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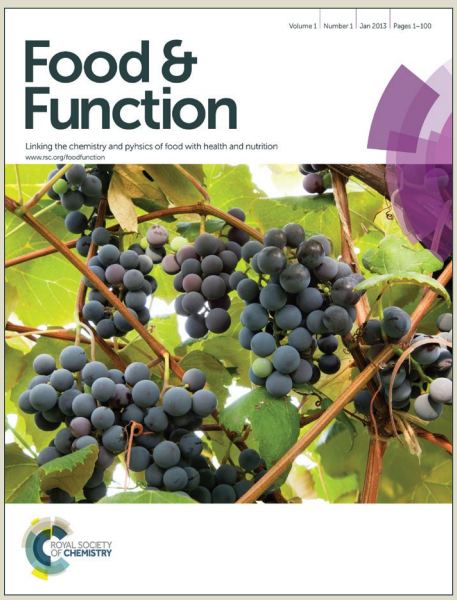

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Modulation of hepatocarcinogenesis in N-methyl-N-nitrosourea treated Balb/c mice by mushroom extracts

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Abstract

The hepatoprotective potential of edible mushrooms from Mauritius, namely *Pleurotus sajor-caju* and *Agaricus bisporus* were evaluated using a N-methyl-N-nitrosourea (MNU)-induced hepatocarcinogenesis Balb/c mice model. Mushroom extracts restored normal weight in MNU treated mice over a 3 month supplementation period. Blood parameter analyses indicated a clear modulation of hemoglobin concentration, leukocyte, platelet, lymphocyte, neutrophil, monocyte and eosinophil counts in MNU-induced mice ($p<0.05$). Mushroom extract supplementation effectively reduced oxidative damage in MNU-primed mice, which was marked by a significant decrease in the extent of lipid peroxidation ($p<0.05$) and a concomitant increase in the enzymatic antioxidants levels, primarily catalase, superoxide dismutase, glutathione reductase and peroxidase, and FRAP values ($p<0.05$). DNA protective effects of the extracts were confirmed by Raman spectroscopy where, the MNU-DNA interaction as evidenced by an intense peak at 1254 cm^{-1} was normalized. The findings demonstrated hepatoprotective, immunomodulatory and anti-carcinogenic effects and suggest the use of mushrooms as potential dietary prophylactics in cancer chemoprevention.

Keywords: N-methyl-N-nitrosourea, *Pleurotus sajor-caju*, *Agaricus bisporus*, Hepatocarcinogenesis.

1. Introduction

Cancer in all its guises continues to undermine global health, and is among the foremost causes of death worldwide, accounting for 8.2 million deaths in 2012, with a predicted rise of 13 millions in the coming decades [1]. Epidemiological studies have convincingly shown that lifestyle plays a major role in the aetiology of human cancers with approximately 90% of all cancers being linked to adverse lifestyle, and 30–40% associated to unhealthy diet [2]. Numerous strategies leading to effective anticancer therapies have been adopted for several decades, with however, mitigated success as the morbidity and mortality from this disease have been unexpectedly high over the past years [3].

The prevention of cancer through dietary intervention has thus gained considerable interest, thereby fueling the need for comprehensive data on natural biofactors with potential therapeutic and chemopreventive properties[4]. Evidence from biochemical and molecular studies have revealed that phytophenolics, in view of their pluripharmacological properties, can exert modulatory actions in cancer cells by interacting with a wide range of cellular and molecular targets that are vital to the cell signaling machinery [5].

There is currently an upsurge of interest in macrofungi (mushrooms) which have been part of the human diet and a source of both healthy food and antioxidants with potential chemopreventive properties. A number of clinical trials have assessed the benefits of medicinal mushrooms in particular *Ganoderma lucidum*, whereby their potential uses, individually and as adjuncts to cancer therapy were revealed [6]. As natural immune-enhancers, mushroom extracts were reported to complement chemotherapy and radiation therapy by countering the side-effects of cancer such as nausea, bone marrow suppression, anemia, and lowered resistance [7].

Pharmacological activities of mushrooms include antioxidant, antidiabetic, hypocholesterolemic, anti-tumor, anti-cancer, immunomodulatory, anti-allergic, nephroprotective, and anti-microbial properties [6,8-9]. Mushrooms contain, in addition to antioxidant phytophenolics, polysaccharides that have been shown to exert antitumor activity against Hela tumour cell[10]. Sarangi et al. [11] and Shah et al. [12] also reported the immunomodulatory and antitumor properties of proteoglycans derived from fruiting body and mycelia of *Pleurotus ostreatus*. *In vitro* and *in vivo* anticancer activities of white button mushrooms (*Agaricus bisporous*) have indicated their possible use in breast cancer treatment as they suppress aromatase activity and estrogen biosynthesis [13].

In the light of rising cancer incidence and growing interest and demand for effective functional foods, the phenolic composition of mushroom extracts and their modulatory effects on hepatocarcinogenic Balb/c mice were studied. The immune-modulating effect of *P. sajor-caju* and *A. bisporus* extracts were examined for a number of haematological and biochemical parameters and the alteration of DNA bases and backbone structure was studied by Laser Raman Spectroscopy.

2. Materials and Methods

2.1. Chemicals and Reagents

MNU was purchased from Sigma-Aldrich (Steinheim, Germany) and superoxide dismutase (from bovine liver, Lot #091M7019V) was from Sigma-Aldrich Co. (St Louis, USA). Epinephrine bitartrate, oxidised glutathione, reduced glutathione, nicotinamide adenine dinucleotide phosphate, reduced tetrasodium salt (NADPH), isoamyl alcohol, 5,5-dithiobis (2-

nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), 2,4,6-tripyridyl-s-triazine (TPTZ), bovine liver catalase enzyme (Lot 000167575), reduced glutathione (Lot 0000046991) and molecular grade saturated phenol (w/10mM Tris, 1mM EDTA) were from Hi-Media (Mumbai, India). All the other reagents used for analysis were of analytical grade.

2.2. Mushroom Extracts

P. sajor-caju (ME_1) was harvested from the Mushroom Unit of the Food and Agricultural Research and Extension Institute (FAREI) and *A. bisporus* (ME_2) was purchased from S.K.C Surat & Co Ltd (Curepipe, Mauritius). The substrate used for the cultivation of *P. sajor-caju* consists of a mixture of sugarcane bagasse (20%), lime (10%) and crushed maize (10%). The lyophilised mushrooms were extracted as per the modified method of Barros et al. (2009). Lyophilised mushroom samples (500g) were subjected to exhaustive extraction using 100% methanol (1:2 w/v). After each extraction and overnight-maceration at 4^0C, filtration and evaporation to dryness was carried out at 37^0C. The extracts were re-dissolved in distilled water prior to lyophilisation.

2.3. Phytophenolic Analyses

2.3.1. Total Phenolic, total flavonoid and total proanthocyanidin contents

The Folin-Ciocalteu method was used to estimate total phenolics, the aluminium chloride method to assess the total flavonoids and the modified 'acid/butanol assay' was used for the quantification of proanthocyanidins in the methanolic mushroom extracts [14]. Results were expressed as mg gallic acid/g dry weight (DW), mg of quercetin/g DW and mg of cyanidin chloride/g DW, for these three assays respectively.

2.3.2. High Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis of mushroom extracts was carried out using a Hewlett Packard 1100 series liquid chromatography system (Waldbronn, Germany). Methanolic mushroom extracts were injected into a Zorbax SB-C18 column (4.6 mm internal diameter x 250 mm length, 3.5 µm pore size) (Agilent Technologies, CA, USA), fitted with a suitable guard column. An injection volume of 65 µl and elution with a flow rate of 0.7 ml/min at 35°C was applied as follows: 0-30 min, 0-10 % B in A; 30-50 min, 10-15 % B in A; 50-60 min, 15-25 % B in A; 60-90 min, 25-100 % B in A; 90-100 min, 100-0 % B in A. (Solvent A- acetonitrile:water, 1:9 (v/v), pH 2.5; Solvent B: acetonitrile:water, 1:1 (v/v), pH 2.5; adjusted with phosphoric acid). Gallic acid, (+)-catechin, chlorogenic acid, 3-coumaric acid, ferulic acid, gentisic acid, 3-hydroxybenzoic acid, protocatechuic acid, pyrogallol and trans-cinnamic acid and ergothioneine were identified and quantified by comparing their retention times and spectral data with those of their authentic standards at 275 nm and 320 nm. Mushroom extracts were analysed in five replicates and results were expressed as mg DW/ml of respective standards.

1.1. Experimental Design

Balb/c mice of either sex were housed under standard conditions (20 $\pm$ 2°C; 65 $\pm$ 15% relative humidity). 120 animals of the same age (7 weeks) and weight (20 $\pm$ 2 g) were selected for the study. The mice were routinely examined for any symptoms of ill health and any visible morphological changes. Furthermore, body weight was monitored throughout the treatment period and liver weight was noted after sacrifice. The animals were studied 24 hours after the last dose. The mice were sacrificed at scheduled periods and complete autopsies were performed. Liver samples were stored and used for DNA isolation. The study protocol was in accordance with the rules and regulations of the Chhatrapati Shahuji Maharaj University and ethical clearance was obtained from the University

Ethical Committee (Ethical Clearance Reference: 1589/PO/a/12/CPCSEA).

1.2. Treatment Groups

After an acclimatization period of 3 weeks, the animals were randomly assigned to 12 groups, each having 10 mice. Group I (control or normal mice) were given only phosphate buffer saline (PBS), while Group II mice were given the carcinogen N-methyl, N-nitrosourea (MNU) at a concentration of 50 mg/kg body weight (b.w.) (Stock: 1g/100ml) intraperitoneally (i.p.). Mice were induced with MNU and simultaneously given oral extract treatment in the Groups III (150 mg/kg ME_1 + MNU, i.p.), IV (300 mg/kg ME_1 + MNU, i.p.), V (450 mg/kg ME_1 + MNU, i.p.), VI (600 mg/kg ME_1 + MNU, i.p.), VIII (150 mg/kg ME_2 + MNU, i.p.), IX (300 mg/kg ME_2 + MNU, i.p.), X (450 mg/kg ME_2 + MNU, i.p.) and XI (600 mg/kg ME_2 + MNU, i.p.). Mice of Groups VII (600 mg/kg ME_1) and XII (600 mg/kg ME_2) were given only mushroom extract treatment. The treatment period for the twelve groups lasted 3 months (92 days).

The dose selection for this study was based on the level of ergothionieine, a natural antioxidant, reported in mushroom species and on its bioavailability in the human body which is estimated within a range of 1 to 4 mg/100 ml blood [15]. The amount of ergothioneine in an average adult of 64 kg would be 44-176 mg (Concentration: 0.69-2.76 mg/Kg body weight). Dose selected for the mice weighing around 20 g was therefore estimated based on this amount. Considering the fact that the mushroom extracts contain a cocktail of metabolites and since the *Pleurotus* sp. (oyster mushrooms) have been reported to contain about 119 mg ergothioneine per kilogram of mushrooms [16], a dose of 150-600 mg/Kg body weight was selected.

1.3. Biochemical Analyses

Blood samples were collected via retro orbital sinus bleeding, without using any anticoagulant. Centrifugation at 4695 x g (5000 rpm) was carried out for 10 minutes to separate the serum. Liver tissue homogenate (10%) was prepared by homogenising 0.1 g of tissue in 1 ml of cold buffer-pH 7.5 (50 mM phosphate buffer and 1 mM EDTA), followed by centrifugation at 10, 285 x g (10,000 rpm) for 10 minutes at 4°C. All the assays used for the biochemical analyses were optimised for mice serum and liver tissue homogenate.

1.3.1. Bradford Protein Test

The protein content of mice serum/liver homogenate samples from different treatment groups was measured by the Bradford Test. To 1 ml of Bradford Reagent, 1 µl of serum/liver homogenate was added and the absorbance of the blue colour formed was read at 595 nm. Protein concentration of the serum samples and liver tissue homogenate were expressed as µg of bovine serum albumin (BSA) protein/ml.

1.3.2. Catalase activity

Colorimetric assay of catalase was carried out as per the adapted method [17]. Serum/liver homogenate samples (200 µl) were added to 500 µl of 0.01M phosphate buffer (pH 7) and 200 µl distilled water. The reaction was initiated by the addition of 250 µl 0.2M hydrogen peroxide (H_2O_2), followed by an incubation period of 1 minute at 37°C. The reaction was stopped by adding 500 µl of 5% dichromate acetic acid reagent and the reaction mixture was incubated in a boiling water bath for 15 minutes. The reaction mixture was cooled and the absorbance was measured at 570 nm. Results were expressed as mmol H_2O_2/min/ml of serum and mmol H_2O_2/min/mg protein of homogenates.

