Fermented papaya preparation modulates the progression of N-methyl-N-nitrosourea induced hepatocellular carcinoma in Balb/c mice

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A R T I C L E   I N F O

A B S T R A C T

Aim and main method: The medicinal properties of fermented papaya preparation (FPP) derived from Carica papaya fruit was investigated in order to determine its ability to modulate the progression of N-methyl-N-nitrosourea induced hepatocellular carcinoma in Balb/c mice.

Key findings: As well as reducing the physical symptoms associated with N-methyl-N-nitrosourea (MNU)-induced hepatocellular carcinoma, supplementation of Balb/c mice with 500 mg FPP/kg BW for 92 days normalized the blood cell count, led to an increased activity of several key antioxidant enzymes (SOD: +20%, CAT: +81%, GPx: +66.1%, GR: +54.4%; P < 0.001 vs. MNU control), increased the ferrous reducing antioxidant potential (+36.7%, P < 0.001 vs. MNU control) and reduced the extent of lipid peroxidation in the liver by 44.3% (P < 0.001 vs. MNU control).

Significance: Results demonstrated the ability of FPP to preserve the integrity of liver against oxidative damage and protect hepatocytes against irreversible DNA structural modifications induced by MNU, highlighting its potential role as an immune-defense modulator during hepatocarcinoma.

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1. Introduction

Mutagens present in commonly consumed food items are an important, yet unrecognized exogenous source of carcinogens [1,2]. Some groups of carcinogens receiving much attention are N-nitrosoamines, N-nitrosamines and alkyl-nitrosoureas. A critical survey of popular household food items have revealed significant levels of several N-nitroso compounds namely: nitrates, N-nitrosodimethylamine-2-hydroxyethylamine-4-carboxylic acid, N-nitrosos-2-hydroxymethylthiazolidine-4-carboxylic acid and N-nitrosodimethylamine to be present in commercially packed sausages, cured luncheon meat, processed cheese, beer and soy sauce [2,3,4]. Moderate quantities of N-methyl-N-nitrosourea (MNU) have also been detected in Chinese white cabbage, smoked fish, Japanese pickled vegetables, Chinese-style fermented fish sauce, shrimp and sardines amongst other seafoods [5,6,7]. Although limited evidence link the intake of Chinese style foods to human cancers, there are suggestions that sustained consumption may lead to a heightened risk of developing nasopharyngeal and stomach carcinoma as well as chronic gastritis [8,9] in Eastern Asia. In coastal areas of China, Japan or Korea where fish and fermented based products form the basis of their diets, these countries appear to have the highest incidence of such cancers, an association that is widely thought to be partially due to the level of nitrosamides which are sufficiently higher in Chinese-style salted fish than any other types of fish [7].

Metabolic breakdown of nitrates by gut microflora release health-threatening nitrosoureas that trigger release of excessive quantities of reactive oxygen/nitrogen species (ROS/RNS) and pro-inflammatory cytokines creating a state of oxidative stress within tissues [11]. Excessive free radicals not only direct damage toward cellular lipids and DNA, but also participate in post-translational modifications of proteins or enzymes involved in carcinogenesis (e.g. activate oncogene products, angiogenesis and inactivate DNA repair genes, tumor-suppressor proteins and pro-apoptotic caspses [12,13]). That oxidative stress can seriously weaken the endogenous antioxidant system has been the
center of on-going discussions amongst the medical community and has evoked many human intervention trials focusing on the effectiveness of dietary antioxidants to reverse the progression and occurrence of several diseases, including cancers. The involvement of ROS-dependent mito-
genic and anti-apoptosis signaling pathways within the cancer microenvironment represents a specific vulnerability that can be selectively targeted by those antioxidants which can modulate the activity of antioxidant enzymes and activate DNA repair genes [14]. Carica papaya has folkloric use in the management of diseases governed by oxidative stress, notably cancer [15,16,17]. Fermented papaya preparation (FPP, Immun’Age®) is a promising source of dietary antioxidants [18,19]. FPP is an ISO 9002 and 14001 certified dietary supplement that is made from the yeast fermentation of ripe C. papaya pulp, its nutritional composition is detailed in Table 1. The anti-inflammatory and immune boosting effects of FPP synergistically modulate the activity of endoge-
nous antioxidant enzymes [20,21]. The antioxidant activity of FPP can also be ascribed to its hydroxyl scavenging and iron chelating prop-
ties. FPP has also been reported to modulate oxidative damage via mito-
gen activated protein kinases through the attenuation of Akt and p38 activation in H2O2-exposed pheochromocytoma (PC12) cells [22,23]. An extensive collection of scientific studies have appraised FPP to be a functional food with a potent potential to be used as an anti-
oxidant. As portrayed in the review of Nguyen et al. [16], FPP is now gaining recognition as a promising anti-cancer agent; however data pertaining to its hepatoprotective role remains limited. Although C. papaya is grown abundantly in tropical countries, the medicinal value of its fruit is greatly underestimated by the modern generation. In this context, the ultimate goal of this study was to assess the anti-
cancer efficacy of FPP against MNU-induced hepatocarcinoma.

