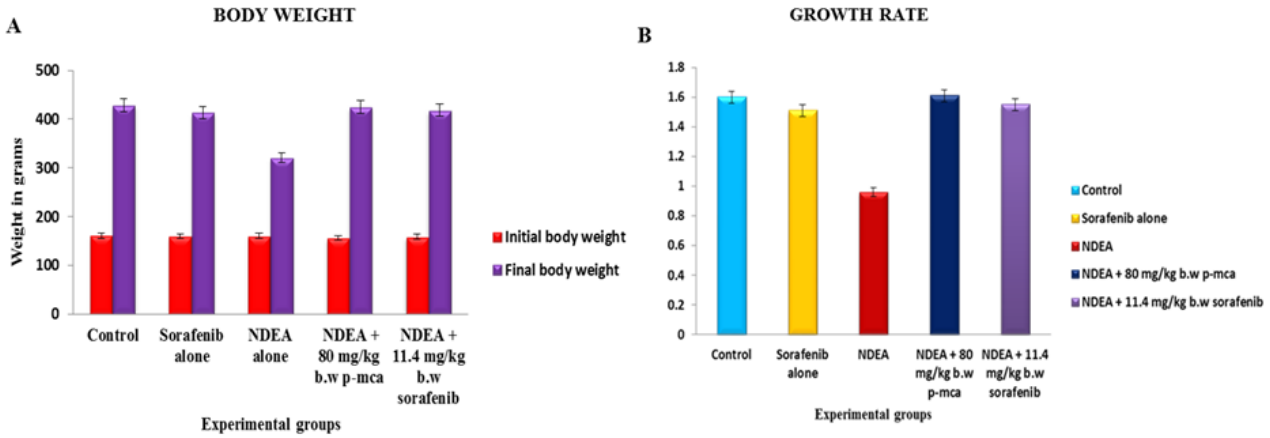

Figure 2: A-Body weight changes of control and experimental rats. B- Growth rate changes of control and experimental rats

3.2. Changes in the liver morphology of control and experimental rats

Figure 3 illustrates the photographical images of the morphological appearance of the control and experimental rats. NDEA treated rats (group 3) showed increased formation of nodules and tumour incidence in the hepatic tissue when compared with control and sorafenib control (group 1 and 2) rats. Administration of p-MCA or sorafenib to NDEA (group 4 and 5) rats showed less number of nodule formation. There were no significant changes in the liver of p-MCA and sorafenib treated rats (group 4 and 5).

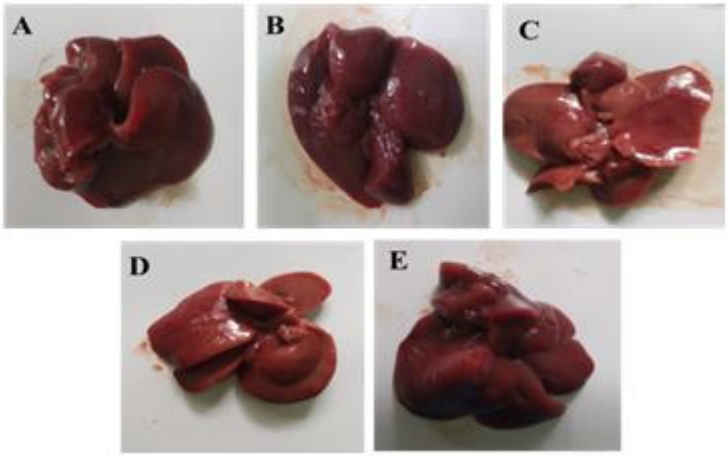

Figure 3: Morphological changes of the liver of control and experimental rats. **A and B –** Liver tissue of control and sorafenib control rats shows normal morphology. **C-**Liver tissue of NDEA treated rats shows nodule formation in their morphology. **D and E-**Liver of NDEA + p-MCA and sorafenib administered rats shows reduced number of nodule formation.

3.3. Effect of NDEA, p-MCA and sorafenib on lung morphology of the control and experimental rats

During NDEA administration, metastatic tumour growth was observed in the lungs after the 24 week experimental period. Figure 4 reveals the metastasis suppressing effect of p-MCA on the lung tissues. Group 3 cancer bearing rats showed high incidence of lung metastasis. Group 1 and 2 rats showed no lung metastasis as well as p-MCA and sorafenib treated rats (group 4 and 5) showed reduced metastatic tumour growth in the lung.

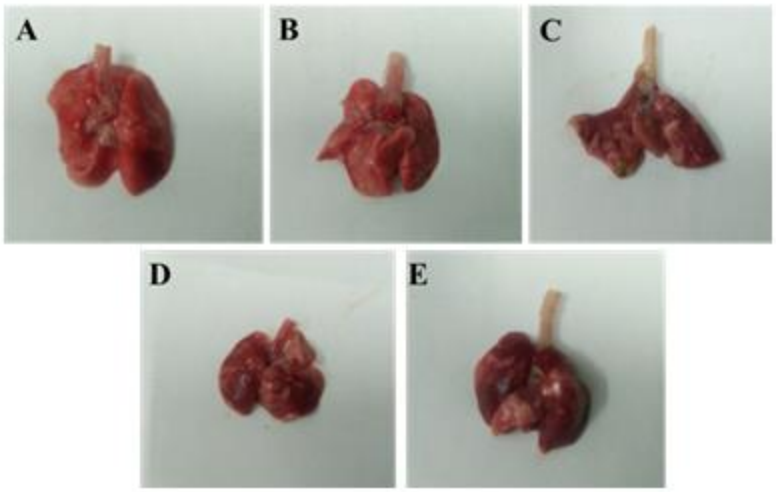

Figure 4: Morphological changes in the lungs of control and experimental rats. **A and B** – lung of control and sorafenib treated rats shows normal morphology. **C**-lung tissues of NDEA treated rats shows decrease in size with tumour growth. **D and E**-lung of NDEA + p-MCA or sorafenib administered rats shows reduced number of tumour formation.

3.4. Effect of NDEA, p-MCA and sorafenib on liver specific enzymes

Figure 5 summarizes the effect of p-MCA and sorafenib on NDEA induced changes in the liver specific marker enzymes. Administration of NDEA leads to hepatic damage, and these cancer bearing rats showed increased activities of the liver specific enzymes. Activities of these enzymes increased in the circulatory system due to the hepatic damage caused on NDEA administration. Group 3 (NDEA) administered rats showed increased levels of hepatic enzymes when compared to the control and sorafenib control rats (groups 2 and 3). Groups 4 and 5 rats treated with NDEA and p-MCA or sorafenib showed a significant decrease in the activities of the liver specific enzymes (ALT, AST and ALP) when compared with group 3 cancer bearing rats. There was no significant decrease between the enzyme activities of the control and sorafenib treated rats.