1.3.3. Superoxide Dismutase (SOD) activity

SOD activity of serum/liver homogenate samples was estimated according to the

modified method [18]. Serum/liver homogenate samples (200 µl) were added to 200 µl 0.3 M sodium carbonate buffer (0.3M, pH 10.2), 200 µl 0.6mM EDTA, 400 µl distilled water and mixed properly. The reaction was initiated by adding 200 µl 1.8 mM epinephrine bitartrate. The increase in absorbance was monitored at 480 nm for 3 minutes, at a 30 second interval. Specific activity of SOD (SOD_{50}) was expressed as Units/ml of serum and Units/mg protein of homogenates.

1.3.4. Glutathione Reductase (GR) activity

Glutathione reductase activity was measured according to the modified method [19]. Serum/liver homogenate samples (100 µl) were added to 1 ml 67 mM phosphate buffer (pH 6.6) and 100 µl 0.06% NADPH. The reaction was initiated by adding 100 µl 7.5 mM oxidised glutathione. The decrease in absorbance was measured at 340 nm for 3 minutes, at a 30 second interval. Results were expressed as nmoles NADPH oxidized/min/ml of serum and nmoles NADPH oxidized/min/mg protein of homogenates, using a molar extinction coefficient of 6.3×10^3 M^{-1} cm^{-1}.

1.3.5. Glutathione Peroxidase (GSH-Px) activity

Glutathione peroxidase activity was monitored according to the modified method of Hafeman et al. [20]. Serum/liver homogenate samples (75 µl) were added to 250 µl 0.4 M phosphate buffer (pH 7), 125 µl 0.01M sodium azide, 250 µl 2 mM reduced glutathione and 300 µl distilled water. The composition of standard was as follows: 250 µl 0.4M phosphate buffer (pH 7), 125 µl 0.01 M sodium azide, 250 µl 2 mM reduced glutathione (GSH) and 375 µl distilled water; and that of blank was: 250 µl 0.4M phosphate buffer (pH 7), 125 µl 0.01M sodium azide and 400 µl distilled water. All reaction mixtures were incubated at room temperature for 5 minutes. H_2O_2 (1.25 mM, 250 µl), pre-warmed at 37^0C was added and the reaction mixtures were further incubated for 3 minutes. After incubation, 250 µl of solution was pipetted out into separate tubes and 1 ml of metaphosphoric acid precipitating solution (1.67 g metaphosohoric acid, 0.2 g EDTA and 30 g NaCl per 100 ml solution) was added. The tubes were centrifuged at 2555 x g (3000 rpm) for 5 minutes at 15°C, in a cooling centrifuge (Remi, India). 500 µl of supernatant was pipetted out and 500 µl phosphate buffer and 250 µl 1% (w/v) Tri-sodium citrate solution of 40 mg DTNB was added. The decrease in absorbance was measured at 412 nm for 3 minutes, at a 30 second interval. Results were expressed as µmol/min/ml of serum and µmol/min/mg protein of homogenates.

1.3.6. Malondialdehyde (MDA) Level

Serum/liver homogenate MDA levels were estimated as per the modified method of Satoh [21]. Serum/liver homogenate samples (200 µl) were added to 300 µl of trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-Hydrochloric acid (HCl) solution. The composition for the TCA-TBA-HCl solution was 15% (w/v) TCA, 0.375% (w/v) TBA and 1 M HCl, which were mixed in equal volumes. The reaction mixture was incubated in a boiling water bath for 10 minutes. After cooling, 500 µl of 1 M sodium hydroxide was added to eliminate the white precipitate formed. The absorbance of the reaction mixture was measured at 535 nm and thiobarbituric acid reactive substances (TBARS) content was expressed as nmol/ml of serum or nmol/mg protein of homogenates, using a molar extinction coefficient of 1.55×10^5 M^{-1} cm^{-1}.

1.3.7. FRAP (Ferric Reducing Antioxidant Power) assay

The Ferric Reducing Antioxidant Power (FRAP) of serum/liver homogenate samples was estimated according to Benzie & Strain method [22]. The ferric-TPTZ reagent was prepared by mixing 10 mM of 2,4,6-tripyrdyl-5-triazine (TPTZ) in 40 mM HCL and 20 mM $FeCl_3$ in 0.25 M acetate buffer of pH 3.6. The FRAP reagent was incubated at 37^0C for 4 minutes. The reaction mixture was prepared by adding 50 µl of serum sample to 1 ml of FRAP reagent. The absorbance was read at 593 nm after

incubation at 37^0C for 10 minutes. Ferrous sulphate was used to prepare standards of concentration 0.2, 0.4, 0.6, 0.8 and 1mM. The reducing power of the mice serum/liver homogenate was expressed as mmol Fe(II)/ml of serum and mmol Fe(II)/mg protein of homogenates.

1.4. Haematological Analysis

Prior to cervical dislocation of mice, blood samples were collected between 9.00 and 10.00 a.m. after overnight fasting and dispensed in EDTA-K2 containing tubes (Sarstedt, Germany) for haematological studies. The following parameters were assessed: Red blood cell (RBC) count, hemoglobin concentration, platelet counts, leucocytes, lymphocytes, neutrophils, monocytes and eosinophils.

1.5. DNA Isolation

DNA from the liver samples of all the experimental mice was isolated using a standard phenol-chloroform protocol [23].

1.6. Raman Analysis

DNA isolated from the liver of Balb/c mice was used for Raman spectral analysis. The spectra of all four experimental DNA samples were recorded on a micro-Raman setup from Renishaw, UK, equipped with a grating of 1800 lines/mm and a peltier cooled CCD. The GRAM-32 software was used for data collection. DNA (10 ml) was placed in a quartz cell on an automated X–Y stage below the Olympus long distance 50X microscope objective. The accumulation time for one window was selected as 60 s and 3 spectra were accumulated in each window. With these experimental parameters, reasonably good quality Raman spectra were obtained. In one window, approximately 800 cm^{-1} regions were covered. The resolution of the spectrometer was slightly better than 1 cm^{-1}. A MX50 A/T Olympus microscope (Florida, USA) was attached to the spectrometer. It focused the laser light onto the sample and collected the scattered light at 180° scattering geometry. The 514.5 nm wavelength line of Ar+ laser was used as an excitation source for DNA samples. The recorded data were first saved in ‘‘.spc’’ extension files and were then converted to ASC II files. The Raman spectra were recorded repeatedly to ensure reproducibility of the results.

1.7. Data Analysis

Mean slope analysis was carried out to assess significant differences in weight gain for the 12 different treatment groups in the animal studies. Trend lines were generated for each replicate of the different groups and the slope analysis was carried out using statistical software STATISTICA (Release 7). The specific activity of the enzymes in the different biochemical assays, mean and standard deviation were calculated and one-way ANOVA, followed by LSD test at 5% significance level was applied to assess any significant differences between the different treatment groups, using STATISTICA. The Raman spectra recorded as ASC II files compatible with Spectra Calc and Excel softwares, and Origin 9.1 (Graphing and Analysis) was used for generating the spectra for the 12 different treatment groups.

2. Results

2.1. Polyphenolic analysis of mushroom extracts

The total phenolic content of mushroom extracts varied widely, with the highest level recorded in *A. bisporus* extract (133.7 ± 3.2 mg/g DW) and the lowest in *P. sajor-caju* (33.3 ± 0.9 mg/g DW). The extracts were generally poor in flavonoids (*P. sajor-caju*- 4.6 ± 0.1 and *A. bisporus*-0.5 ± 0.02 mg/g DW) and negligible in proanthocyanidins. Gallic acid (*P. sajor-caju*-356.9 ± 38.9 µg/g DW; *A. bisporus*-726.2 ± 4.3 µg/g DW), pyrogallol (*P. sajor-caju*-2831.3 ± 105.8 µg/g DW; *A. bisporus*-2354.7 ± 88.6 µg/g DW) and protocatechuic acid (*P. sajor-caju*-

630.0 ± 15.2 µg/g DW; *A. bisporus*-84.9 ± 16.1 µg/g DW) were the major phenolic acids detected in the mushroom extracts. L-Ergothioneine, a potent antioxidant, was identified in micro amounts in the two species (*P. sajor-caju*- 2518.9 ± 22.2 µg/g DW; *A. bisporus*-2261.2 ± 14.6 µg/g DW).

2.2. Effect on body and liver weight

The animals in all the groups experienced an increase in weight during the first week of treatment with visible differences in growth patterns observed as from day 14. MNU treated mice (group II) showed a significant decrease in body weight after 22 days compared to the PBS and extract treated groups. MNU-primed mice showed ill health symptoms like loss of weight, uneven shedding of hairs, decreased activity, hunched posture, thin appearance, labored and rapid breathing, at the end of the experimentation period while control mice remained healthy.

Moreover, liver from MNU treated mice, showed formation of several micronodular lesions appearing as small islands or large stripes. These lesions eventually altered the normal architecture of the liver lobes with continued carcinogen exposure (Figure 1a,b). The 56% decrease in body weight of MNU-primed mice by the end of the treatment period, was modulated by extract supplementation in groups III (48%), IV (36%), V (45%), VI (42%), VIII (46%), IX (55%), X (51%) and XII (51%) (Table 1).

Mice treated with ME_1 and ME_2 at 600 mg/kg b.w showed an increase in weight close to the control group. Treatment with ME_2 (450 mg/kg) and ME_1 (300 mg/kg) along with MNU, restored near normal growth and considerably reduced uneven shedding of hairs in Balb/c mice. Moreover, supplementation at these concentrations reduced the formation of micronodular lesions and restored the dark red colour of a normal liver (Figure 1c,d).

The liver/body weight ratio is highly indicative of tumor presence. In MNU treated mice, the liver weight was 275 mg higher than the control mice, thus resulting in an increase of 2.8 folds in liver/body weight ratio. Significant differences in organ weight were observed for liver isolated from the different treatment groups ($p<0.05$). Mushroom extracts (groups VII and XII) restored liver weight close to the control mice. Treatment in groups III, V, VIII, X and XI, showed protective effects by reducing the high liver/body weight ratio of MNU by 53% ($p<0.05$). Overall, the ratios for all the treatments groups except that of Group II were within the range of 3-5 %.

2.3. Protein Concentration and Biochemical Analysis

Serum protein levels were lowest in mice from groups II and IX ($p<0.05$). MNU treated mice, showed comparatively lower levels of protein in both serum and liver homogenate. Protein levels in mice serum from groups VII and XII, where solely extracts were given, were comparable to that of the control (Table 2). In the liver homogenate samples, the lowest protein concentration was observed in the group VIII and the highest in group V ($p<0.05$).

Investigation of CAT activity in both serum and liver homogenate samples showed significant differences for the different treatment groups ($p<0.05$) (Figure 2a). MNU treatment reduced both serum and liver CAT activity ($p<0.05$). Highest liver CAT activity was noted in Group VII (495.8 ± 7.6 mmol H_2O_2/ml/mg protein), followed by control (469.4 ± 5.8 mmol H_2O_2/ml/mg protein) and Group XII (412.4 ± 8. mmol H_2O_2/ml/mg protein) ($p<0.05$). Extract supplementation in both groups upregulated serum and liver CAT activities in mice whereby administration of MNU decreased the enzymatic activity by 78% and 56% respectively, compared to healthy control.

In comparison to healthy controls, mice injected with MNU showed a decline of 20% and 52% in serum and liver SOD activity respectively. Mushroom treatment had a modulating effect

with highest superoxide dismutase (SOD_{50}) activity being assayed in serum of Group XII (10.8 ± 0.4 U/ml) serum and Group I (165.5 ± 4.1 U/mg protein) liver homogenate ($p<0.05$). Lowest activity (SOD_{50}) occurred in MNU treated mice for both serum (4.5 ± 0.9 U/ml) and liver homogenate samples (131.9 ± 4.4 U/mg protein) ($p<0.05$). No significant differences were observed in serum SOD_{50} activity for Groups I, VII, VIII and IX; while the liver SOD_{50} activity for Groups VII, V, XI and XII were similar ($p>0.05$) (Figure 2b).

Administration of MNU decreased liver and serum GR activity (serum-166.0 ± 14.4 nmol NADPH oxidized/min/ml); liver-1865.8 ± 14.0 nmol NADPH oxidized/min/mg protein) by 48-49% compared to healthy mice, while extract treatment upregulated GR activity. Highest serum GR activity was noted in Group I (322.4 ± 17.0 nmol NADPH oxidized/min/ml) while in the liver, highest GR activity was obtained for Group VII (4099.6 ± 62.8 nmol NADPH oxidized/min/mg protein) ($p<0.05$). Lowest GR activity was measured in MNU treated mice (Figure 2c). No significant differences in serum GR activity of Groups IX, XI and XII was observed, while for liver GR activity, groups VI and III; V and XI were similar ($p>0.05$).