2. Methodology

2.1. Experimental design

Sixty Balb/c mice of same age (7 weeks) and weight (20 ± 2 g) were selected and housed under standard conditions (20 ± 2 °C; 65 ± 15% relative humidity). After an acclimatization period of 3 weeks, the animals were randomly assigned to 6 groups. Group I (control) were given only phosphate buffer saline (PBS), while Group II were injected with MNU at a concentration of 50 mg/kg body weight (BW). Groups III to VI were simultaneously given MNU (50 mg/kg BW) and FPP (Immun’Age®, Osato Research Institute, Japan) at a dose of 300 to 1000 mg/kg FPP/kg BW for a period of 92 days (Table 2). MNU was admin-
istered intraperitoneally, while a cannula was used to release the dissolved FPP into the oesophagus of the animal. A cannula was used to release the dissolved FPP into the oesophagus of the animal. All mice were routinely monitored for any signs and symptoms of ill health and body weight re-
corded. At the end of the supplementation period, blood samples were collected via retro sinus bleeding without the use of anti-coagulant and centrifuged at 5000 rpm for 10 min to separate serum. Mice were then sacrificed and complete autopsies performed on liver samples. A 10% liver tissue homogenate was prepared in cold buffer (50 mM phosphate buffer and 1 mM EDTA, pH 7.5) and centrifuged (10,000 rpm, 4 °C) for 10 min. All biochemical assays were carried out using serum and liver tissue homogenate. The study protocol was carried out in ac-
cordance to the rules and regulations of the Chhatrapati Shahuji Maharaj University (Kanpur, India). Ethical clearance was obtained from the University Ethical Committee (Ethical Clearance Reference: 1589/PO/a/12/CPSEA).

2.2. Histopathology of liver tissues

Liver samples were washed, blotted dry, fixed in 10% buffered for-
malin solution and dehydrated with stratified ethanol solution from 50% to 100% for the preparation of paraffin blocks. Sections of 4 μm in thickness were cut and stained with haematoxylin and then counter-
stained with eosin. The histopathological slides were observed and doc-
umented by light photomicroscope at a magnification X20.

2.3. Hematological analysis

Prior to cervical dislocation of mice, fasting blood was dispensed into EDTA-K2 tubes (Sarstedt, Germany) for hematological studies. The red blood cell, platelet count and hemoglobin concentration was deter-
mined within whole blood. Also, cell counts of lymphocytes, neutro-
phils, monocytes and eosinophils were recorded.

2.4. Ferrous reducing antioxidant potential (FRAP)

An optimized variant of the Benzie and Strain method [24] was used to estimate the FRAP levels of liver and serum homogenate samples. Re-
ducing power of liver/serum homogenate was expressed as mM Fe (II)/
ml/mg protein.

2.5. Malondialdehyde (MDA) level

Extent of lipid peroxidation in terms of malondialdehyde (MDA) for-
mation was estimated as per the modified method of Satoh [25]; MDA levels were expressed as mM thiobarbituric acid substances (TBARS)/
ml/mg protein.