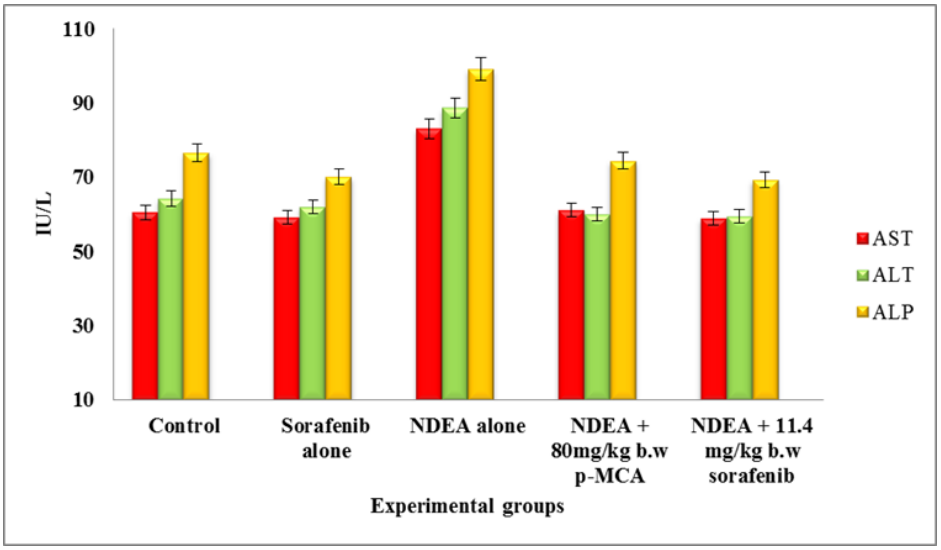

Figure 5: Effect of p-MCA and sorafenib on liver specific enzymes AST-aspartate transaminase, ALT-

alanine transaminase, ALP-alkaline phosphatase

3.5. Effect of NDEA, p-MCA and sorafenib on lipid peroxidation profile

Figure 6 summarizes the effect of p-MCA and sorafenib on lipid peroxidation (LPO) by products (TBARS and LOOH) in the control and experimental rats. In cancer bearing rats (NDEA alone), increased levels of lipid peroxidation was noted in the liver when compared with the control and sorafenib control rats (group 2 and 3). Supplementation with NDEA to p-MCA or sorafenib treated rats restored the levels of lipid peroxidation in the liver and was comparable to that of the control rats. There was no significant difference between the levels of lipid peroxidation in the control and sorafenib control rats.

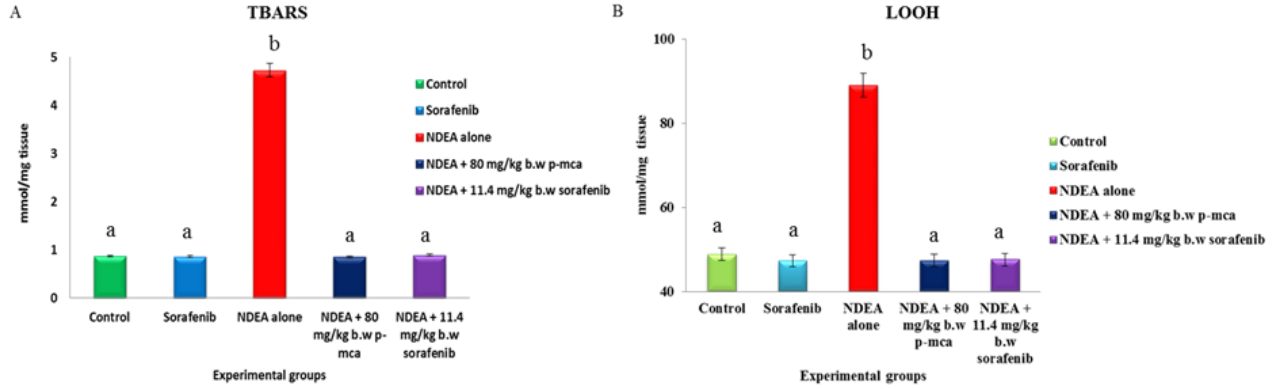

Figure 6: Effect of lipid peroxidation levels in the control and experimental rats. TBARS-thiobarbituric acid reactive substances (mmol/mg tissue); LOOH-lipid hydroperoxides (mmol/mg tissue)

3.6. Effect of NDEA, p-MCA and sorafenib on antioxidant status

The antioxidant property of p-MCA and sorafenib was identified by evaluating the activities of the enzymic and non-enzymic antioxidants in the liver of control and experimental rats. Figure 7 illustrates the effect of p-MCA and sorafenib on the activites of the antioxidant enzymes (SOD, CAT and GSH). The activities of SOD, CAT and GSH were significantly decreased in the NDEA alone administered rats when compared to the control and sorafenib control rats (group 1 and 2). Supplementation with p-MCA or sorafenib to NDEA administered rats (group 4 and 5) significantly elevated the activities of SOD, CAT and GSH. Supplementation with p-MCA or sorafenib to the cancer bearing rats seemed to stabilize the antioxidant status.

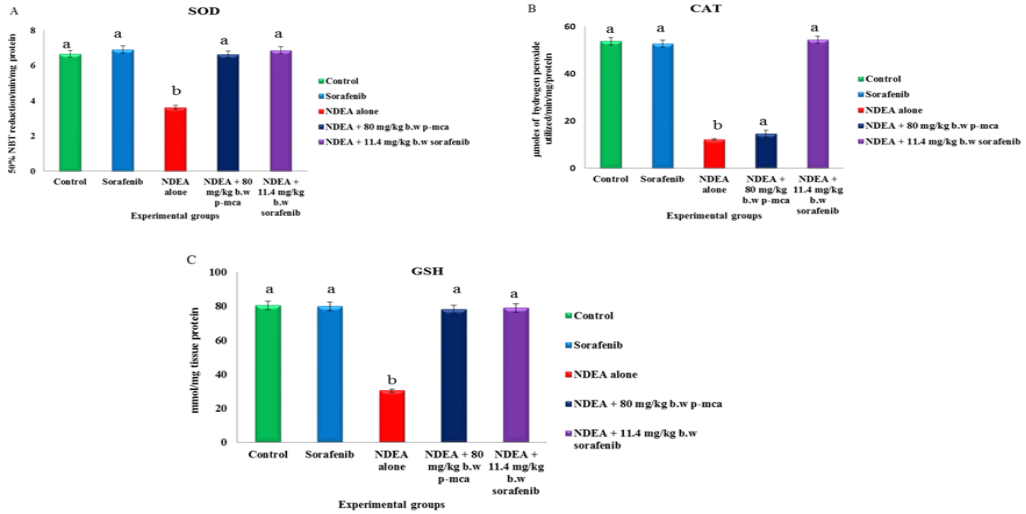

Figure 7: Effect of p-MCA and sorafenib on NDEA induced hepatic enzymic and non-enzymic antioxidants of the control and experimental rats

3.7. Effect of NDEA, p-MCA and sorafenib on phase I and phase II xenobiotic metabolizing enzymes

Figure 8 and 9 shows the effect of p-MCA and sorafenib on NDEA induced phase I enzymes (cytochrome P450, cytochrome P4502E1, cytochrome b5, NADH-cytochrome b5 reductase and NADPH-cytochrome P450 reductase) and phase II enzymes (GST, DTD and UDP-GT) in the liver. The activities of phase I enzymes in NDEA alone administered (group 3) cancer bearing rats were significantly increased whereas the activities of phase II enzymes in this group were significantly decreased when compared with the p-MCA and sorafenib rats (groups 1 and 2). Oral supplementation with p-MCA and sorafenib (groups 4 and 5) to NDEA administered rats significantly decreased the phase I enzymes and increased the activites of phase II enzymes. There was no significant difference between the control and sorafenib control rats.