Extract supplementation also modulated liver GSH-Px activity in MNU induced mice with a fall of 50% and 45% in serum and liver respectively. Serum GSH-Px activity was similar in groups VII, X and I; while liver GSH-Px activity was comparable for control and Group XII ($p>0.05$) (Figure 2d). Significant levels of GSH-Px were noted in Group XII serum (200.6 ± 9.8 µmol/min/ml) and Group VII liver (1271.9 ± 60.7 µmol/min/mg protein) samples ($p<0.05$). GSH-Px activity was lowest in serum (88.5 ± 10.0 µmol/min/ml) and liver homogenate (456.9 ± 32.1 µmol/min/mg protein) of MNU treated mice ($p<0.05$).

Lipid peroxidation, as measured by TBARS levels, was important in MNU-treated mice serum (0.3 ± 0.0 nmol TBARS/ml) and liver homogenate (3.0 ± 0.1 nmol TBARS/mg protein), while its occurrence was lowest in serum of Group VII (0.04 ± 0.0 nmol TBARS/ml) and XII (0.5 ± 0.1 nmol TBARS/ mg protein), and liver sample of Group V (0.6 ± 0.1 nmoles TBARS/mg protein) ($p<0.05$). Lower levels of liver TBARS were measured for control, and extract treated groups (XII and VII) with no statistical difference amongst them ($p>0.05$) (Figure 2e). Mushroom treatment reduced lipid peroxidation in MNU induced mice, whereby a rise in serum (75%) and liver (70%) MDA levels, was apparent compared to normal mice.

Extract treatment without carcinogen more effectively reduced circulating levels of TBARS, compared to PBS-treated mice, thus indicating an improved antioxidant status. The highest ferric reducing potential however was recorded in serum and liver homogenate samples of Groups I (serum: 80.4 ± 0.2 mmol Fe(II)/ml; liver: 275.4 ± 5.7 mmol Fe(II)/mg protein), VII (serum: 72.5 ± 1.0 mmol Fe(II)/ml; liver: 263.8 ± 3.8 mmol Fe(II)/mg protein) and XII (serum: 70.5 ± 0.6 mmoles Fe(II)/ml; liver: 249.6 ± 4.4 mmol Fe(II)/mg protein) ($p<0.05$). FRAP levels in serum (39.9 ± 0.6 mmoles Fe(II)/ml) and liver (151.9 ± 5.2 mmol Fe(II)/mg protein) of MNU treated mice were very low ($p<0.05$) (Figure 2f). Extract supplementation upregulated serum and liver FRAP levels in mice whereby administration of MNU decreased the FRAP value by 50% and 45% respectively compared to the healthy mice.

2.4. Haematological analyses

Analysis of blood parameters showed that the haemoglobin concentration of MNU treated mice was 31% lower than the control group and was well below the normal range of 12-17 g/dL. The highest haemoglobin level was measured in the control mice and groups IV, VI, VII and XII (12.5-13.3 g/dL). The leucocyte counts for all the groups were within the normal range of (4-10) x 10^3 cell/cumm, except for MNU (39% higher than control). Furthermore, significantly high levels of eosinophils (82%), monocytes (76%), and red blood cell count (27%) were observed in the MNU treated mice in comparison to control ($p<0.05$) (Table 3).

Extract treatments in groups VII and XII (without carcinogen), gave a blood parameter profile close to that of normal mice. When these mushroom extracts were administered along with MNU, protective effects were observed at all concentrations tested ($p<0.05$). The decline in haemoglobin concentration was prevented in extract treated groups: III (25%), IV (28%), V (26%), VI (28%), VIII (20%), IX (18%), X (23%) and XII (26%) and lymphocyte levels were also reduced in these groups: III (28%), IV (5%), V (3%), VI (6%), VIII (22%), IX (21%), X (31%) and XII (33%).

Moreover, mushroom supplementation reduced the elevated levels of leukocytes in MNU-induced mice in groups: III (32%), IV (28%), V (33%), VI (26%), VIII (20%), IX (22%), X (17%) and XII (21%). Abnormally high eosinophil levels were also reduced in groups III (75%), IV (75%), V (74%), VI (75%), VIII (79%), IX (77%), X (73%) and XII (75%) groups. The high percentage of monocytes was downregulated by extract treatment: III (71%), IV (75%), V (74%), VI (80%), VIII (81%), IX (76%), X (80%) and XII (71%).

Administration of MNU led to a decline in platelet counts in group II mice, which was restored to certain extent by mushroom treatment in groups: III (34%), IV (30%), V (31%), VI (26%), VIII (30%), IX (21%), X (22%) and XII (20%). In general, haematologcial parameters were comparable when MNU primed mice were treated with ME_1 at concentrations 150 mg/kg b.w and 450 mg/kg b.w and ME_2 at concentrations 300 mg/kg b.w and 600 mg/kg b.w ($p>0.05$).

2.5. Raman Analysis

Raman Spectroscopy was performed to evaluate the effects of mushroom extracts ME_1 and ME_2, in reducing MNU carcinogenic damage to DNA structure at nucleic bases and phosphate backbone levels. Figure 3 and 4 show the spectra of liver DNA samples at the end of 3 months treatment period. Peak intensities are studied by using the standard analysis Software Spectra Calc. Peaks appearing in each region were superimposed to the mixture of Lorenz Ian and Gaussian bands to evaluate the extent in intensity, frequency and peak area changes (Figure 3(iii)). To assess the precise change in speak intensity and shift, an internal standard (1434 cm^{-1} band) was selected. This band 1434 cm^{-1} does not appear to change in intensity in any of the four samples. The peak normalization eliminated the effect of variation in DNA sampling. Each measurement was done separately on the same day of DNA isolation. The Raman band peaks were analysed and compared to literature data, as given in Table 4.

The general features of the Raman spectra of liver DNA of control (PBS), MNU, ME_1 (300 mg/kg b.w.) and ME_1 (600 mg/kg b.w.) mice were relatively similar according to their band assignments and the regions in which the peaks were obtained. However differences in their relative intensity were apparent. For the analysis of the Raman bands, the complete spectra (600-1600 cm^{-1}) were divided into six regions (Table 4). A total of ten peaks including 763 cm^{-1}, 840 cm^{-1} and 911 cm^{-1} for phosphodiester stretching; 959 cm^{-1}, 1007 cm^{-1}, 1066 cm^{-1}, 1136 cm^{-1} and 1257 cm^{-1} peaks for deoxyribose and 1327 cm^{-1}, 1405 cm^{-1} and 1461 cm^{-1} for purine and pyramidine bases were characterised. These assigned peaks increased in intensity (shown at Y-axis) by 50-70% for MNU treated (Group II) liver DNA samples (Figure 3(ii)).

The region 1200–1600 cm^{-1} corresponds to nucleic bases which are prone to any type of alkylation by MNU. Mushroom protective effect was confirmed by Raman spectroscopy where, the MNU-DNA interaction as evidenced by an intense peak at 1254 cm^{-1} was normalised and was not apparent in any of the mushroom-treated DNA samples. The increase in peak intensity value was within the same range of 0-200 (a.u.) in Group I (PBS treated) and MNU + ME_1 (300 mg/kg b.w.) (Group IV).

Mushroom extract treatment with ME_1 at a concentration of 300 mg/kg b.w. was more effective in reducing DNA damage after 92 days of supplementation compared to 600 mg/kg b.w.

The combined effect of carcinogen and ME_1 extract (600 mg/kg b.w.) significantly increased the overall intensity of the Raman peaks by one fold compared to MNU treated mice (Figure 3(ii) and 4(ii)). The major change in spectra was observed at peak 959 cm^{-1}, 1007cm^{-1} and 1188 cm^{-1} where the latter was more intense and prominently increased (2 folds) in ME_1 (600 mg/kg b.w.) treated samples (Figure 4(ii)). In the case of ME_2 extract treatment, the Raman spectra for MNU + ME_2 (450 mg/kg b.w.) (Group X) showed an increase in peak intensity comparable to control mice (Figure 4(v)).

Extract treatment with MNU + ME_2 (300 mg/kg b.w.) showed a less prominent increase in peak intensity compared to MNU + ME_2 (150 mg/kg b.w.) (Figure 4(iv), (iii)). The protective effects of extract treatment in ME_2 groups seemingly increased in a dose dependent manner, with major change in spectra at peaks 911 cm^{-1}, 959 cm^{-1}, 1247 cm^{-1}, 1066 cm^{-1} and 1188 cm^{-1}. The intensity of these peaks was much lower in Group X, and rose sequentially in Groups IX and VIII. The rise in intensity of the peaks in the Raman spectra of Groups X, IX, VIII and IV were much lower than that of Group VI. The biochemical scenario demonstrated that treatment by both *P. sajor-caju* and *A. bisporus* effectively reduced oxidative damage in MNU primed mice, as evidenced by a significant decrease in the extent of lipid peroxidation and a concomitant increase in the antioxidant enzyme activities notably CAT, SOD, GR and GSH-Px and FRAP levels.

3. Discussion

Mounting evidence increasingly suggests the putative role of mushrooms in the management of health and disease and support the use of mushrooms for the development of functional food ingredients [24-26]. Reports have ascribed their protective effects to the pluripharmacological effects of their bioactive constituents such as polysaccharides, amino acids, polyphenols and terpenoids [27-29], subsequently prompting a number of research initiatives into their anti-cancer properties. In this vein, mushrooms consumed in Mauritius, where cancer incidence has increased to account for 12.1% deaths in 2009 [30], were investigated for their prophylactic chemopreventive potential.

Locally consumed mushrooms were rich in phenolics ($p<0.05$). Smolskaite et al. [26] reported total phenol contents (TPC) of 4.2 ± 0.1 mg gallic acid equivalent (GAE)/g dry weight (DW) for *A. bisporus* and 5.3 ± 1.0 mg GAE/g DW for *P. ostreatus*. These values are much lower than those observed for *P. sajor-caju* (33.3 ± 0.9 mg GAE/g DW) and *A. bisporus* (133.7 ± 3.2 mg GAE/g DW) in this study.

HPLC analyses of the extracts confirmed the predominance of phenolic acids in the extracts. Several studies have also confirmed that major antioxidants in mushrooms, characterised in numerous species from Finland [31], India [32], Korea[33] and Portugal [34] , were phenolics. In our study, gallic acid, protocatechuic acid and pyrogallol were the most prominent compounds identified, in line with several other studies on *P. sajor-caju*, *P. ostreatus* and *A. bisporus* [31-33].

The modulatory effects of these phenolics in cancer have been well expounded in literature. Maurya et al. [35] and Yumnam et al. [36] reported anti-cancer activity of gallic acid in human colon cancer (HCT15), breast cancer (MDA MB 231) and lung adenocarcinoma (A549) cell lines. Protocatechuic acid has been shown to inhibit *in vitro* chemical carcinogenesis and exert proapoptotic and antiproliferative effects in F344 male rats with DEN (diethylnitrosamine)-induced liver carcinogenesis [37]. This phenolic is also an inhibitor of free radicals, plays a vital role in phases 1 and 2 of the metabolism of certain carcinogens and behaves like a blocking agent at the site where these carcinogens bind with DNA molecules [38].

Mice liver cancer models represent a valuable practical tool to study the mechanisms involved in experimental tumour proliferation and the development of therapeutic strategies in the assessment of the bioefficacy of natural extracts. MNU used as a mutagen to induce cancer in the mice, is a direct-acting alkylating agent

interacting with DNA by transferring its methyl group to nucleobases in nucleic acids. This leads to the creation of mutagenic lesions and accumulation of mutations that play an important role in cancer initiation [39]. The intake of this nitrostable compound develops a conducive environment for the activation of inflammatory cytokines (IL-1β, IL-6) with the increased expression of NF-kB, leading to hepatocarcinogensis in Balb/c mice [40].

The evaluation of the modulatory effects of *P. sajor-caju* (ME_1) and *A. bisporus* (ME_2) revealed protective effects against MNU. The morphological changes and weight records for mice in this study are consistent with those made by Verma et al. [23,40] under similar conditions. MNU-treated mice experienced a decrease of 15.2 % in body weight compared to the control after 4 weeks of treatment as the carcinogenic effects of MNU started to manifest. Verma et al. [23] reported a decrease of 40% in body weight of Balb/c mice treated with MNU after a longer period of 28 weeks compared to control.