2.6. Assessment of the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR)

Colorimetric assay of catalase was carried out using an adapted method of Sinha [26]. CAT activity was expressed as mM H2O2/min/
mg protein. SOD activity was estimated according to the methods of Mishra and Fridovich [27] and Poliodoro et al. [28]. Specific activity of
SOD was expressed as units per ml enzyme/mg protein. Glutathione peroxidase activity was estimated according to the method essentially described by Hafeman et al. [29]. GPX activity was expressed as μM/min/mg protein. Glutathione reductase activity was measured according to the method of Carlberg and Mannervik [30]. GR activity was expressed as nm NADPH oxidized/min/mg protein.

2.7. Raman analysis

DNA from the liver samples of all the experimental mice was isolated using a standard phenol-chloroform protocol (Verma et al. [31]). DNA spectra for all four experimental groups were recorded on a micro-Raman setup (Renishaw, UK) equipped with a grating of 1800 lines/mm and a peltier cooled CCD. The GRAM-32 software was used for data collection. The accumulation time for one window was 60 s and 3 spectra were accumulated in each window. In one window, approximately 800 cm⁻¹ regions were covered. The 514.5 nm wavelength line of Argon ion laser was used as an excitation source for DNA samples.

2.8. Data analysis

Results are expressed as mean ± standard deviation of 5 replicates, where values in brackets ( ) represent percentage change and error bars represent standard deviation. Student’s paired t-test was used to compare the mean values of two samples. Differences were considered significant when value of P < 0.05 (two-tailed). Raman spectra data was saved as .spc extension compatible with Spectra Calc and Excel software. Origin 8 (v8.0951) was used for generating the spectra for the 6 different treatment groups.

3. Results

3.1. Body weight and physical characteristics

All animals experienced an increase in body weight during the first 2 weeks of treatment. After intraperitoneal administration of MNU, prominent decreases in body weight were observed as from day 22 (Fig. 1A). At the end of the experimentation period, body weight of Group II decreased by 15.2% compared to its initial weight. In contrast to Group I (Fig. 1B), mice from Group II demonstrated many characteristics associated with aggressive carcinoma such as decreased alertness, shedding of hair, difficulty in breathing and a loss of appetite. Red swollen inflamed growths were also apparent on the abdominal area (Fig. 1C). While mice in Group I remained healthy, those which were supplemented with FPP had less apparent symptoms of ill health. At the end of the study, significant increases in body weight of 71.6% (Group III), 81.7% (Group IV), 77% (Group V) and 65.8% (Group VI) were recorded in groups supplemented with 300, 500, 700 and 1000 mg FPP/kg respectively (vs. Group II MNU control). An autopsy of livers from MNU-treated mice revealed them to be discolored with several micronodular lesions (Fig. 1E); whereas in FPP-supplemented mice, fewer micronodular lesions were observed and the dark red coloration was restored. Panel F of Fig. 1 shows the effect of FPP supplementation on tumor burden. Expressed as percentage ratio of liver to whole body weight, the tumor burden of Group II was 10.1% (vs. 3.6% Group I), whereas that of Groups III to VI ranged between 4.0 and 5.0%, representing a 50.7 to 61% decrease. Greatest decrease was recorded in Group IV mice (P < 0.01 vs. Group II).

3.2. Histopathology of liver tissues

Fig. 2 clearly shows changes in liver morphology after 92 days of MNU treatment compared to control. Panels B to E of Fig. 2 reveal hepatocytes to be enlarged, vacuolated and altered in cell structure in all MNU exposed mice. No alterations were observed in PBS control group (Fig. 2A) but, in mice where FPP was supplemented, liver tissues showed vacuolated hepatocytes to a much lesser extent.