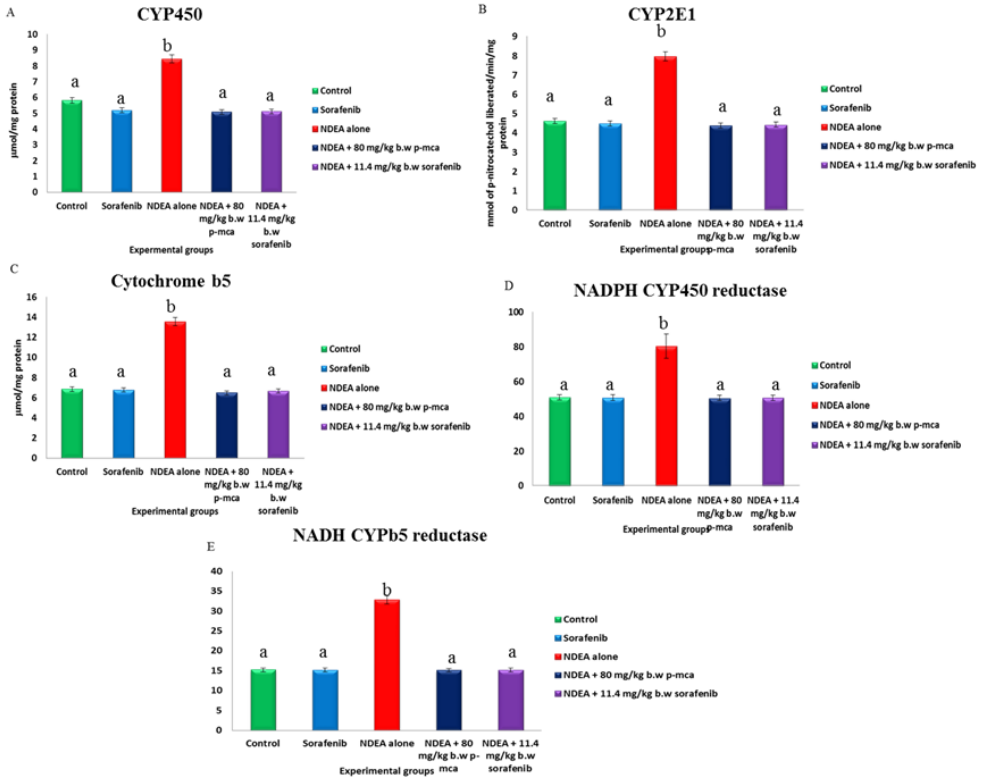

Figure 8: Effect of p-MCA and sorafenib on phase I enzymes in the liver of control and experimental rats.

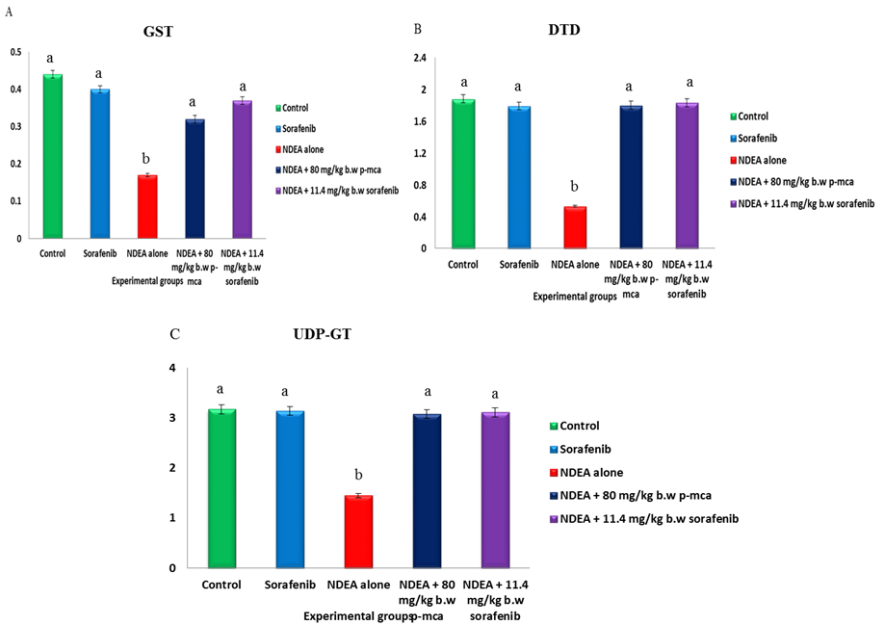

Figure 9: Effect of p-MCA and sorafenib on phase II enzymes in the liver of control and experimental rats.

3.8. Effect of NDEA, p-MCA and sorafenib on liver histopathology: Hematoxylin and eosin staining

Figure 10 represents the histological image of the liver sections of control and experimental rats stained with hematoxylin and eosin. NDEA alone administered rats (group 3) showed distorted liver architecture with pleomorphism and hepatic fibrosis. Control and sorafenib control rats (group 1 and 2) showed normal architecture with portal vein, periportal region, portal triad and hepatic artery. NDEA + p-MCA and sorafenib treated rats (groups 4 and 5) showed normal hepatic organization, central portal veins and restored liver architecture.

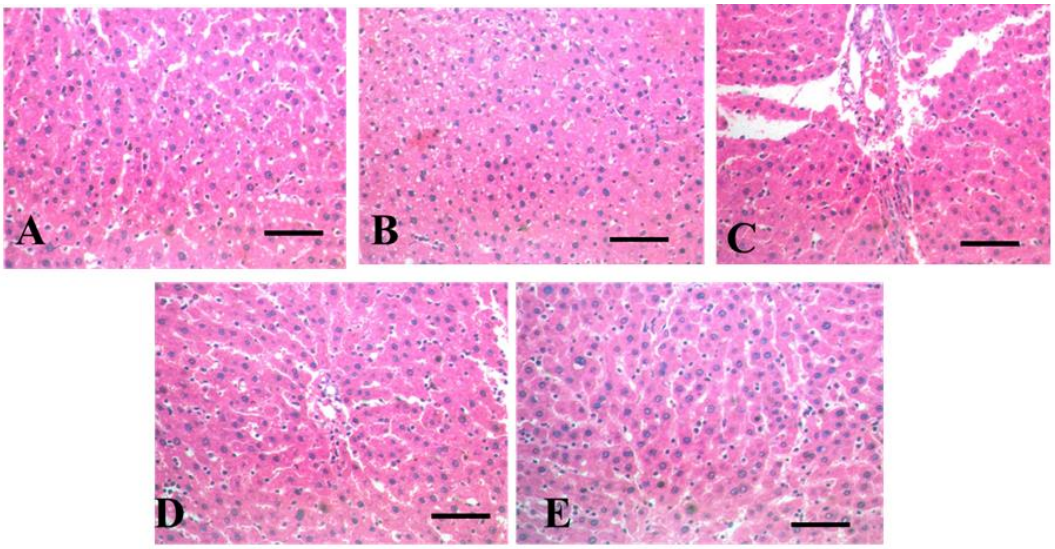

Figure 10: H & E staining of liver cancer on control and experimental rats. A and B – control and sorafenib control rats shows normal architecture of the liver. C – NDEA alone administered rats shows distorted liver

architecture, liver necrosis, and congestion of sinusoids with neutrophilic infiltration. D and E – NDEA + p-MCA or sorafenib treated rats shows restored liver architecture.

3.9. Lung histology

Figure 11 illustrates that histological images of the lung of control and experimental rats stained with hematoxylin and eosin. NDEA alone administered rats (group 3) showed alveolar fibrosis and metastatic foci. Lung sections of control and sorafenib control rats (group 1 and 2) showed normal lung architecture with proper branching and well stained alveoli. NDEA + p-MCA or sorafenib treated rats (group 4 and 5) showed normal lung alveoli.