Mushroom treatment in MNU-induced hepatocarcinogenesis effectively restored body weight of the mice after 22 days, and this beneficial effect can most probably be attributed to the anti-mutagenic and anti-cancer properties of the mushroom extracts as reported by Sarangi et al. [11] and Shah et al. [12]. Data from Raman spectroscopy analysis support these observations, thereby clearly demonstrating a consequent reduction in the peak intensities of MNU liver DNA spectra as a result of extract treatment. *P. sajor-caju* and *A. bisporus* consistently reduced uneven shedding of hairs and formation of micronodular lesions in the liver of the animals by the end of the 3-months supplementation period. Moreover, high liver/body weight ratio of MNU-treated mice (2.8 folds greater than control mice) was significantly lowered by extract treatment. Jayakumar et al. [41] reported hepatoprotective effects of *P. ostreatus* extracts in improving the antioxidant status and reverting hepatic damage in carbon tetrachloride (CCl_4)-treated male Wistar rats. Moreover, Nada et al. [42] also demonstrated that polysaccharides from *P. ostreatus* mycelium protected hepatocytes from CCl_4-induced damage in Sprague Dawley rats. *In vitro* antiproliferative effects of *P. ostreatus* extracts have also been reported in other cancers, e.g. breast (MCF-7), colon (COLO-205), and kidney (ACHN) human cancer cell lines [43].

Mushroom treatment on serum and liver antioxidant enzymes of Balb/c mice clearly showed an upregulation of the latter and consequently a reduction in oxidative stress. MNU considerably decreased the protein level and antioxidant enzymes like CAT, SOD, GR and GPx in serum and liver of the mice. However, the protein level was restored by *P. sajor-caju* at 150mg/kg and *A. bisporus* at 300 and 450 mg/kg respectively. Similar observations were made by Llauradó et al. [44], where the total serum protein levels of malnourished Balb/c mice supplemented with cold-water extract from the fruiting bodies of *Pleurotus* spp. were higher than for those mice without supplementation.

This was substantiated by low reducing power and elevated MDA levels (as measured by circulating levels of TBARS) in both serum and liver tissues of MNU treated mice. Mohd Ali et al. [45] reported a significant decrease in SOD activity and FRAP levels in liver tissues of male Balb/c mice with ethanol-mediated liver damage. This decrease in cellular enzyme activities was largely due to the impairment of antioxidant enzymes that protect cells against reactive oxygen species [46]. Prolonged exposure to potent carcinogens has been shown to decrease enzyme activity in targeted tissues of organ-specific carcinogenesis [47,48].

Nonetheless, in the initial phase of acute toxicity and carcinogenesis, a concomitant increase in enzyme activities curbing down the ROS levels was noteworthy. Lukaszewicz-Hussain & Moniuszko-Jakoniuk [47] assessed CAT, GPx and GR activities, hydrogen peroxide and MDA levels in liver tissues of male Wistar rats after intoxication with chlorfenvinphos (an organophosphate insecticide). An initial increase in rat liver CAT, GPx and GR activity, followed

by a considerable decrease by the end of the treatment, and a sharp rise in liver MDA levels were noted. In this study, a decrease in enzyme activity and high rate of lipid peroxidation in MNU treated mice occurred by the end of the 3 months supplementation. Decline in SOD activities has been associated with an increase in circulating lipid peroxides which can result in an steady state of superoxide anion, a highly diffusible and potent oxidizing radical capable of traversing membranes and causing deleterious effects far from the tumour sites [49].

CAT and GPx detoxify significant amount of hydrogen peroxide produced during electron transport chain and protect mitochondrial membranes from oxidation [50]. The decreased activities of catalase found in the cancerous condition of MNU treated mice, could be attributed to exhaustion of these enzymes, leading to accumulation of hydrogen peroxide by cancerous cells [48,51]. Decrease in GPx activities in various cancerous conditions has also been reported in literature [52]. The enormous production of free radicals, as is the case of several cancers, could have altered the antioxidant defense system in mice with MNU-induced hepatocarcinogenesis, thereby accounting for the decline observed in the serum and liver GPx activities in the present study.

Polyphenols, flavonoids and anthocyanins content have been suggested to exert strong antioxidant activity, which contribute to the protective effect against liver injury in mice and rats [53]. However, most dietary polyphenols being in their native form of esters, glycosides or polymers, cannot be absorbed directly and need to be hydrolyzed by intestinal enzymes or by colonic microflora, which reduces bioavailability [54,55]. An indirect evidence of their absorption through the gut barrier is given by an increase in the antioxidant capacity of plasma after the consumption of foods rich in polyphenols (Young et al., 1999; Pandey & Rizvi, 2009).

Phenolics like gallic acid and protocatechuic acid, identified in the mushrooms have been reported to have excellent antioxidant and antitumour properties [35-37], potentiating a protective effect against hepatocarcinogenesis. It is noteworthy that the biological functions of polyphenols such as anti-mutagenicity, and anti-carcinogenicity is partly a function of their antioxidant activity [57,58]. Mice groups, given only extract treatment of ME_1 and ME_2 respectively, showed better or comparable CAT and SOD activity ($p<0.05$).

Treatment with *A. bisporus* reduced TBARS levels in breast tissue of 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in Sprague-Dawley rats [48]. Fruiting body extracts of *P. sajor-caju* have also been reported to reduce the number of neoplastic cells in female Swiss mice inoculated with Ehrlich ascitic tumour [59]. In the present study, extract treatment of ME_1 (150 mg/kg) in serum and ME_2 (300 mg/kg) in liver tissues showed appreciable FRAP levels. The FRAP assay can be used as a simple screen for assessing the ability to maintain redox status in cells and tissues, as it detects redox potentials of less than 0.7 V (the redox potential of Fe^{3+}-TPTZ).

However, since this assay does not measure activity of thiol antioxidants, such as glutathione, it may not reflect antioxidant activity mechanistically and physiologically in complex systems [60]. Measuring the *in vivo* antioxidant enzymes like CAT, SOD, GR and GPx, provided a better assessment of antioxidant status in Balb/c mice induced with hepatocarcinogenesis. A more effective reduction of circulating TBARS levels was achieved by ME_1 (600 mg/kg) and ME_2 (600 mg/kg) compared to control mice and these groups showed an improved antioxidant status.

Hepatic antioxidant status was restored with ethanolic extract of a wild edible mushroom (*Macrocybe gigantea*) treatment in carbon tetrachloride-induced hepatic damage in Swiss albino mice [61]. Wu et al. [62] reported the hepatoprotective effects of aqueous extracts of medicinal mushroom *Ganoderma lucidum* on liver injury induced by α-amanitin in mice, where a significant increase in SOD and CAT activities, and decreased MDA content in liver

tissues were observed. In line with these observations, the protective effects of both *P. sajor-caju* and *A. bisporus* on biochemistry of Balb/c mice serum and liver tissues are clearly evident, and can be attributed to their antioxidant properties *in vitro*.

The immunomodulatory effects of the mushroom extracts were obvious on hemoglobin, leukocyte and platelet as well as lymphocyte, neutrophil, monocyte and eosinophil counts in MNU-treated mice. MNU-treated samples showed an abnormal elevated level of leucocytes, monocytes and neutrophils. A strong association between abnormally high neutrophil, monocyte or leucocyte counts and poor survival rates in patients with metastatic carcinoma has been reported [63,64]. The sharp drop in lymphocyte count in MNU-treated sample compared to other extract-treated groups strongly confirms this association and suggests this parameter as a valuable marker in cancer prognosis [65,66].

MNU-treated mice were found with a significantly high platelets count (Table 3). This hike in platelet counts is linked to an increased expression of inflammatory cytokine (IL-1b, IL-6) as observed in various types of cancer patients [67]. The inflammatory response within the microenvironment of tumours is characterised by tumour-associated macrophages and tumour-infiltrating lymphocytes. These lead to the overproduction of several proinflammatory cytokines, in particular tumour necrosis factor, chemokines, transcription activators and interleukin-1β and -6 [68]. Mushroom extract treatment also normalised the leucocyte, neutrophil, monocyte and eosinophil counts after 3 months of treatment.

The haematological profile of groups III and IV, and IX and XI were comparable ($p>0.05$), thus providing additional evidence of the beneficial effects of ME_1 and ME_2 mushroom extracts when administered at these concentrations. Verma et al. [23] also reported beneficial properties of natural extracts of *A. marmelos* which normalised leucocyte, neutrophil, monocyte and eosinophil counts and increased the lymphocyte count after 28 weeks of treatment in MNU-induced hepatocarcinogenesis in Balb/c mice. Haemoglobin concentrations was best normalised in MNU + ME_1 (300 mg/kg) groups and by in other ME_1 treated groups. Similar observations were reported by Morris et al. [69] where protective effects of lypohilised extracts of *Pleurotus* spp. fruiting bodies on cyclophosphamide-treated Balb/c mice were assessed. Mice that were treated with the *Pleurotus* spp. extract prior to cyclophosphamide administration showed haemoglobin concentrations similar to that of the control mice at the end of treatment period.

Raman spectroscopy analysis provided a valuable indication in determining the best performing extracts in terms of their ability to protect mice liver DNA from damage at the level of nucleic bases and phosphate backbone. The changes observed in terms of peak intensity increases in MNU-primed mice were clearly evident (Figure 4). Increase in peak intensity in Raman spectroscopy is reflective of a highly cooperative conformational change occurring within different portions of MNU-treated DNA samples [40]. Raman spectra for MNU + ME_2 (450 mg/kg b.w.) showed a rise in peak intensity comparable to control mice while ME_1 at a concentration of 300 mg/kg b.w. proved to be more effective in reducing DNA damage after 92 days of supplementation (Figure 5). The 1252 cm^{-1} peak assigned for adenine and cytosine (Table 4) is prominent and intense in MNU-treated samples. The 1200–1600 cm^{-1} region has been assigned to purines and pyramidines, corresponding to ring electronic structures that are very sensitive to base unstacking and metal binding at these ring sites [70]. Rizvi and Hadi [71] and Ruiz-Chica et al. [72] demonstrated that MNU had a strong affinity for guanine and adenine bases in synthesised calf DNA.

The 1252 cm^{-1} band might be a consequence of alkylation at adenine bases, resulting in a strong interaction between MNU and DNA structure. Verma et al [40] reported a peak value of 1256 cm^{-1} that was assigned to adenine and cytosine. In this study, MNU-DNA interaction evidenced by an intense peak at 1254 cm^{-1} in the MNU Raman

spectra, was normalised by mushroom extract supplementation. Prolonged exposure to potent hepatocarcinogens, may lead to an accumulation of interactions between MNU and DNA, and their relative adducts, thus causing mutations and eventually leading to cancer [23]. This MNU–DNA interaction was markedly inhibited by mushroom extracts in the treatment groups MNU + ME_2 (450 mg/kg) and MNU + ME_1 (300 mg/kg) respectively. The normal structure and confirmation of nucleic acid backbone and sugar bases in DNA samples were maintained, thereby strongly suggesting the anti-carcinogenic properties of *P. sajor-caju* and *A. bisporus* against hepatocarcinogenesis in MNU-treated Balb/c mice.

Non-cytotoxic nutrients and/or pharmacological agents have the ability to target multiple aberrant cellular signaling circuits [73]. Several polyphenols have been reported to correct DNA methylation imbalances. The results of Raman spectroscopy demonstrate that one of the possible mechanism of action employed by the mushroom extracts consists of preventing and/or reversing the alteration in DNA caused by an interference of MNU in the structure of nucleic acid bases, reflected by a peak shift in the Raman spectra.

4. Conclusion

The rise in liver cancer will continue to undermine health due to the increased exposure to environmental pollutants, dietary sources of carcinogens and xenobiotics as indicated by health statistics. With the rising incidence of cancer, functional foods rich in specific antioxidants, showing promising results in vivo and in clinical trials may provide a practical alternative approach to disease control with reduced morbidity and mortality. The findings of this study are highly demonstrative that *Pleurotus sajor-caju* and *Agaricus bisporus* are liable to modulate hepatocarcinogenesis in Balb/c mice. Mushroom extracts with modulatoty effects on biochemical, haematological and biophysical parameters could therefore provide efficient points of intervention at early cancer stages.