3.3. Hematological analysis

Erythrocytes were observed to significantly increase from 7.85 to 10.74 x 10⁶ cells/cm³ in Group II (P < 0.001 vs. PBS control). FPP intake normalized levels to 7.06–8.09 x 10⁶ cells/cm³. A significant decrease of 34.3% was noted in the group supplemented with 500 mg/kg (P < 0.001 vs. MNU control). However, hemoglobin levels were seriously compromised upon exposure to MNU as levels were observed to drop by 30.9% (P < 0.001 vs. PBS control) (Table 3). FPP treatment normalized erythrocyte and hemoglobin levels, especially in Group IV. As shown in Table 3, MNU greatly affected platelet cell count. Levels increased considerably from 5.81 to 9.55 lac/mm in mice treated with MNU only (P < 0.001 vs. PBS control). A 92-day supplementation with FPP brought levels down to 5.85–7.12 lac/mm. Greatest decreases were recorded in Group IV, where levels dropped by 38.7% (P < 0.001 vs. MNU control). Experimental mice also demonstrated a significantly altered white blood cell profile (total leukocyte count + 53.4% vs. PBS control, P < 0.001). Administration of MNU caused the lymphocyte count to drop 2-fold, whereas monocyte and eosinophil levels multiplied by 4.2– 5.6-fold respectively compared to untreated mice (Table 3).

3.4. Ferric reducing antioxidant potential activity

Compared to the PBS control, the overall antioxidant potential in terms of the Fe³⁺ reducing capacity of liver/serum homogenates from MNU-exposed mice was greatly compromised. The FRAP value for Group II control mice was 151.86 mM (vs. 277.51 mM Fe(II)/ml/mg protein in PBS control) and 399.2 mM/ml (vs. 803.45 mmol Fe(II)/mg protein in PBS control) for liver and serum samples respectively, representing a loss of 45.3% and 50.3% compared to healthy controls of Group I. As shown in Fig. 3, a dose of 500 mg FPP/kg had the greatest influence on FRAP activity (liver: 188.47 mM Fe(II)/ml/mg protein; serum: 772.42 mM Fe(II)/ml/mg protein).

3.5. Malondialdehyde levels

Using MDA formation as an index of oxidative stress, the antioxidant activity of FPP measured as a function of its ability to reduce lipid peroxidation in MNU-exposed mice is illustrated in Fig. 4. Compared to Group I control mice, MDA levels peaked to 3.03 (liver) and 2.62 (serum) nM/ml/mg protein in PBS control) and 399.2 mM/ml in MNU-only treated mice (P < 0.001 vs. PBS control). Treatment of mice with varying doses of FPP caused MDA levels to decrease considerably, where minimum levels were recorded in Group IV (500 mg FPP/kg BW). At this particular dose, MDA dropped by 44.3 (P < 0.01) and 68% (P < 0.001) in the liver and serum respectively.

3.6. Activity of endogenous antioxidant enzymes

MNU exposure caused SOD activity to significantly drop by 20.3% (liver) and 52.4% (serum) compared to Group I healthy controls (P < 0.001) (Table 4). However, a significant improvement of + 20% (P < 0.01 vs. MNU control) and + 18.9% was noted in liver and serum.
homogenates of the 500 mg FPP/kg group ($P < 0.001$ vs. MNU control). Acute damage inflicted by MNU within liver tissues was suspected as CAT activity significantly reduced by 56.1% (liver) and 78.1% (serum) compared to MNU control. FPP had a modulating effect on CAT. A maximum of 372.71 mM H$_2$O$_2$/min/mg protein (liver; vs. 205.85 mM H$_2$O$_2$/min MNU control) and 232.07 mM H$_2$O$_2$/min/mg protein (serum; vs. 54 mM H$_2$O$_2$/min MNU control) was recorded in Group IV. Similarly, administration of MNU lead to the alteration in the activities of GPx ($-60.9\%$) and GR ($-47.8\%$) (Table 4) in Group II. FPP at an optimal dose of 500 mg/kg proved to restore redox balance within Balb/c mice, allowing regeneration of GPx and GR (Table 4).