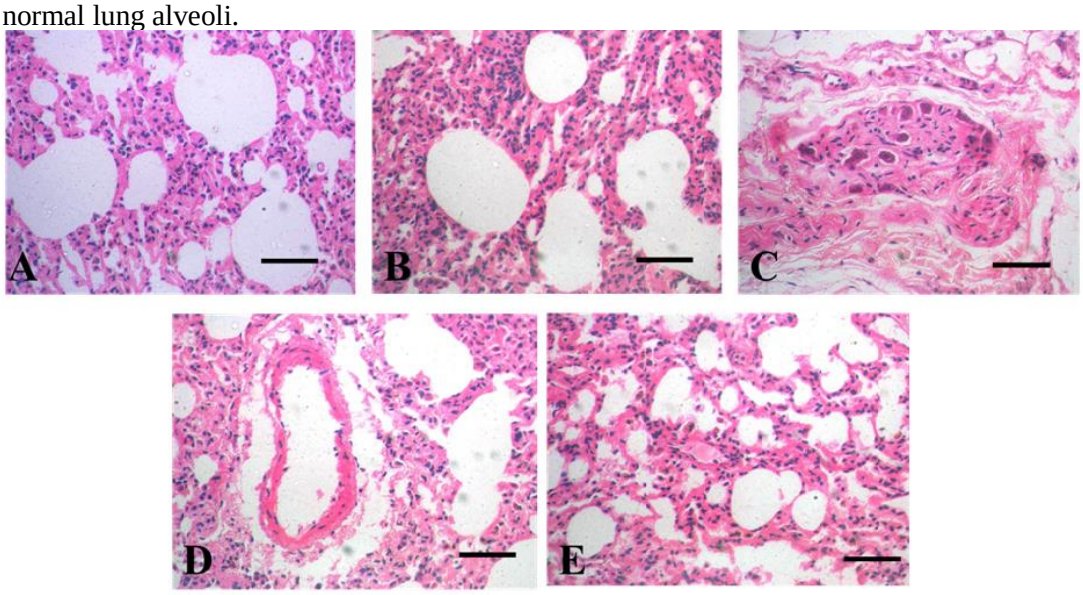

Figure 11: Microscopic images of hematoxylin and eosin stained lung sections of control and experimental rats. A and B – control and sorafenib alone treated rats shows normal architecture. C – NDEA administered rats shows alveolar sac filled inflammatory cells and damaged alveoli. D and E – NDEA + p-MCA or sorafenib treated rats shows restored lung architecture with normal alveoli.

3.10. Effect of NDEA, p-MCA and sorafenib on collagen deposition

Figure 12 illustrates the collagen accumulation in the liver of control and experimental rats stained with picro-sirius red. NDEA alone administered rats (group 3) showed progressive accumulation of collagen deposition in the around the tissues with signs of hepatic fibrosis. Control and sorafenib control rats (groups 1 and 2) showed reduced levels of collagen deposition in the tissues with no signs of hepatic fibrosis. Decreased level of collagen deposition was seen in NDEA + p-MCA or sorafenib treated rats (group 4 and 5) when compared with NDEA alone administered rats.

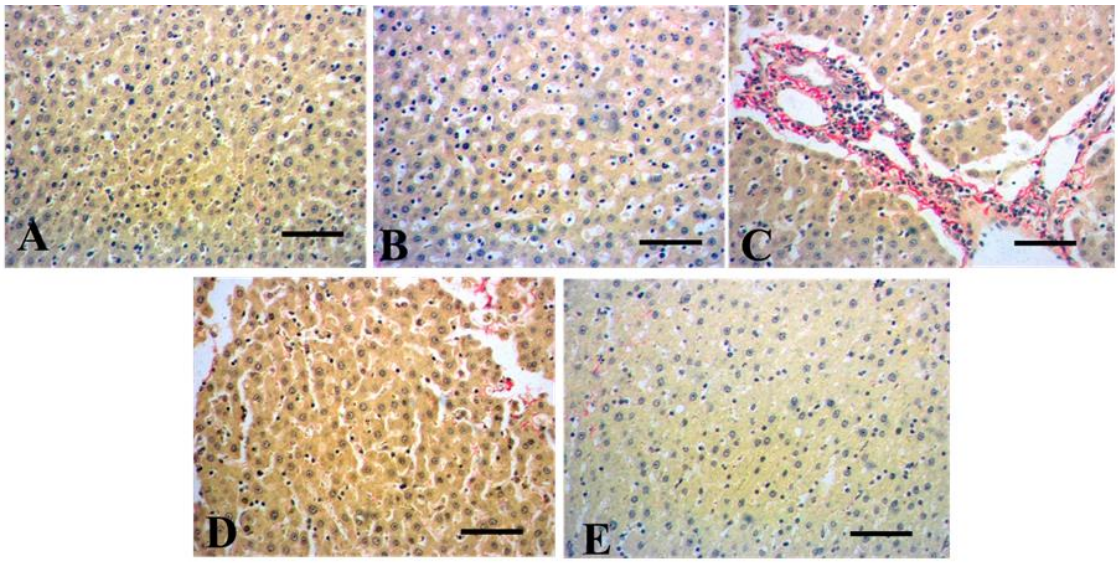

Figure 12: The photomicrographs of collagen deposition of the liver tissues stained with picro-sirius red. A and B – minimal collagen deposition in the liver sections of control and sorafenib control rats. C- NDEA alone administered rats shows marked collagen accumulation. D and E – NDEA + p-MCA and sorafenib treated rats shows reduced collagen deposition.

3.10.1. Effect of NDEA, p-MCA and sorafenib on Milligan's trichrome staining

Figure 13 represents the collagen accumulation in the liver tissues of the control and experimental rats stained with Milligan's trichrome. The hepatic sections of NDEA administered rats (group 3) showed progressive collagen accumulation around the central vein and portal triad in the tissues with hepatic damage. Supplementation with p-MCA or sorafenib to NDEA treated rats (group 4 and 5) showed reduced collagen deposition with minimal hepatic damage. There were no signs of hepatic fibrosis and minimal collagen deposition was seen in the control and sorafenib alone treated rats (group 1 and 2).

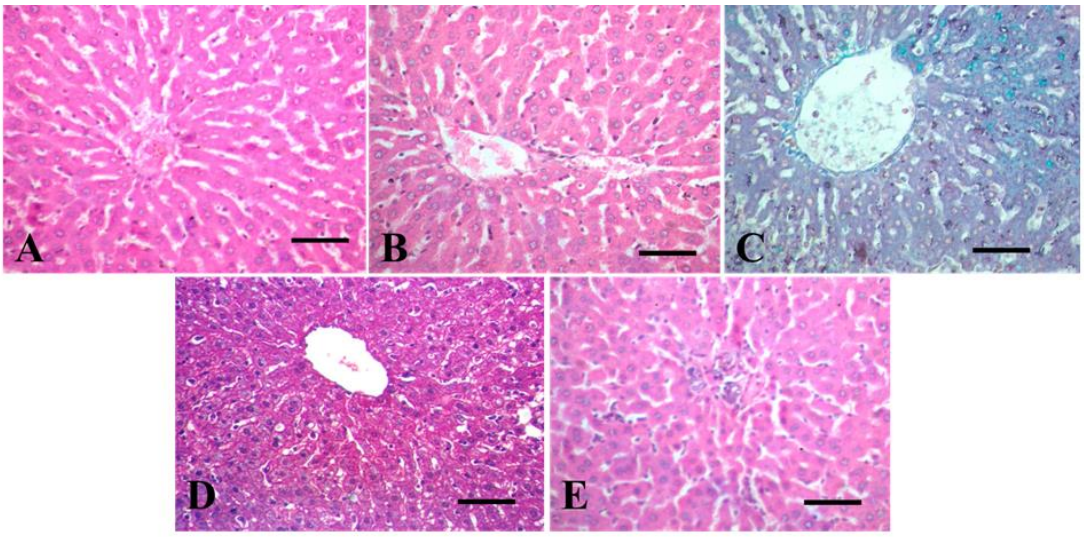

Figure 13: Microscopic images of the liver sections of control and experimental rats stained with Milligan's trichrome. A and B – control and sorafenib control tissue sections shows minimal accumulation of collagen. C – NDEA alone administered rats shows progressive accumulation of collagen. D and E – Hepatic sections of NDEA + p-MCA or sorafenib rats shows reduction in the accumulation of collagen.