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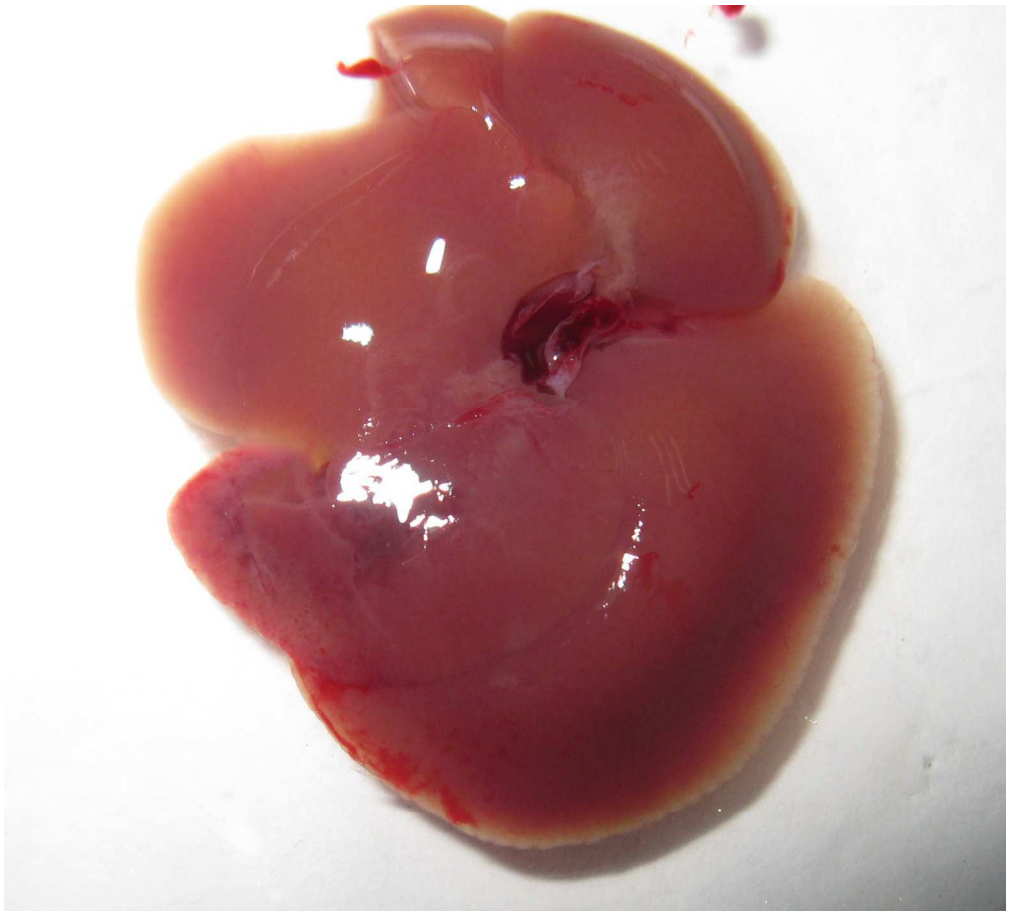

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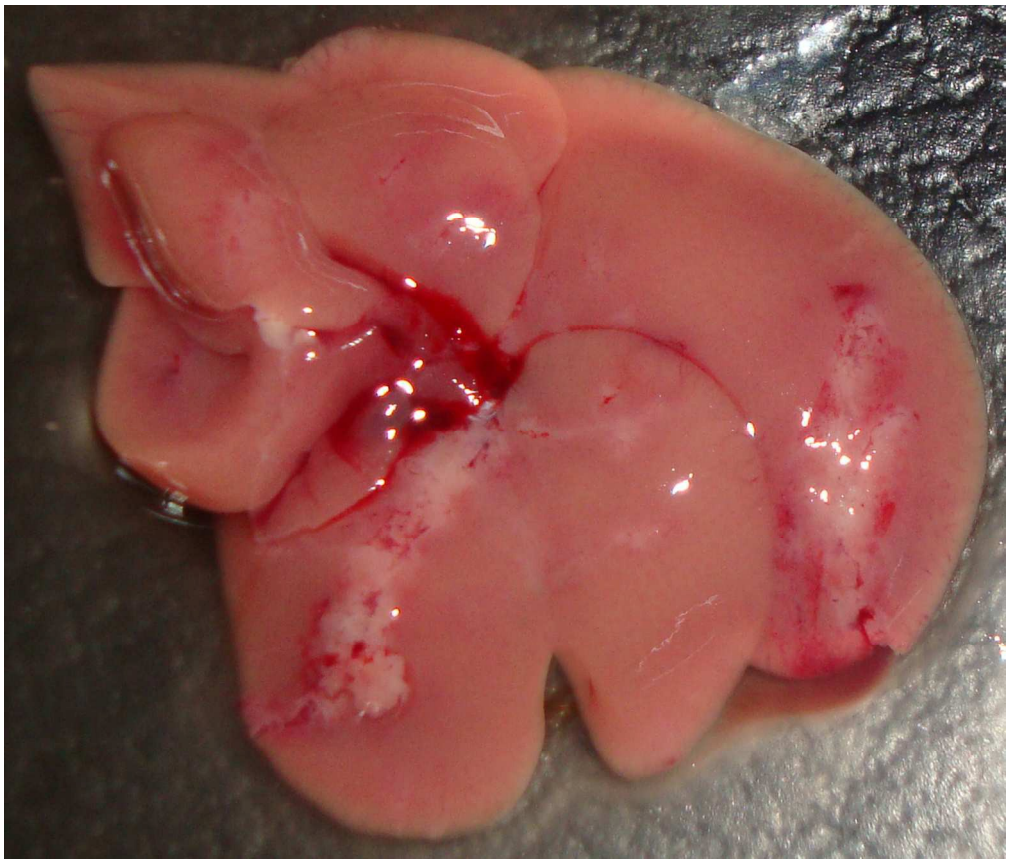

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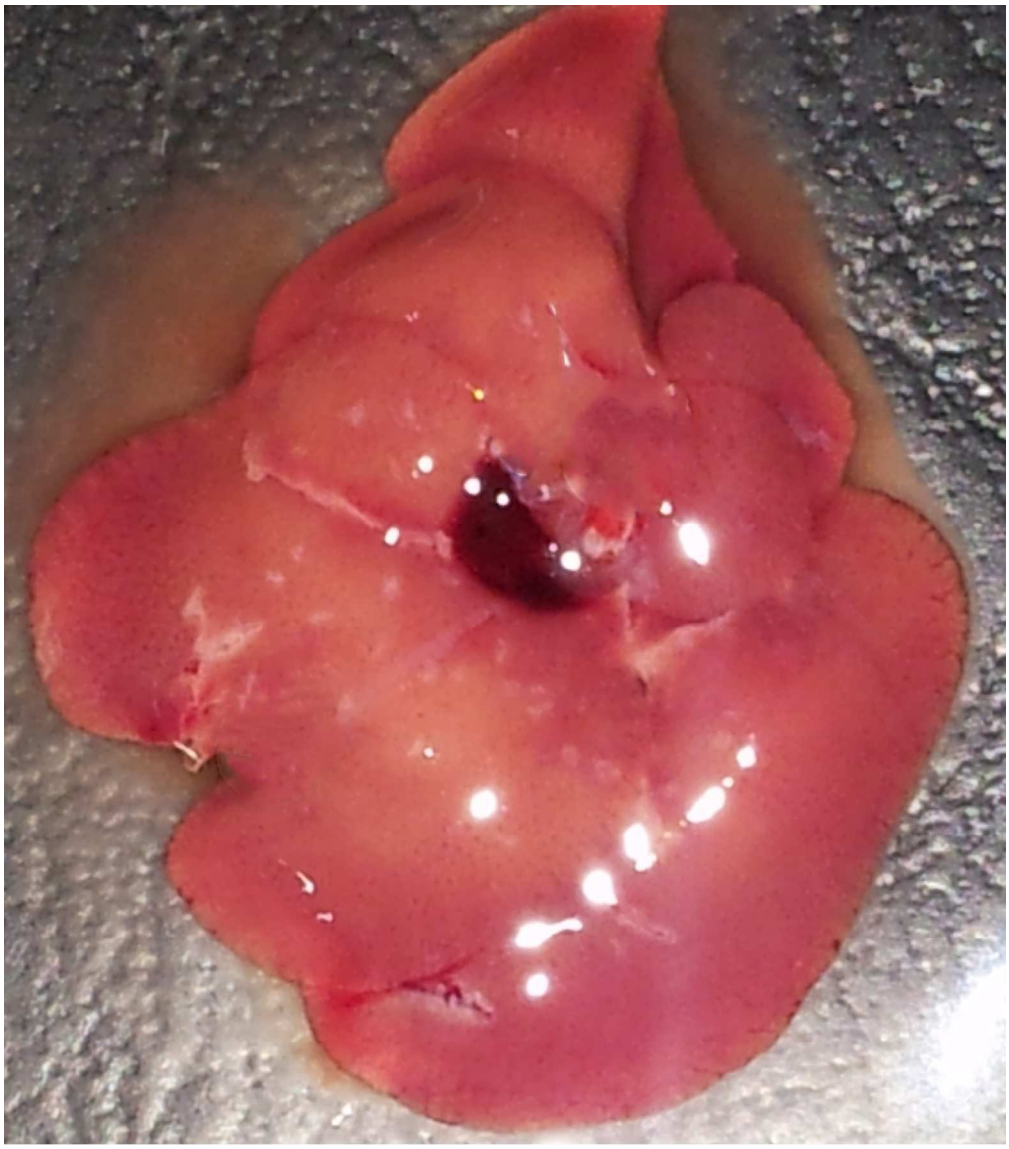

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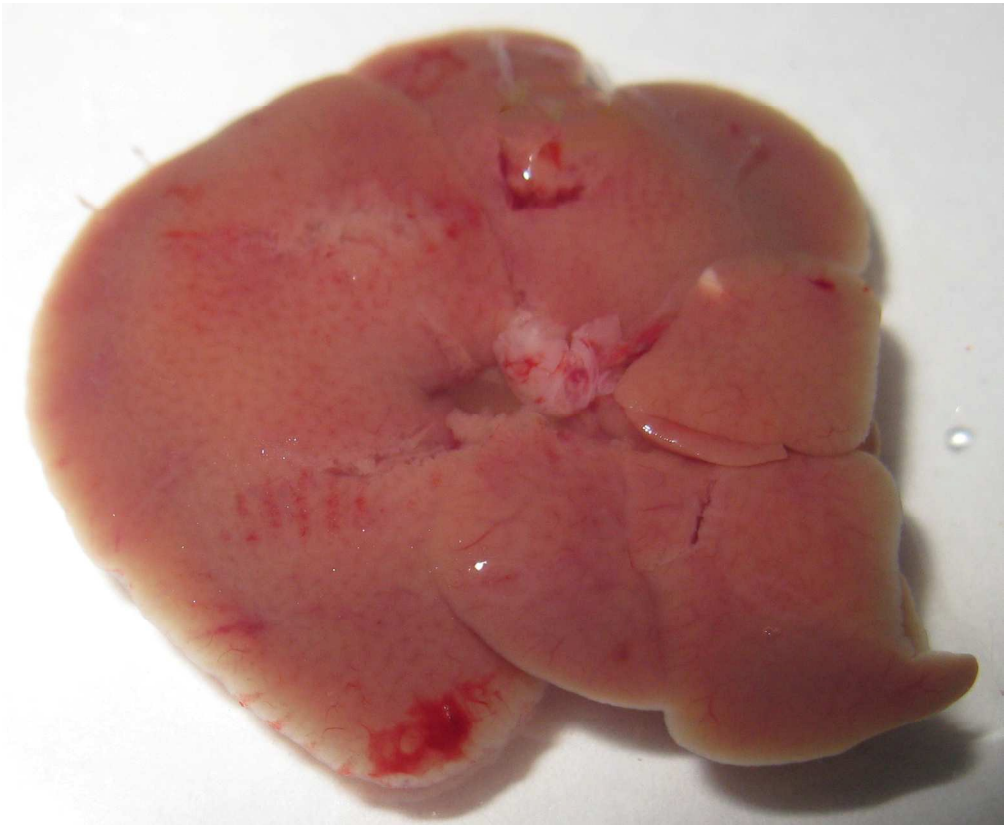

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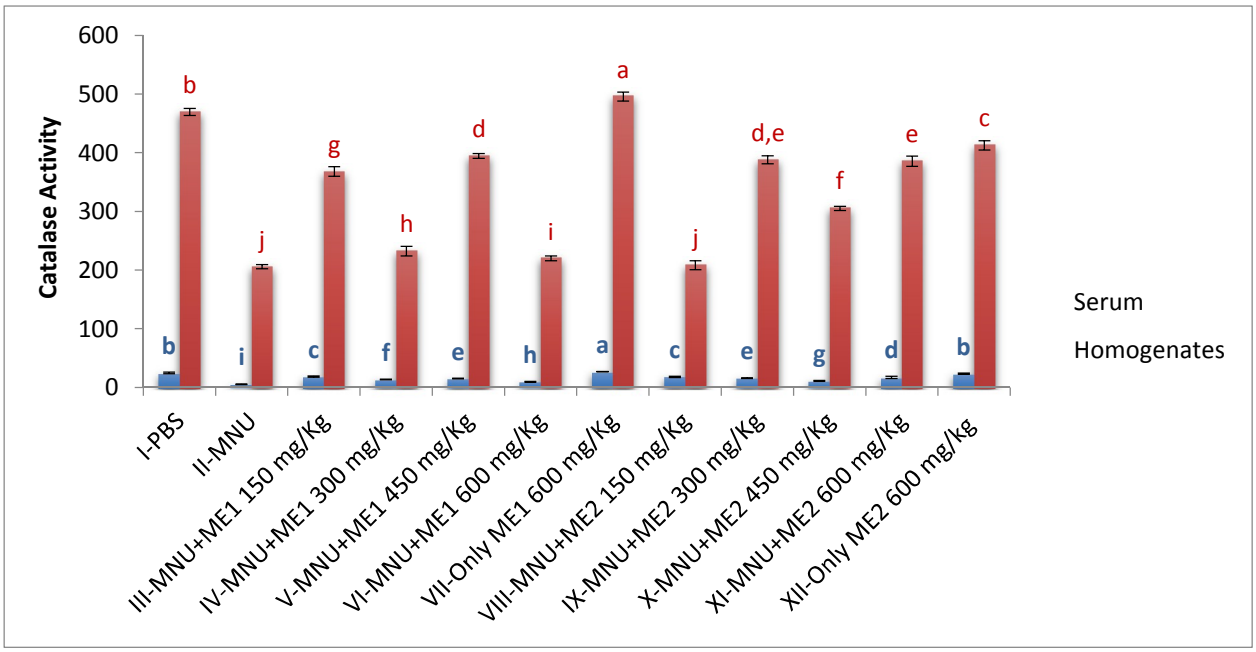