3.7. Raman analysis

Spectral analysis of DNA for all experimental groups is presented in Panels (A) to (F) of Fig. 5. Intensities of all major peaks were compared to that of the controls and general differences noted within groups. Data were normalized to that of the PBS control to eliminate variations in DNA sampling. Major peak bands identified were 763, 835, 910, 970, 1090, 1138, 1190, 1254, 1322, 1405 and 1463 cm$^{-1}$. From Fig. 5D, the spectral data of Group IV bears similar resemblance to that of Group I PBS control, whereas the peak intensities of Group II MNU control were much greater. Compared to Group II, a pronounced decline in intensity was noted at 763 cm$^{-1}$ in Group IV mice: as levels dropped by almost 1.4-fold. At 835 cm$^{-1}$ peak intensity was amplified 2.4-fold by MNU exposure. Treatment of mice with various doses of FPP greatly reduced the magnitude by approximately 12.5–57.9%, notably in amongst mice of Group IV. Elevated intensities recorded at bands 970 cm$^{-1}$ and 1463 cm$^{-1}$ in the MNU control group indicate biophysical alterations in the structure of deoxyribose (Fig. 5B). FPP (500 mg/kg BW) could suppress intensity elevation by 37.4%. In the MNU control group, this peak dropped by 21.6% (vs. PBS control). Treatment with FPP generally caused peak intensity at 1090 cm$^{-1}$ to normalize to 155–194 a.u. (vs. 164 a.u. in Group I PBS control). Elevated peaks were also recorded at 1190, 1254, 1322, 1405 and 1463 cm$^{-1}$ in MNU-primed mice, however values were much lower in those co-treated with FPP.

4. Discussion and conclusion

In contrast with data from Group II, FPP-supplemented mice clearly showed reduced symptoms associated with metastatic carcinoma. Reduced shedding of hair, improved alertness and a gain in both weight and appetite were the most evident improvements amongst those of the 500 mg $\text{kg}^{-1}$ kg BW group (Group IV). All H&E stained sections of MNU-treated liver had enlarged vacuolated hepatocytes, giving them a ‘leaky’ appearance. Similar observations were reported by Verma et al. [46,47] in Balb/c mice after a carcinogenic treatment of 40 days. The same authors observed marked fatty changes in addition to altered cell structure. In addition to a significant reduction in tumor burden in Group IV mice, the intake of FPP modulated the integrity of whole blood, including the hemoglobin concentrations, leukocyte and platelet counts in MNU-primed mice– an observation that is consistent with the reported immune-modulatory role of FPP [32]. The simultaneous rise in erythrocytes can be interpreted as an adaptive mechanism of the bone
Table 3

Hematological analysis of N-methyl-N-nitrosourea (MNU)-treated Balb/c mice after a 92-day supplementation with FPP.

<table>
<thead>
<tr>
<th></th>
<th>Group I PBS control</th>
<th>Group II MNU only</th>
<th>Group III MNU + 300 mg/kg BW</th>
<th>Group IV MNU + 700 mg/kg BW</th>
<th>Group V MNU + 1000 mg/kg BW</th>
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</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td></td>
<td></td>
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<tr>
<td>Erythrocytes (10^6 cells/cmm)</td>
<td>7.85 ± 0.99</td>
<td>10.74 ± 1.40**</td>
<td>7.24 ± 0.82</td>
<td>7.06 ± 0.63</td>
<td>7.06 ± 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+36.8%)</td>
<td>(-32.6%)</td>
<td>(-34.3%)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.04 ± 0.83</td>
<td>9.01 ± 0.67***</td>
<td>12.79 ± 1.79</td>
<td>12.17 ± 0.99</td>
<td>12.42 ± 1.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-30.9%)</td>
<td>(+41.9%)</td>
<td>(+37.8%)</td>
</tr>
<tr>
<td>Platelets (lac/mm)</td>
<td>5.81 ± 0.61</td>
<td>9.55 ± 0.82***</td>
<td>7.12 ± 1.21</td>
<td>5.85 ± 1.62</td>
<td>6.37 ± 1.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+64.5%)</td>
<td>(+25.5%)**</td>
<td>(+38.7%)</td>
</tr>
<tr>
<td>Total leukocytes (cells/cmm)</td>
<td>8620 ± 0.70</td>
<td>13,224 ± 623***</td>
<td>10,577 ± 2275</td>
<td>10,123 ± 1708</td>
<td>10,869 ± 1929</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-53.4%)</td>
<td>(-20%)</td>
<td>(-17.8%)</td>
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<tr>
<td>Leukocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lymphocytes (%)</td>
<td>41.9 ± 2.99</td>
<td>20.8 ± 2.62***</td>
<td>27.9 ± 3.51</td>
<td>30.5 ± 4.30</td>
<td>28.9 ± 5.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-50%)</td>
<td>(+34.1%)**</td>
<td>(+46.8%)**</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2.9 ± 1.10</td>
<td>12.2 ± 1.14***</td>
<td>2.9 ± 1.66</td>
<td>2.5 ± 1.35</td>
<td>2.9 ± 1.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+320.7%)</td>
<td>(-76.2%)**</td>
<td>(-79.5%)**</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>2.4 ± 1.17</td>
<td>13.4 ± 1.26***</td>
<td>3.1 ± 1.66</td>
<td>3.1 ± 1.79</td>
<td>3.5 ± 1.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+458.3%)</td>
<td>(+76.9%)**</td>
<td>(+73.9%)**</td>
</tr>
</tbody>
</table>