3.10.2. Reticulin staining

Figure 14 illustrates the reticulin framework present in the liver tissues stained with reticulin stain. Reticulin fibers, type 3 collagen is present in the extracellular matrix. NDEA alone administered rats (group 3) showed compressed reticulin framework in the nodular region as compared with the control and sorafenib control rats (group 1 and 2). Supplementation with NDEA + p-MCA or sorafenib (group 4 and 5) restored reticulin framework. Hepatic sections of control and sorafenib alone treated rats showed well-arranged reticulin framework.

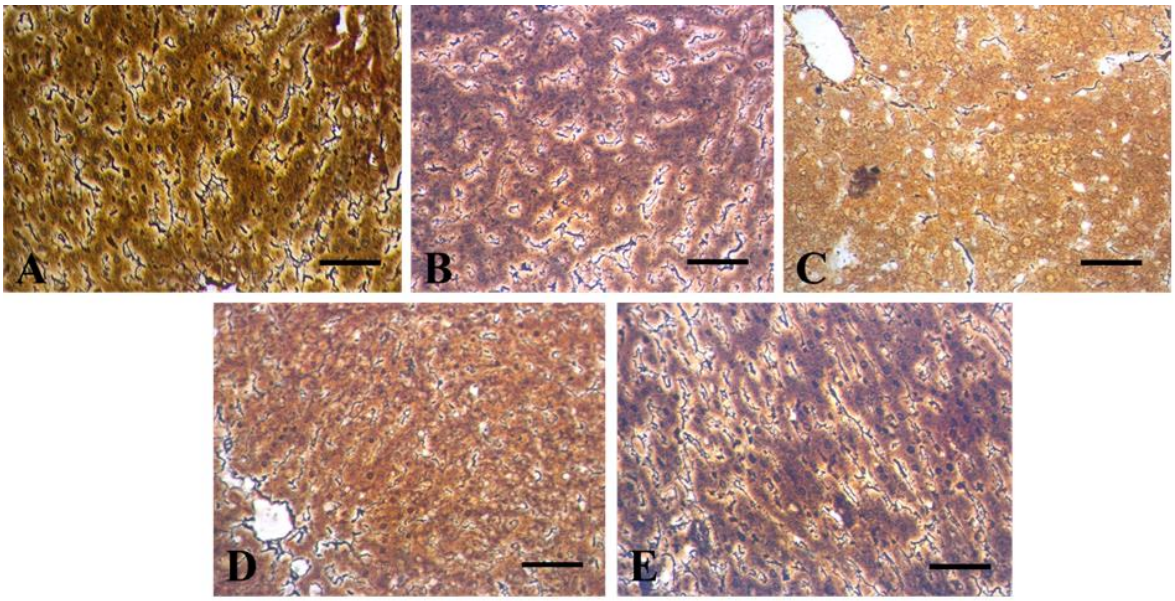

Figure 14: Histological sections of control and experimental rats stained with reticulin stain. A and B – control and sorafenib control tissue sections shows well-arranged reticulin framework. C – hepatic sections of NDEA alone administered rats shows collapsed reticulin framework. D and E – NDEA + p-MCA and sorafenib supplemented rats restored reticulin framework.

3.10.3. Effect of NDEA, p-MCA and sorafenib on glycogen content

Best's Carmine staining

Figure 15 showed the glycogen level in the liver of control and experimental rats stained with Best's carmine. Control and sorafenib control rats (group 1 and 2) showed normal glycogen content. NDEA alone administered rats (group 3) showed marked accumulation of glycogen in the liver. NDEA + p-MCA and sorafenib supplemented rats (group 4 and 5) showed reduced glycogen accumulation in the liver.

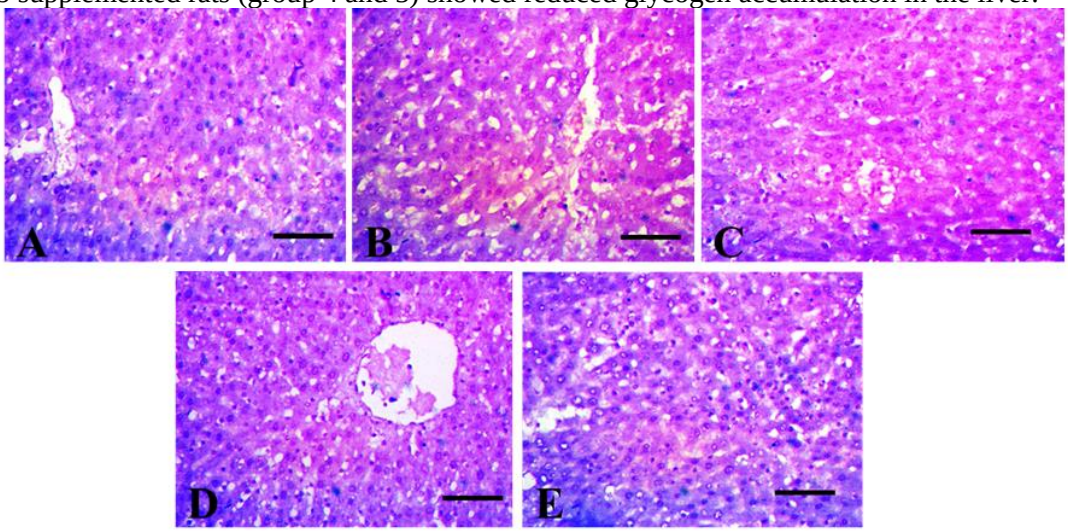

Figure 15: The photomicrographs of hepatic glycogen accumulation, stained with Best's carmine reagent. A and B – control and sorafenib control rats shows normal level of glycogen in the liver. C – NDEA alone administered rats shows increased glycogen in the liver. D and E – NDEA + p-MCA or sorafenib rats shows reduced levels of glycogen accummulation.

3.10.4. Effect of NDEA, p-MCA and sorafenib on lipid accumulation in the liver

Sudan Black staining

Figure 16 illustrates the hepatic sections of control and experimental rats stained with Sudan Black B. NDEA alone administered rats (group3) showed increased lipid accumulation in the form of fats and triglycerides (stained in black colour). Supplementation with p-MCA or sorafenib to NDEA treated rats (groups 4 and 5) showed reduction in the level of lipid accumulation in the hepatocytes. Control and sorafenib control rats (groups1 and 2) showed very low lipid deposition in the hepatocytes.