Figure 2a: Catalase activity of PBS, MNU and extract treated mice

Catalase activity expressed as mmol H_2O_2/min/ml of serum and as mmol H_2O_2/min/mg protein of homogenates; Data expressed as mean $\pm$ standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

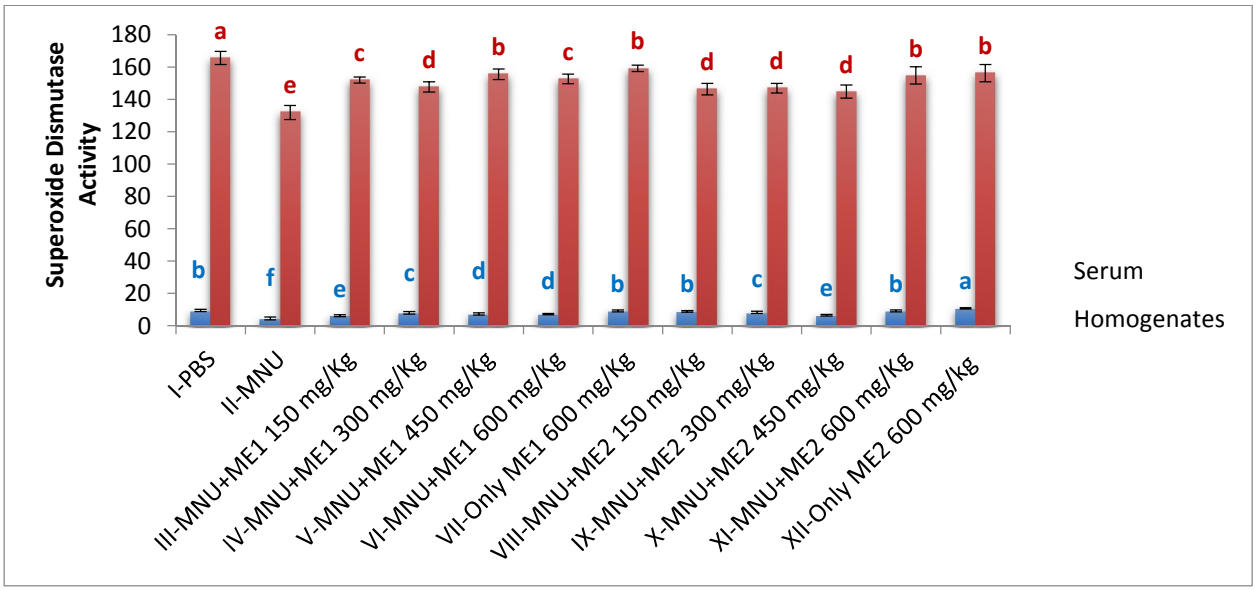


Figure 2b: Superoxide dismutase activity of PBS, MNU and extract treated mice

Superoxide dismutase activity expressed as Units/ml of serum and as Units/mg protein of homogenates; Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

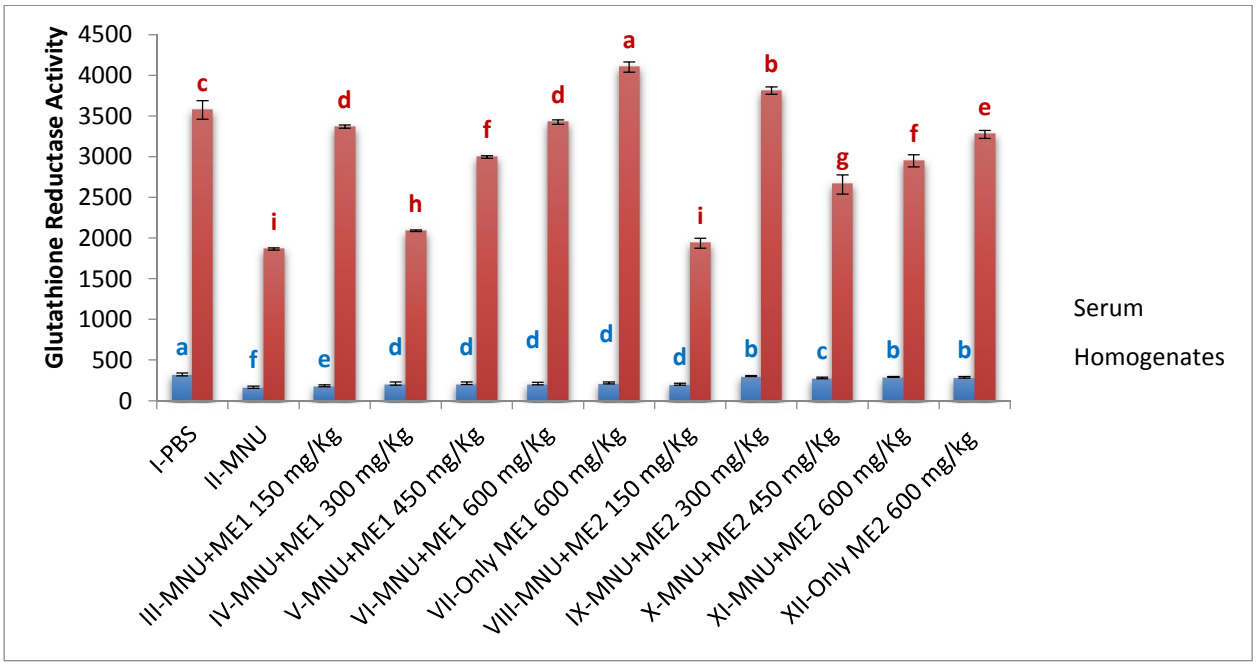


Figure 2c: Glutathione reductase activity of PBS, MNU and extract treated mice

Glutathione reductase activity expressed as nmol NADPH oxidized/min/ml of serum and as nmol NADPH oxidized/min/mg protein of homogenates; Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

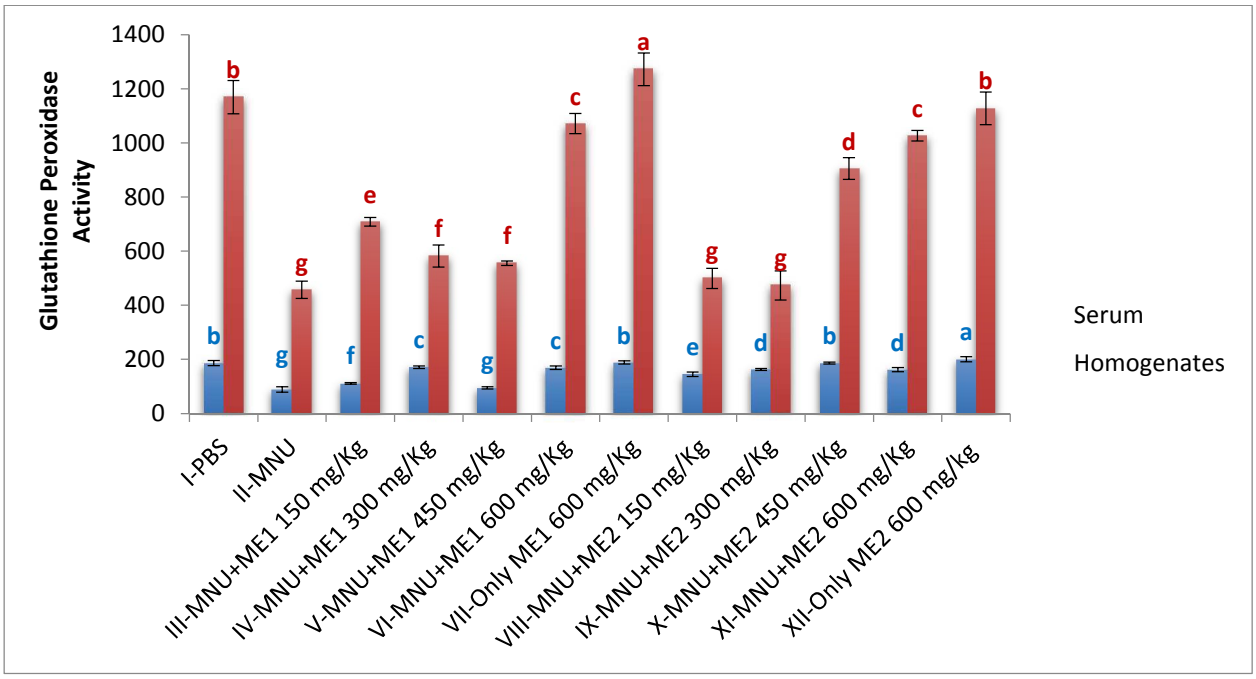


Figure 2d: Glutathione peroxidase activity of PBS, MNU and extract treated mice

Glutathione peroxidase activity expressed as µmol/min/ml of serum and µmol/min/mg protein of homogenates; Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

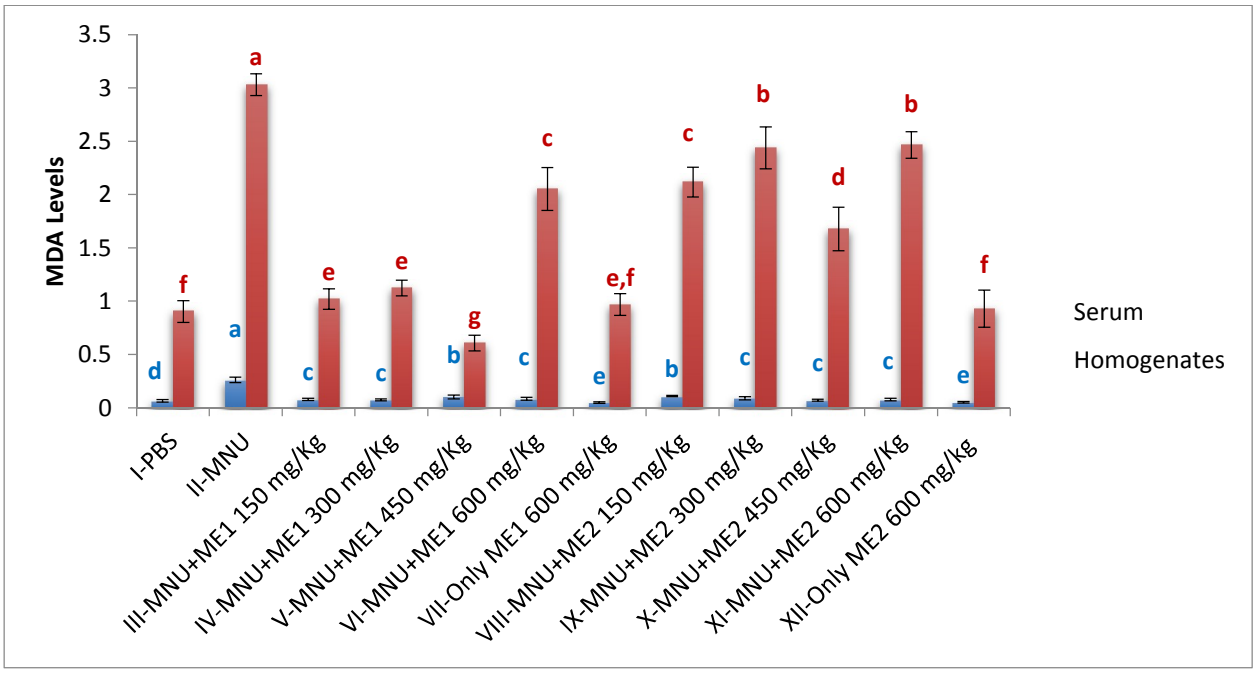


Figure 2e: Malondialdehyde levels in serum and liver homogenate of PBS, MNU and extract treated mice

MDA levels expressed as nmol TBARS/ml of serum and nmol TBARS/mg protein of homogenates; Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