Data is expressed as mean cell count ± standard deviation of 5 replicates; percentage changes to control are indicated in brackets ( ).

** P < 0.01.
*** P < 0.001 vs. MNU-only control.
**** P < 0.001 vs. PBS control.

 marrow in attempt to compensate for the state of anemia and premature cell death of erythrocytes brought upon by MNU exposure. Reversal of these changes upon FPP supplementation suggests that FPP can protect the integrity of erythrocyte membranes against oxidative stress-induced hemolysis [33,34]. Positive correlations between platelet count and expression of pro-inflammatory cytokines (IL-6, IL-1β), anti-apoptosis (Bcl-2) and tumor promoter genes (p53) have been detected in hepatocellular carcinoma [31], thus can be collectively used as markers for cancer prognosis. Active platelets usually congregate at sites of damaged blood vessels. That platelet count was exceptionally high (+64.4% vs. PBS control) in Group II control mice suggests the formation of metastatic lesions within the liver, as evidenced by the appearance of red, swollen and inflamed growths apparent on the abdominal area. Experimental mice also demonstrated a significantly altered white blood cell profile (total leukocyte count +53.4% vs. PBS control, P < 0.001). Abnormal counts of lymphocytes, monocytes and eosinophils are typical characteristics associated with aggressively growing tumors and chronic oxidative-inflammation.

Oxidative stress (as a consequence of defective antioxidant defense mechanisms) plays a critical role in the pathogenesis of toxic liver diseases has been consistently linked to a number of diseases, including cancer [35,36,37]. The inactivation of key antioxidant enzymes such as SOD, CAT, GPx and GR by MNU is one of the major contributory factors to explain the cytotoxic levels of MDA (malondialdehyde) in the liver and serum of experimental mice. MDA can thus be considered an indirect tumor promoter and co-carcinogenic agent. A previous study by Marot et al. [51] showed that a total of 9 g FPP/day for 6 months could lower serum MDA levels of patients by 1.8-fold [51]. A similar trend was also reported by Imao et al. [38].

Defective SOD expression has been experimentally proven in cancer states induced by mutagens such as CCl4, γ-radiation, acrylamide and H2O2 [39,40,41,42]. According to findings of Suman et al. [35] excessive levels of circulating MDA can form cross links with SOD, distorting its active binding site. It is interesting that Korkindel et al. [43] have suggested that FPP could boost the antioxidant status of cancer patients undergoing radiotherapy by stimulating over expression of Mn-SOD. Catalase levels have been found to be depressed during advanced stages of cancer [44,45]. Acute inflammation within liver tissues onset by MNU may also create an unfavorable environment, whereupon catalase may become inactivated. Consumption of 6 g FPP/day for a period of 4 weeks boosted the CAT gene expression (P < 0.05) and the effect is thought to be a likely outcome of the interaction of FPP with post transcriptional or translational protein modifications related to CAT synthesis [21]. The close relationship between the reduction in activity of GPx and GR in the liver indicates that these enzymes were rapidly depleted upon accumulation of MNU within liver tissues. The proactive role of
such enzymes in the neutralization of lipid peroxides or other related radicals is evidenced by a simultaneous drop in MDA levels in Groups III—VI. The current data reported is in accordance with the unequivocal notion that papaya peel, pulp, seed and FPP normalizes integrity of the antioxidant defense enzymes SOD, CAT and GPx [40,42,46]. Thus, FPP can be used as a dietary supplement for the management of oxidative stress and inflammation injury such as that arises during hepatocellular carcinoma.