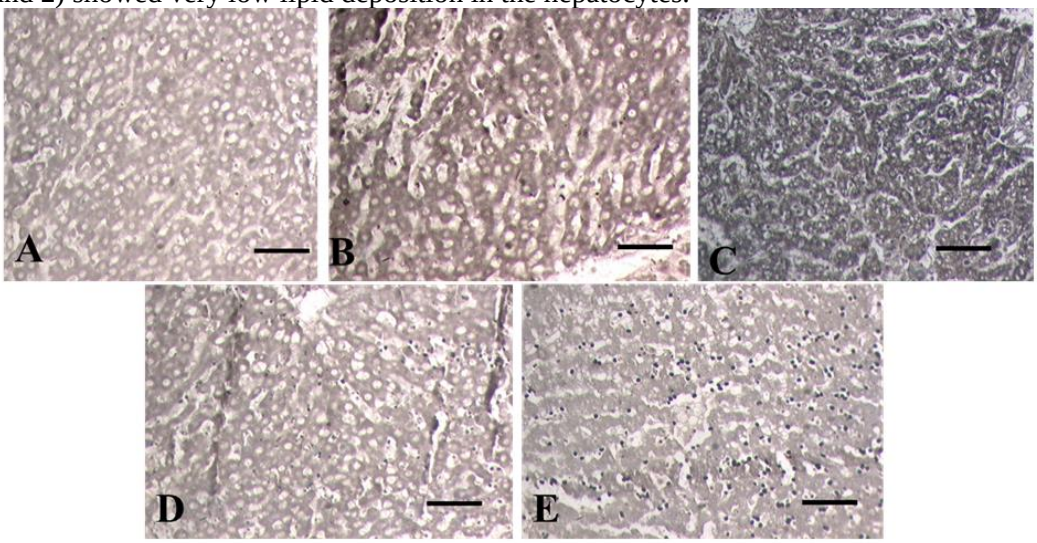

Figure 16: Microscopic images of lipid accumulation in the control and experimental rats. A and B – control and sorafenib control liver sections shows low level of lipid accumulation. C – NDEA alone administered rat shows increased lipid deposition in the hepatic tissue. D and E – NDEA+ p-MCA and sorafenib treated rats shows reduced lipid deposition.

3.11. Effect of NDEA, p-MCA and sorafenib on liver specific marker; alfa – fetoprotein (AFP)

AFP expression in the liver of control and experimental rats were analyzed using immunohistochemistry, represented in figure 17. NDEA alone administered rats (group 3) showed increased expression of AFP when compared with control and sorafenib control rats (groups 1 and 2). Oral supplementation with p-MCA or sorafenib to NDEA treated rats showed decreased AFP expression.

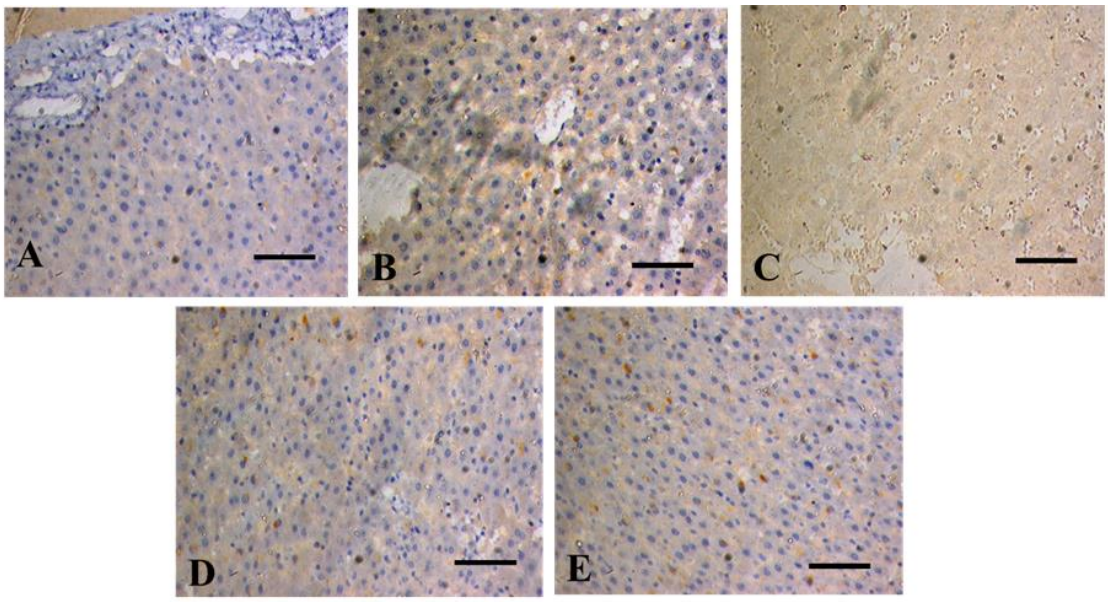

Figure 17: Immunohistochemical staining for AFP. A and B – control and sorafenib control rats shows weak expression of the protein AFP. C – NDEA alone administered rats shows enhanced AFP expression. D and E – p-MCA and sorafenib to NDEA treated rats shows reduced AFP expression.

3.12. Effect of NDEA, p-MCA and sorafenib on transcription factor (Akt)

Figure 18 immunohistochemical images represent the Akt expression in the liver of control and experimental rats. NDEA alone administered rats (group 3) showed increased Akt expression when compared with control and sorafenib control rats (groups 1 and 2). Oral supplementation with p-MCA or sorafenib to NDEA treated rats (group 4 and 5) showed decreased Akt expression as compared to the NDEA alone administered rats.

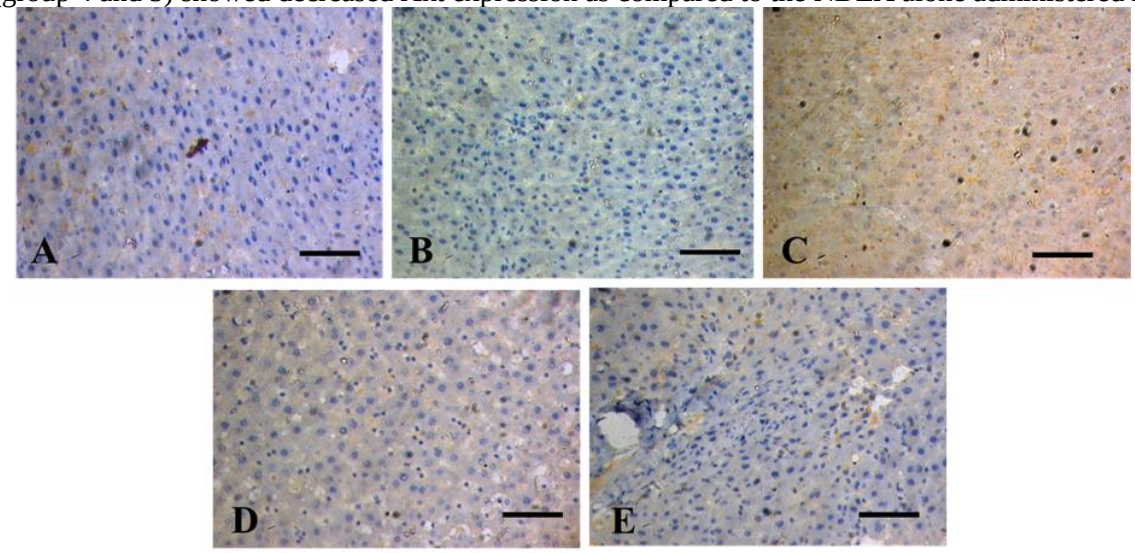

Figure 18: Immunohistochemical staining for Akt. A and B – control and sorafenib control rats shows minimal Akt expression. C – Hepatic section of NDEA alone treated rats shows increased Akt expression. D and E – p-MCA and sorafenib to NDEA treated rats shows weak Akt expression.

3.13.Effect of p-MCA and sorafenib on angiogenesis marker (VEGF)

The imunohistochemical images of the protein expression of the angiogenesis marker VEGF in the liver of control and experimental rats is represented in figure 19. NDEA alone administered rats (group 3) showed elevated expression of VEGF when compared to the control and sorafenib control rats (group 1 and 2). Oral supplementation with p-MCA or sorafenib to the NDEA treated rats showed decreased expression of VEGF when compared to the NDEA alone treated rats.