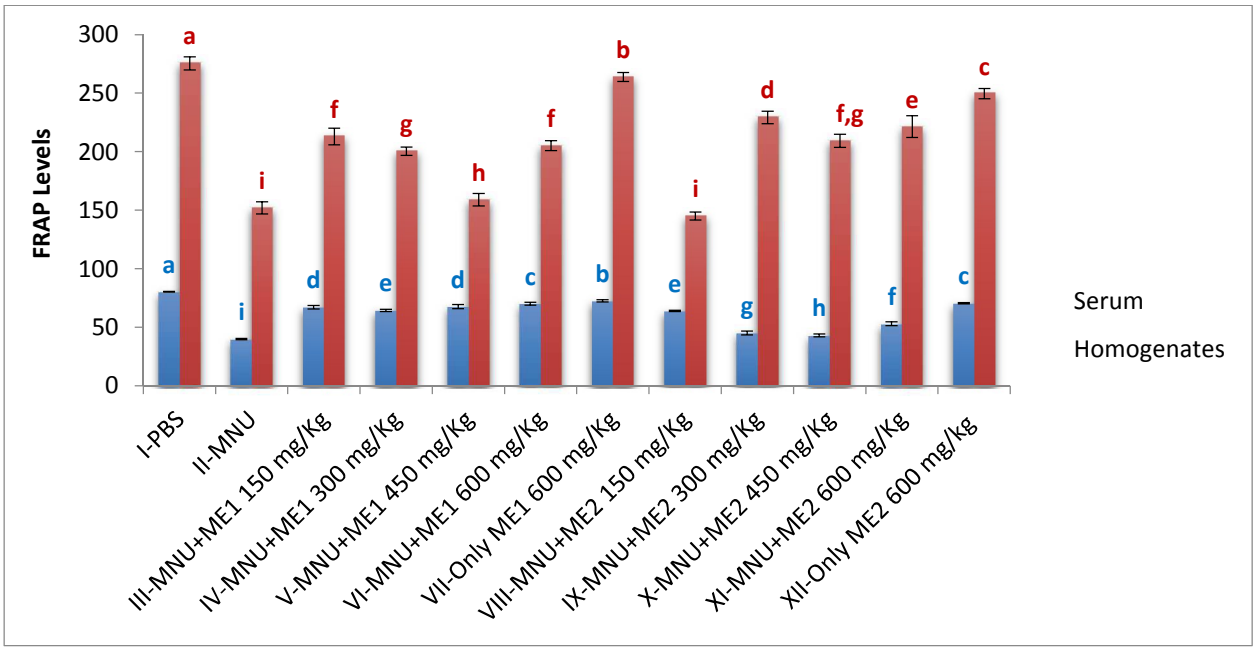


Figure 2f: Ferric Reducing Antioxidant Power of serum and liver homogenate of PBS, MNU and extract treated mice

FRAP levels expressed as mmol Fe(II)/ml of serum and as mmol Fe(II)/mg protein of homogenates; Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and LSD were carried out at 5% significance; Similar superscripts on the mean values represent no significant differences between serum samples or liver tissue homogenate respectively.

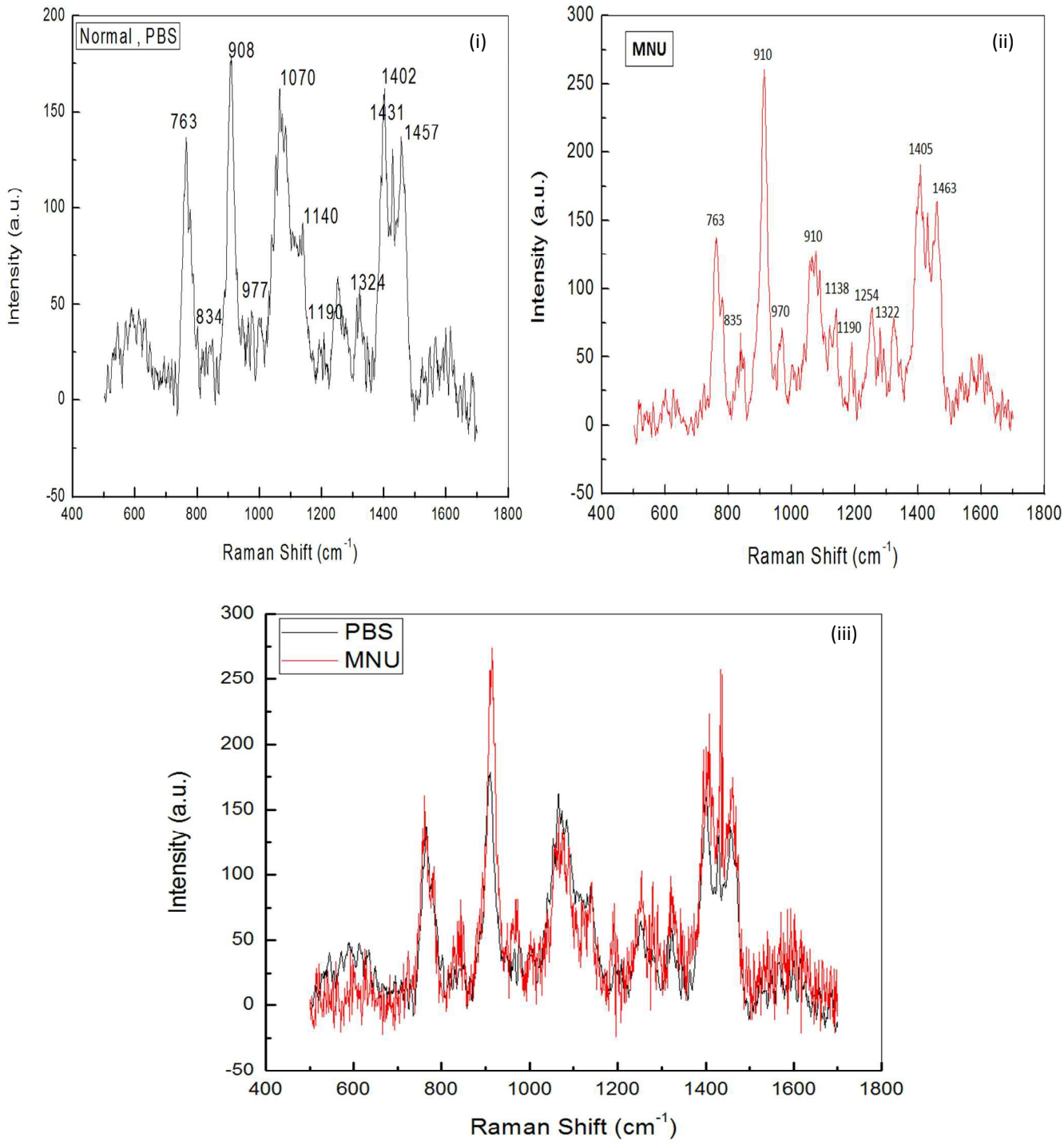


Figure 3: Raman spectra for Normal (PBS) and MNU treated mice, with comparison of Raman laser spectra of DNA in the region 600–1600 cm^{-1} (n=8).

Raman conditions and parameters: resolution cm^{-1}, laser power < 5 mW. Exciting source was 514.5 nm Argon ion laser.

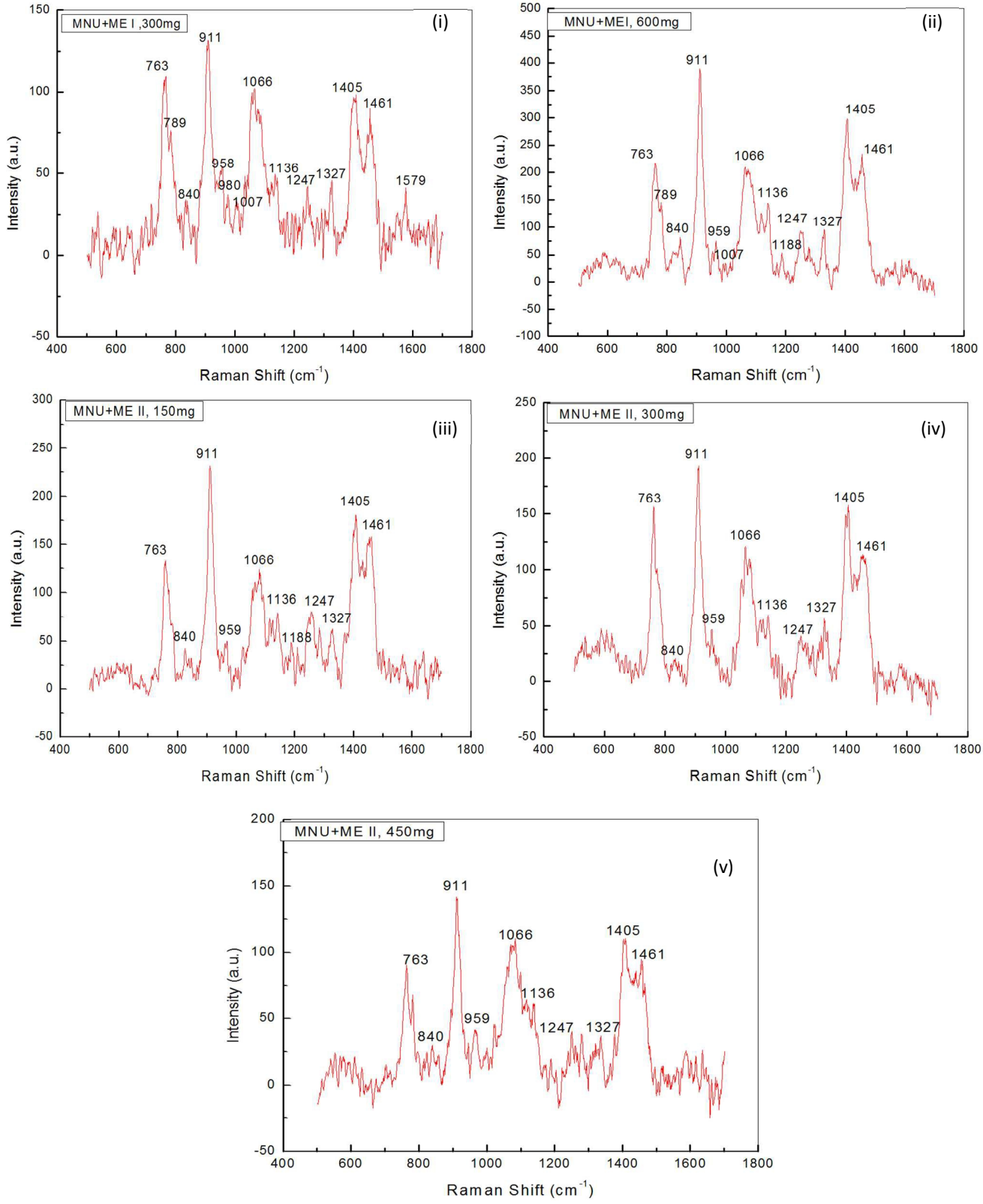


Figure 4: Raman spectra for Extract treated mice, with comparison of Raman laser spectra of DNA in the region 600–1600 cm^{-1} (n=8).

Raman conditions and parameters: resolution cm^{-1}, laser power < 5 mW. Exciting source was 514.5 nm Argon ion laser.

Table 1: Liver/Body weight ratio for the 12 treatments groups after sacrifice at the end of 3 months supplementation period.

Treatment Groups	Liver Weight (g)	Body Weight (g)	Liver/Body weight Ratio (%)
I-Normal (PBS only)	1.359 ^{j} ± 0.019	38.0 ^{a} ± 2.8	3.6 ^{d} ± 0.3
II-MNU only	1.634 ^{a} ± 0.005	16.6 ^{d} ± 2.8	10.1 ^{a} ± 2.0
III-ME1 (150 mg/Kg) + MNU	1.544 ^{c} ± 0.005	31.6 ^{b} ± 2.7	4.9 ^{c} ± 0.5
IV-ME$_1$ (300 mg/Kg) + MNU	1.452 ^{g} ± 0.002	25.8 ^{c} ± 2.9	5.7 ^{b} ± 0.6
V-ME$_1$ (450 mg/Kg) + MNU	1.494 ^{f} ± 0.005	29.8 ^{c} ± 4.8	5.1 ^{c} ± 0.8
VI-ME$_1$ (600 mg/Kg) + MNU	1.553 ^{b} ± 0.002	28.5 ^{c} ± 2.9	5.5 ^{b} ± 0.5
VII-ME$_1$ only (600 mg/kg)	1.323 ^{l} ± 0.009	31.7 ^{b} ± 2.8	4.2 ^{c} ± 0.4
VIII-ME$_2$ (150 mg/Kg) + MNU	1.533 ^{d} ± 0.002	30.4 ^{c} ± 0.9	5.0 ^{c} ± 0.2
IX-ME$_2$ (300 mg/Kg) + MNU	1.397 ^{h} ± 0.005	36.8 ^{a} ± 3.0	3.8 ^{d} ± 0.3
X-ME$_2$ (450 mg/Kg) + MNU	1.374 ^{i} ± 0.003	33.7 ^{b} ± 2.1	4.1 ^{c} ± 0.2
XI-ME$_2$ (600 mg/Kg) + MNU	1.511 ^{e} ± 0.008	33.5 ^{b} ± 2.7	4.5 ^{c} ± 0.4
XII-ME$_2$ only (600 mg/kg)	1.340 ^{k} ± 0.001	38.0 ^{a} ± 1.1	3.5 ^{d} ± 0.1

Data expressed as mean ± standard deviation (n=5); ANOVA and Fisher's LSD were carried out at 5% significance; Similar superscripts within the columns represent no significant differences between the treatment groups.