Raman laser spectroscopy was utilized to detect any structural alterations to DNA. As shown in Fig. 5, a substantial peak was apparent at 910 cm$^{-1}$ in Groups II, III, V and VI, which is thought to correspond to asymmetrical stretching of phosphodiester bonds in DNA [48,49]. According to Chan et al. [48] and Verma et al. [31,46], any changes detected between the region of 1190 and 1600 cm$^{-1}$ can be interpreted as conformational changes to the structures of purines and pyrimidines. Treatment of Balb/c mice with FPP could reduce the intensity of all peaks, especially at a dose of 500 mg FPP/kg BW (Group IV), providing firsthand evidence of its ability to protect liver DNA against genotoxin-induced structural modifications. This is the first study to demonstrate the potential anti-carcinogenic properties of FPP against MNU using Balb/c mice as an experimental model of hepatocarcinoma. Genotoxins like MNU, benzo(a)pyrene, Fe-NTA and H$_2$O$_2$ attack DNA and distort its stability through two basic pathways either by reaction with a DNA nucleophile or electrophile or by reaction with the pi (π) or C—H bonds located within nucleotides [48]. In the case of H$_2$O$_2$-derived radicals, FPP has been extensively studied for its successful anti-radical activity. It is thought that FPP is able to divert hydroxyl radicals away from the π bonds of C$_3$—C$_5$ pyrimidines and N$_7$ or C$_4$—C$_6$ bonds of purines- thus protecting the vulnerable areas of DNA from any major structural alterations [23,50,51]. Nucleotide 8-hydroxy-

| Table 4 |

| Activity of endogenous antioxidant enzymes SOD, CAT, GPx and GR in N-methyl-N-nitrosourea (MNU)-treated Balb/c mice after a 92-day supplementation with FPP. |

<table>
<thead>
<tr>
<th>SOD (U/ml/mg protein)</th>
<th>Group I PBS control</th>
<th>Group II MNU only</th>
<th>Group III MNU + 300 mg/kg BW</th>
<th>Group IV MNU + 500 mg/kg BW</th>
<th>Group V MNU + 700 mg/kg BW</th>
<th>Group VI MNU + 1000 mg/kg BW</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>165.49 ± 4.07</td>
<td>131.90 ± 4.38</td>
<td>156.91 ± 4.38 (+19%)***</td>
<td>158.34 ± 4.07 (+20%)***</td>
<td>154.06 ± 6.87 (+16.8%)***</td>
<td>149.77 ± 4.38 (+13.5%)***</td>
</tr>
<tr>
<td>Serum</td>
<td>95.45 ± 4.87</td>
<td>45.44 ± 8.53</td>
<td>80.45 ± 4.79 (+286.1%)***</td>
<td>90.46 ± 4.07 (+196.5%)***</td>
<td>76.16 ± 8.23 (+67.6%)***</td>
<td>90.46 ± 4.07 (+34.6%)***</td>
</tr>
<tr>
<td>CAT (nmH$_2$O$_2$/min/mg protein)</td>
<td>469.43 ± 5.80</td>
<td>205.85 ± 3.63</td>
<td>313.82 ± 6.72 (+52.5%)***</td>
<td>372.71 ± 9.20 (+81.1%)***</td>
<td>351.18 ± 5.48 (+70.6%)***</td>
<td>286.32 ± 8.55 (+39.1%)***</td>
</tr>
<tr>
<td>Liver</td>
<td>246.48 ± 15.40</td>
<td>54 ± 7.21</td>
<td>208.51 ± 11.61 (+329.7%)***</td>
<td>232.07 ± 15.89 (+246.9%)***</td>
<td>187.33 ± 9.88 (+196.5%)***</td>
<td>160.10 ± 5.74 (+196.5%)***</td>
</tr>
<tr>
<td>Serum</td>
<td>1169.55 ± 61.66</td>
<td>54 ± 7.21</td>
<td>208.51 ± 11.61 (+329.7%)***</td>
<td>232.07 ± 15.89 (+246.9%)***</td>
<td>187.33 ± 9.88 (+196.5%)***</td>
<td>160.10 ± 5.74 (+196.5%)***</td>
</tr>
<tr>
<td>GPx (nm/ml/mg protein)</td>
<td>1863.04 ± 97.96</td>
<td>885.30 ± 99.53</td>
<td>1530.22 ± 83.15 (+69.8%)***</td>
<td>1704.73 ± 65.48 (+92.6%)***</td>
<td>1245.08 ± 102.53 (+40.6%)***</td>
<td>1107.56 ± 91.95 (+25.1%)***</td>
</tr>
<tr>
<td>Liver</td>
<td>13572.65 ± 114.81</td>
<td>1865.69 ± 14.06</td>
<td>2753.67 ± 90.63 (+47.6%)***</td>
<td>2880.69 ± 57.85 (+54.4%)***</td>
<td>2225.71 ± 44.70 (+19.3%)***</td>
<td>2902.02 ± 94.17 (+55.5%)***</td>
</tr>
<tr>
<td>Serum</td>
<td>3223.73 ± 170.31</td>
<td>1658.89 ± 144.03</td>
<td>1671.19 ± 36.78 (+0.7%)</td>
<td>1826.67 ± 29.90 (+10%)</td>
<td>1669.71 ± 35.26 (+6.5%)</td>
<td>1767.51 ± 24.89 (+6.5%)</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± standard deviation of 5 replicates. Percentage change to control is indicated in brackets ( ). ***P < 0.001 vs. Group I PBS control; **P < 0.01 and *P < 0.001 vs. Group II MNU control. SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GR: glutathione reductase.