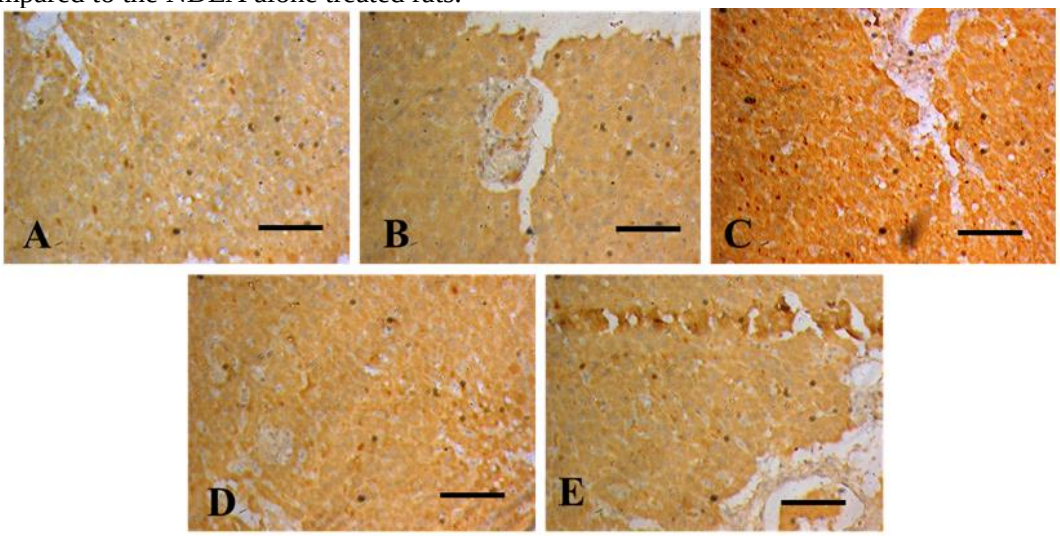

Figure 19: Immunohistochemical staining for VEGF. A and B – control and sorafenib control rats shows minimal VEGF expression. C – NDEA alone administered rats shows increased VEGF expression. D and E – p-MCA and sorafenib to NDEA treated rats shows reduced VEGF expression.

4. Discussion

The present study evaluates the effect of p-MCA and sorafenib on NDEA induced hepatocellular carcinoma (HCC) in male wistar rats. In line with the previous reports, [26] our study emphasizes that administration of NDEA provokes the growth of neoplasms in the liver. p-MCA is a cinnamic acid derivative present in rice bran, brown rice, turmeric and *Kaemperfia galangal*. It has several pharmacological properties especially, the anticancer and antihyperglycemic activities [9].

Sorafenib is a tyrosine protein kinase inhibitor and thereby, controls tumour growth. It has antitumour activity across various types of malignancies including hepatocellular, breast, melanoma and colorectal cancers. Sorafenib is a standard drug, approved for the treatment of advanced HCC (primary liver cancer) [27].

In the 24 week experimental period designed in our study, hepatic tumour growth in NDEA administered rats (group 3), was obvious as evidenced by their physical appearance such as continuous hair loss, decreased body weight, poor growth, abdominal bulging and gradual decrease in food intake. These symptoms were similar to those of the hepatic cancer patients [28], proving that NDEA administered rats developed HCC. However, p-MCA supplemented rats showed optimal growth rats, and thus marked protection against NDEA induced carcinogenicity.

Hepatic damage is caused by NDEA which disrupts the membrane permeability. This leads to release of the liver specific enzymes such as aminotransferases (AST, ALT) and alkaline phosphatase (ALP) into the circulatory system [29]. These enzyme activities are used to diagnose the extent of hepatic damage and injury.

In citric acid cycle, aminotransferases such as AST and ALT play a major role in catalyzing 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. For this reaction these enzymes require pyridoxal-5'-phosphate (vitamin B6), even though the effect of pyridoxal-5'-phosphate deficiency is greater on ALT activity than on that of AST. Deficiency of pyridoxal-5'-phosphate may increase the AST/ALT ratio in the cancer patients [30]. The observations of the present study concur with the above findings, revealing increase in the activities of liver-specific enzymes (AST, ALT and ALP) in the circulation, which indicates NDEA induced hepatocarcinogenesis [31]. These results also correlate our own previous reports [26] and with those of Fazal Khan et al.,[1]. Supplementation with p-MCA under the experimental conditions greatly inhibits hepatic injury/ carcinogenesis, decreases AST, ALT and ALP activities by altering the efficacy of NDEA in initiating the neoplastic changes. These results establish the hepatoprotective effects of p-MCA against NDEA induced hepatocarcinogenesis.

Free radicals are the foremost cause for cell and tissue damage due to lipid peroxidation. It leads to degeneration of membrane lipids, which inturn results in further production of reactive oxygen species (ROS) resulting in tissue damage. Oxidative stress is caused by an imbalance between the reactive oxygen species and antioxidant interaction [32]. Damage due to oxidative stress have been implicated in numerous disease processes, including inflammation, degenerative diseases, and tumor formation. The important primary products are the thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH). These reactive species form cross-links with macromolecules and promotes carcinogenesis. The extent of oxidative stress can be gauged by the amount of lipid hyperperoxides present in the serum [33]. Our results showed that administration of NDEA resulted in an increase in the levels of lipid peroxidation products when compared with the control rats. In this context, NDEA is well known to generate free radicals, distract the antioxidant status, thereby induce oxidative stress and subsequently carcinoma [34]. Several studies have demonstrated increased lipid peroxidation in the liver during NDEA- induced hepatocarcinogenesis [35]. However we observed decreased lipid peroxidation in the liver of NDEA treated rats supplemented with p-MCA and sorafenib, as evidenced by the reduced concentrations of thiobarbituric acid reactive substances.

Antioxidants are the substances that inhibit oxidation. Important enzymic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase. Generally SOD present in our body catalyzes the dismutation of superoxide. Hydrogen peroxide, a byproduct of this reaction helps to conduit in transmission of the injury caused by free radicals. Catalase is an antioxidant enzyme that acts as a catalyst for the conversion of hydrogen peroxide to oxygen and water. It nullifies the effect of hydrogen peroxide that is present intracellularly. Our study proved that the activity of SOD and CAT were decreased in the liver of cancer bearing rats. These symptoms are similar to those presented by the hepatocarcinogenesis patients [36].

In our experiment, the activities of SOD and CAT were decreased in NDEA alone administered rats when compared with the control and sorafenib control rats. Similar results were also obtained by Sudhanshu J et al., [37] who proposed that inflammation in the liver may contribute to the decreased activity of CAT, resulting in an increased concentration of intracellular H2O2 and the promotion of cancer. A significant increase in SOD and CAT activities were observed in the liver of NDEA plus p-MCA treated rats, showing that p-MCA offers protection against NDEA induced liver injury.

GSH is an antioxidant that protects cells from oxidative damage by maintaining redox homeostasis. There are two forms of glutathione, the oxidized and the reduced form. The reduced form of glutathione is self-protective and is involved in neutralizing hydrogen peroxide produced inside the cell. It counters the excessive generation of ROS. However, the oxidized form is not protective [38]. Our study proved that the GSH concentration decreased markedly in the NDEA alone administered (cancer bearing) rats. Huang et al. have reported that GSH is increased in human HCC and this increased GSH level facilitates the growth of liver cancer cells. In this context Ferruzzi E et al., [39] have also reported that GSH concentration is higher in tumor tissues than in the normal tissues. A significant increase in GSH content was observed in NDEA plus p-MCA treated rats, which may be due to increased synthesis of this antioxidant during enhanced cell proliferation.