Table 2: Protein concentration of mice serum samples and Liver tissue homogenate

Treatment Groups (n=5)	**Serum Protein Concentration (µg Protein/ml)**	**Liver Tissue Homogenate Protein Concentration (µg Protein/ml)**
I-Normal (PBS only)	21.5 ^{a,b} ± 1.6	9.0 ^{c} ± 0.1
II-MNU only	19.0 ^{c} ± 1.1	5.7 ^{g} ± 0.1
III-MNU + ME_1 (150mg/kg)	20.0 ^{b} ± 0.5	8.5 ^{d} ± 0.2
IV-MNU + ME_1 (300mg/kg)	20.9 ^{b} ± 1.9	4.9 ^{h} ± 0.2
V-MNU + ME_1 (450mg/kg)	21.4 ^{b} ± 0.9	10.0 ^{a} ± 0.2
VI-MNU + ME_1 (600mg/kg)	20.9 ^{b} ± 2.8	7.6 ^{f} ± 0.2
VII-ME_1 only (600mg/kg)	19.8 ^{b} ± 1.9	9.3 ^{b} ± 0.1
VIII-MNU + ME_2 (150mg/kg)	22.5 ^{a} ± 1.1	3.8 ^{i} ± 0.1
IX-MNU + ME_2 (300mg/kg)	18.8 ^{c} ± 1.1	8.1 ^{e} ± 0.2
X-MNU + ME_2 (450mg/kg)	21.0 ^{b} ± 0.5	8.0 ^{e} ± 0.1
XI-MNU + ME_2 (600mg/kg)	20.8 ^{b} ± 1.0	9.2 ^{b} ± 0.2
XII-ME_2 only (600mg/kg)	23.2 ^{a} ± 0.4	9.4 ^{b} ± 0.2

Data expressed as mean ± standard deviation (error bars) (n=5); ANOVA and Fisher's LSD were carried out at 5% significance; Similar superscripts within the columns represent no significant differences between the treatment groups.

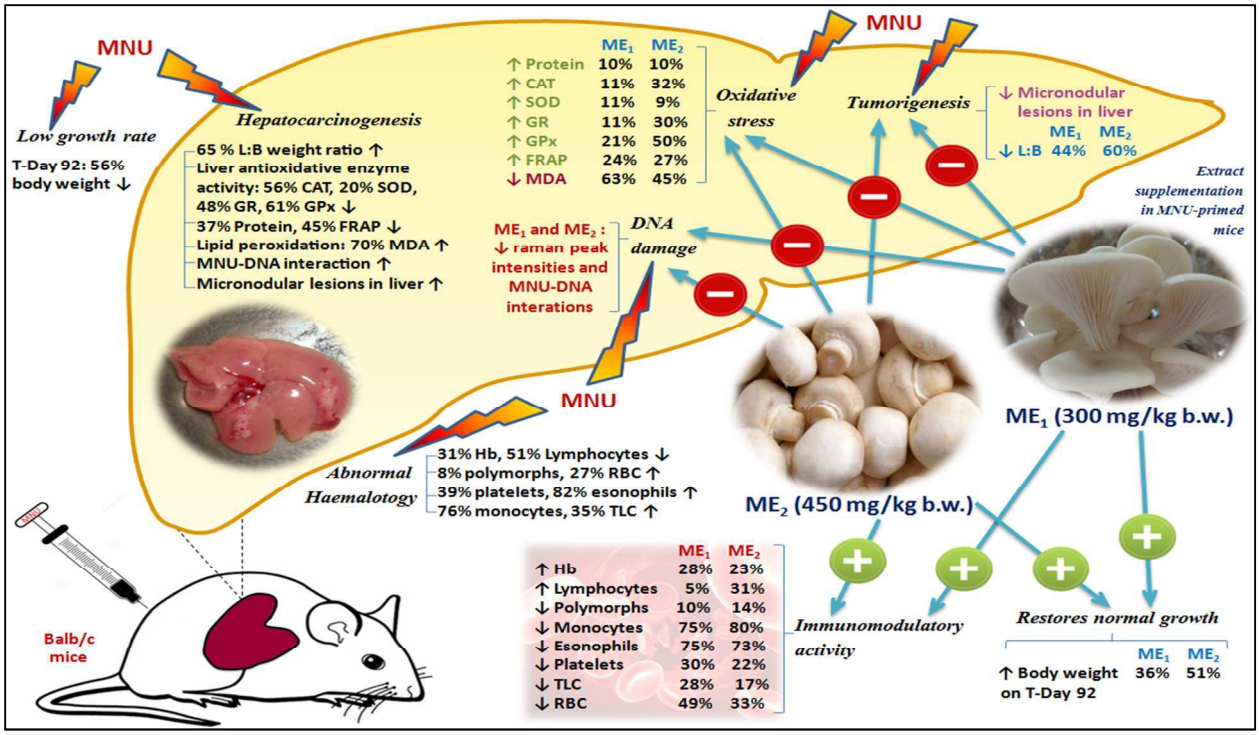
Table 3: Haematological analysis of mice blood samples

Treatment Groups (n=10)	Hb (g/dL)	TLC x 10^3 (cell/ cumm)	Polymorphs (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)	Basophils (%)	Platelets (lacs/ mm)	RBC (mill cells/ cumm)
I-Normal (PBS only)	13.0^{b} ± 0.8	8.6^{d} ± 0.7	67.6^{b} ± 3.3	41.6^{a} ± 3.0	2.4^{c} ± 1.2	2.9^{b} ± 1.1	0 ± 0.00	5.8^{c} ± 0.6	7.9^{b} ± 1.0
II-MNU only	9.0^{d} ± 0.7	13.2^{a} ± 0.6	73.5^{a} ± 4.3	20.8^{e} ± 2.6	13.4^{a} ± 1.3	12.2^{a} ± 1.1	0 ± 0.00	9.6^{a} ± 0.8	10.7^{a} ± 1.4
III-MNU + ME_1(150mg/ kg)	12.0^{b} ± 0.8	9.0^{d} ± 0.8	60.6^{c} ± 7.4	29.0^{c} ± 2.4	3.4^{b} ± 1.7	3.5^{b} ± 1.7	0 ± 0.00	6.3^{c} ± 1.3	6.5^{c} ± 1.5
IV-MNU + ME_1(300mg/ kg)	12.5^{b} ± 1.1	9.5^{c} ± 0.6	66.4^{b} ± 7.7	21.8^{e} ± 1.9	3.3^{b} ± 1.5	3.0^{b} ± 1.9	0 ± 0.00	6.7^{b} ± 1.1	5.5^{d} ± 1.1
V-MNU + ME_1(450mg/ kg)	12.1^{b} ± 1.6	8.8^{d} ± 0.8	62.0^{c} ± 8.9	21.5^{e} ± 2.3	3.5^{b} ± 1.8	3.2^{b} ± 1.3	0 ± 0.00	6.6^{b} ± 1.0	5.0^{d} ± 0.8
VI-MNU + ME_1(600mg/ kg)	12.5^{b} ± 1.6	9.7^{c} ± 1.0	67.8^{b} ± 8.5	22.1^{e} ± 2.3	3.4^{b} ± 1.6	2.5^{b} ± 1.3	0 ± 0.00	7.0^{b} ± 0.8	5.0^{d} ± 1.2
VII-ME_1 only (600mg/kg)	13.3^{a} ± 1.4	8.8^{d} ± 0.5	66.6^{b} ± 7.5	24.2^{e} ± 1.6	3.7^{b} ± 1.8	2.7^{b} ± 1.4	0 ± 0.00	6.4^{c} ± 0.9	5.8^{d} ± 1.1
VIII-MNU + ME_2(150mg/ kg)	11.3^{c} ± 1.0	10.6^{b} ± 1.0	66.0^{b} ± 1.4	26.8^{d} ± 3.0	2.8^{b} ± 1.3	2.3^{b} ± 1.1	0 ± 0.00	6.7^{b} ± 0.8	6.8^{c} ± 0.8
IX-MNU + ME_2(300mg/ kg)	10.9^{b} ± 1.0	10.4^{b} ± 1.0	72.2^{a} ± 5.1	26.2^{d} ± 6.9	3.1^{b} ± 1.7	2.9^{b} ± 1.0	0 ± 0.00	7.6^{b} ± 1.4	6.1^{c} ± 1.0
X-MNU + ME_2(450mg/ kg)	11.7^{c} ± 1.6	10.9^{b} ± 0.7	63.1^{c} ± 3.3	30.0^{c} ± 4.5	3.6^{b} ± 1.5	2.4^{b} ± 1.0	0 ± 0.00	7.4^{b} ± 0.6	7.2^{b} ± 0.7
XI-MNU + ME_2(600mg/ kg)	12.2^{b} ± 1.4	10.5^{b} ± 1.0	67.7^{b} ± 4.2	30.9^{c} ± 4.4	3.4^{b} ± 1.7	3.5^{b} ± 1.8	0 ± 0.00	7.6^{b} ± 1.1	7.8^{b} ± 1.0
XII-ME_2 only (600mg/ kg)	14.1^{a} ± 0.5	8.9^{d} ± 0.5	68.3^{a} ± 3.2	38.1^{b} ± 4.3	3.9^{b} ± 1.7	3.3^{b} ± 1.3	0 ± 0.00	7.0^{b} ± 1.0	6.1^{c} ± 0.7

Data expressed as mean ± standard deviation (error bars) (n=10); ANOVA and Fisher's LSD were carried out at 5% significance; Similar superscripts within the columns represent no significant differences between the treatment groups.

Table 4: Comparison of experimental liver DNA peak values in Raman spectra of control, MNU and extract treated mice, with reported literature peak values in Raman spectra of DNA.

	Raman spectra peak as per our experimental DNA sample and their assignments as per observations		Raman spectra peaks and their assignments as per earlier reports and references	
Region of peak position (cm^{-1}) in Raman spectra.	**Peak position (cm^{-1}) in Raman spectra.**	**Peak assignments for Raman spectra of liver DNA.**	**Literature Reference of Peak assignments in DNA Raman spectra**	**Literature value for the reported Peak position in DNA Raman spectra**
Region-1 (740 – 900)	763	O-P-O stretching	Prescott *et al.* (1984) Duguid *et al.* (1996)	807
	840	Phosphodiester stretching	Prescott *et al.* (1984) Duguid *et al.* (1996)	838
Region-2 (900-1040)	911	Deoxyribose	Ke *et al.* (1999)	920
	959	Deoxyribose	Ke *et al.* (1999)	956
	1007	Deoxyribose	Sipro (1987)	1012
Region -3 (1080-1190)	1066	Deoxyribose–phosphate DNA backbone	Ke *et al.* (1999)	1153
	1136		Gao *et al.* (2005)	1167
Region -4 (1190- 1280)	1247	O-P-O stretching	Guan and Thomas (1996)	1220
	1254	Adenine, Cytosine	Ke *et al.* (1999); Ruiz-chica *et al.* (2006); Verma *et al.* (2013)	1252
				1256
Region -5 (1280-1400)	1327	Adenine, Guanine	Ruiz-chica *et al.* (2006)	1339
Region-6 (1400-1525)	1405	Deoxyribose	Ruiz-chica *et al.* (2006)	1422
	1461	Deoxyribose	Ke *et al.* (1999)	1462



MNU: *N-methyl*-N-*nitrosourea;* L:B-Liver:Body weight ratio; b.w.: body weight; CAT: Catalase; SOD: Superoxide dismutase; GR: Glutathione reductase; GPx: Glutathione peroxidase; MDA: Malondialdehyde; FRAP: Ferric reducing antioxidant power; Hb: Haemoglobin; RBC: Red blood cell count; TLC: Total leukocyte count; T-Day 92: Last day of treatment-Day 92.

Biochemical, haematological and biophysical parameters assessed in this study are highly demonstrative that *Pleurotus sajor-caju* and *Agaricus bisporus* mushrooms are liable to modulate hepatocarcinogenesis in Balb/c mice.