Fig. 4. Malondialdehyde (MDA) levels in FPP-supplemented Balb/c mice treated with or without N-methyl-N-nitrosourea (MNU). Data is presented as the mean of 5 replicates where error bars represent ± standard deviation. ***P < 0.001 vs. Group I PBS control; **P < 0.01 and *P < 0.001 vs. Group II MNU control.
Deoxyguanosine (8-OHdG) is a common biomarker used to quantify oxidative damage to DNA [50]. Circulating levels of 8-OHdG were reported to be abnormally high in smokers and those suffering from chronic cirrhosis [51], an oral supplementation of FPP (9 g FPP/day for 3 months) was reported to decrease 8-OHdG, demonstrating its antioxidant efficacy to surpass that of vitamin E [34,51]. Although many studies have claimed secondary metabolites such as polyphenolics to be major contributors to the antioxidant activity of *C. papaya* [42,52], the exact source of FPP’s bioactivity still remains undetermined. However, an alteration in ratio of its free amino acids, the appearance of novel oligosaccharides and its elevated vitamin B levels are highly suspected to have boosted the functional qualities of FPP [53]. The elucidation of the exact mechanism of action of FPP in the cancer microenvironment may have a nutrigenomic implication, thus warrants further investigation.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>8-OHdG</td>
<td>8-oxo-2′-deoxyguanosine</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CAT</td>
<td>catalase</td>
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<tr>
<td>CCD</td>
<td>charge coupled device</td>
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<tr>
<td>CCl₄</td>
<td>carbon tetrachloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPP</td>
<td>fermented papaya preparation</td>
</tr>
<tr>
<td>FRAP</td>
<td>ferrous reducing antioxidant potential</td>
</tr>
<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hemoxynin and eosin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneal injection</td>
</tr>
<tr>
<td>ISO</td>
<td>international organization for standardization</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NTA</td>
<td>nitritolactric acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
</tbody>
</table>

rpm rounds per minute  
SOD superoxide dismutase  
TBARS thiobarbituric acid reactive substances

**Contributor statements**

J.S., S.R., S.V. and T.B. designed research; J.S., S.R., S.V., P.S. conducted research; J.S. analyzed data and wrote the paper. R.K.S. supervised the biophysical work. T.B., O.I.A., E.B. and A.K. had the primary responsibility for final content. All authors read and approved the final manuscript.

**Conflict of interest**

Okezie I. Aruoma is actively involved in biomedical research involving fermented papaya preparation with the Osato Research Institute, Gifu, Japan. All authors declare that there are no conflicts of interest.

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**References**