Oxidation is the most common phase I reaction involved in xenobiotic metabolism. Cytochrome P450 is a key enzyme involved in oxidation systems present in the endoplasmic reticulum of hepatocytes. It is a member of the family of monoxygenases that catalyzes oxidative metabolism. Similarly CYP2E1 is also one of the xenobiotic metabolic enzyme that plays an important role in the pathogenesis of liver disease and non-alcoholic steatohepatitis (NASH). The activity of CYP2E1 is associated with the production of reactive oxygen species (ROS) with secondary damage to cellular membranes and mitochondria. Due to its ability to activate carcinogens, increased activity of CYP2E1 is reported to be one of the factors in the pathogenesis of HCC [40].

Glutathione S-transferases (GST), a phase II enzyme plays a primary role in the detoxification of drugs, cell signaling, post-translational modification and drug resistance. It acts as a defense mechanism against toxic and reactive electrophiles that are generated by cellular oxidative reactions catalyzed by CYP450. In our present study, the activities of phase I enzymes were significantly increased and those that of the phase II enzymes 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 and suppressed the activities of phase I enzymes. This underlines the protective effect of p-MCA, playing a significant role in detoxifying and inhibiting the metabolic activation of NDEA in the liver.

NDEA alone administered (cancer bearing rats) rat liver showed some pathological changes such as atypical nuclei, thickened hepatic plates, dispersed chromatin, completely damaged hepatocytes, absence of portal tracts and hepatic lobules. Several similar studies also report the presence granular eosinophilic cytoplasm and rounded nuclei with dispersed chromatin and prominent nuclei in HCC bearing rats [16]. The hepatic architecture of p-MCA and sorafenib treated rats showed normal appearance with portal vein and hepatic artery surrounded by hepatocyte cord, periportal region and portal triad similar to the control and sorafenib control rats. In addition, p-MCA and sorafenib supplemented NDEA treated rat lung showed normal architecture with alveoli. It proves that p-MCA not only has an ability to suppress NDEA induced hepatic carcinogenesis, but also prevents lung metastasis.

Milligan's trichrome stain is mainly used to determine the type of extracellular material that accumulates in the hepatic tissue such as collagen, fibrin, muscle and elastic fiber. Collagen is an important macromolecule present in the extracellular matrices. Picrosirius red is a strong, linear anionic dye containing six sulphonate groups along with cationic collagen fibers [41]. Picrosirius red staining is used to determine the amount of fibrillar collagen accumulation in the liver tissue [42]. In normal liver, collagen occupies only a small space whereas in cancer conditions it is excessively deposited, which in turn disrupts the liver architecture. During liver injury, degradation and deposition of extracellular matrix leads to excessive collagen accumulation in

the liver. The excessive collagen deposition proves that the hepatic tissue is completely damaged and cirrhotic.

In our study, NDEA alone administered rats showed excessive accumulation of collagen in the central vein and the portal traid of the liver indicating hepatic fibrosis which could be due to increased collagen synthesis. Our study showed that p-MCA and sorafenib treat NDEA rats showed reduced collagen accumulation and restoration of hepatic appearance probably due to optimal collagen synthesis. This result proves that administration of NDEA causes increased collagen accumulation in the liver leading to liver injury or cirrhosis. Our results also correlate with those reported by Mcgee JOD [43].

Reticulum fibers are fine fibers which include both reticular fibers (type III collagen) and basement membrane material (type IV collagen and laminin). In the liver, it is present outside the endothelium of the hepatic sinusoids and provides support to the vessels and lymphatic sinuses in the tissues. According to Singhi et al., [44], loss or reduced reticulin is associated with liver cirrhosis. In our present study, NDEA alone administered rats showed collapsed reticulin structures in the nodular region, indicating the presence of HCC. p-MCA and sorafenib treatment to cancer bearing rats showed intact fine reticuin framework similar to those of the control and sorafenib control rats which indicates the preventive effect p-MCA against HCC.

Glucose is stored in the liver as glycogen which gets distributed to other tissues during energy requirements. The alterations in the glycogen levels indicate the diseased condition of the liver [45]. The distribution of glycogen in the liver was determined by the Best's carmine staining. Though NDEA alone administered rats showed increased glycogen accumulation in the liver, p-MCA and sorafenib treatment revealed decreased glycogen levels in the liver. Our study proves that p-MCA is effective in preventing glycogen accumulation may be due to its ability to stimulate the key enzymes of glycogenesis in the liver.

Sudan stain is a special stain used to stain fat and fat droplets and examine the presence of lipids in the liver. Hepatic lipid accumulation is a noticeable feature of non-alcoholic fatty liver disease (NAFLD). Hepatic steatosis is characterized by accumulation of lipids in the cytoplasm of hepatocytes. In hepatocytes, triglycerides are synthesized in the ER and stored in the cytoplasm surrounded by a monolayer of phospholipid in distinct lipid droplet structures [46]. The accumulation of lipids in the liver is a hallmark of NAFLD, which leads to cellular stress and hepatic damage, eventually leading to chronic liver disease [47]. Our study showed that NDEA alone administered rat liver showed revealed lipid deposition in the form of fat vacuoles, indicating fatty liver one of the hall marks of hepatocarcinogenesis. Similar findings were observed by Nisha ST et al., [26]. p-MCA and sorafenib supplementation to NDEA treated rats showed significant reduction in hepatic lipid accumulation thereby protecting liver damage.

The hallmark of NAFLD is the hepatic accumulation of lipids, which subsequently leads to cellular stress and hepatic injury, eventually resulting in chronic liver disease.

AFP is an oncofetal protein produced by the hepatocytes. It is an immunomodulatory glycopreotein present in the immature fetal hepatocytes. The extent of expression of AFP is based on the tumour grade. In HCC, higher grade tumour expresses more AFP than the lower grade tumours. In this context, several studies have reported that AFP affects cell proliferation and gene expression in hepatomas [48]. In our present study NDEA alone administered rats showed increased AFP expression indicating HCC. Oral supplementation with p-MCA and sorafenib to NDEA treated rats showed decreased AFP expression, underlining the protective effect of p-MCA/ sorafenib.

Vascular endothelial growth factor (VEGF) is a homo-dimer of 34-42 kDa heparin-binding glycoproteins is an effective angiogenic factor that stimulates endothelial cell proliferation and improves vascular permeability [49]. VEGF promotes extravasation of plasma fibrinogen, leading to the formation of fibrin scaffolding that facilitates cell migration during invasion. Several studies reported higher levels of VEGF during HCC when compared to the other forms of cancer [50]. Our study correlates with these results in that NDEA alone administered rats showed increased expression of VEGF when compared to the control and sorafenib control rats. p-MCA or sorafenib supplementation to NDEA treated rats showed reduced expression of VEGF, thereby protecting against invasion and metastasis.

5. Conclusion

Sorafenib, a standard drug was recently approved for the treatment of hepatocelluluar carcinoma (HCC) which has proved to be effective in patients with advanced HCC [51]. p-MCA is an active prinicipal present in natural products such as rice bran and is known to have minimal side effects. In this particular long term study, 80 mg/ kg.b.w. p-MCA supplementation markedly reduced NDEA induced liver damage, lipid peroxidation, hepatic fibrosis, angiogenesis in addition to its role in modulating the activities of phase I and phase II enzymes. Our study concludes that p-MCA acts as an effective chemotherapeutic and chemopreventive agent against HCC, at the same time prevents lung metastasis. Besides our study reveals that p-MCA and sorafenib are equally effective against hepatocarcinogenesis.

6. Acknowledgements

The author declare no technical or editorial assistance.

7. Conflicts of interest

The authors declare that there are no conflicts of interest

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