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Full length Research Article

Protocatechuic Acid Modulates Hepatic Oxidative Stress and Inflammation Linked to Dimethyl Nitrosamine Exposure in Rat

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Summary: Dimethyl nitrosamine (DMN), a potent hepatotoxin, exerts carcinogenic effects and induces hepatic necrosis in experimental animals via CYP2E1 metabolic activation, and generation of reactive oxygen species (ROS). Protocatechuic acid (PCA), a plant-based simple phenolic compound and potent antioxidant, has been shown to affect the development of neoplasia in the rat liver and inhibit the initiation or progression phases of most cancers. In this study, the modulatory effects of PCA on DMN-induced hepatotoxicity, oxidative stress, inflammation, and selected phase I xenobiotic metabolizing enzymes were investigated in male Wistar rats. This study assessed biomarkers of hepatic injury (alanine transaminase, aspartate aminotransferase, alkaline phosphatase, and gamma- glutamyl transferase); oxidative stress (hydrogen peroxide concentration, lipid peroxidation, and reduced glutathione levels); measured activities of antioxidant enzymes (catalase, sodium dismutase, glutathione peroxidase, glutathione S-transferase); and inflammation (Tumor necrosis factor (TNF)-α, interleukin-1-Beta (IL-1β) and iNOS). The results of our investigation demonstrated that pretreatment with PCA at 50 and 100 mg/kg body weight p.o. reduced DMN (20 mg/kg bw) i.p. mediated hepatic injury, oxidative stress, and inflammation in a dose-dependent manner. In addition, the activities of phase I metabolizing enzymes were significantly induced except for aminopyrine-N-demethylase in the DMN-treated rats when compared with the DMN alone control group. This induction was also reversed by pre-treatment with PCA. The result of this study suggests that PCA is hepatoprotective against DMN-induced hepatic damage by its ability to suppress oxidative stress, inflammation, and modulate the activities of the selected phase I drug metabolizing enzymes. Thus, PCA may prove useful in combating DMN-induced hepatic damage.

Keywords*: Protocatechuic Acid, Hepatotoxicity, Antioxidant, Oxidative Stress, Dimethyl nitrosamine*

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INTRODUCTION

Dimethyl nitrosamine (DMN) belongs to the family of compounds known as nitrosamines an important environmental carcinogen (Ray *et al*., 2014). Scientists have been motivated to investigate the role of DMN in the etiology and pathophysiology of liver damage following the report of two cases of liver cirrhosis in men working in the research laboratory of a large firm that introduced DMN as a solvent (Barnes and Magee, 1954). Several research reports have indicated that DMN can also affect the kidney, in addition to being a potent hepatotoxicant (Usunomena *et al*., 2012; Saricicek *et al*., 2016; Usunobun and Okolie, 2016; Shamsi *et al*., 2017; Abdur-Rahman *et al*., 2022).
Experimental studies have shown that DMN causes oxidative stress; a major factor implicated in the etiology of cancer, via reactive oxygen species (ROS) production which occurs during its CYP2E1-mediated metabolism and alteration of the antioxidant defense system in tissues (Farombi *et al*., 2008; Usunomena *et al*., 2012). These reactive oxygen species have the potential to damage DNA which can lead to mutations and chromosomal damage, and they also oxidize cellular thiols and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Halliwell and Gutteridge, 2015).

Under normal physiological conditions, the production of reactive oxygen species (ROS) is balanced by the activity of antioxidant enzymes and other molecules that help to reduce oxidation. Antioxidants play a crucial role in safeguarding the body against damage resulting from oxidative stress induced by free radicals (Ozsoy *et al*., 2008; Halliwell and Gutteridge, 2015). Excessive ROS is promptly removed from the cell to prevent their potentially harmful effects. Numerous antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR), as well as non-enzymatic endogenous antioxidants like ascorbic acid, tocopherols, reduced glutathione (GSH), and uric acid, participate in mechanisms responsible for eliminating or regulating the levels of ROS within the cell (Halliwell and Gutteridge, 2015). Similarly, exogenous antioxidants like phenolic

phytochemicals have been shown to possess antioxidant activities owing to their capacity for scavenging free radicals (Costa *et al*., 2017; Engwa, 2018; Ortega and Campos, 2019; Shah *et al*., 2021; Abdur-Rahman *et al*., 2022).

Phenolic phytochemicals are secondary metabolites produced by plants; either as a component of their regular growth and maturation process or as a response to pathogen invasion or stress (Falcone Ferreyra *et al*., 2012; Gasmi *et al*., 2022). Consuming diets abundant in phenolic phytochemicals may have the potential to decrease the risk of diseases associated with oxidative damage, such as cancer, and could even act as a preventative measure (Singh *et al*., 2014; Sandoval-Yañez *et al*., 2018; Rojas and Buitrago, 2019; Rudrapal *et al*., 2022). One of such phytochemicals is 3, 4-dihydroxybenzoic acid, a simple phenolic compound; also known as protocatechuic acid (PCA). Like many other phenolic acids, PCA is present in most plants (especially fruit and vegetables) and thus a common component of the human diet (Liu *et al*., 2002; Liu, 2004; Tanaka *et al*., 2011; Khan *et al*., 2015; Singh *et al*., 2017; Liu *et al*., 2020). PCA has been shown to have strong antioxidant and antitumor promotion effects (Tseng *et al*., 2000; Lin *et al*., 2007; Xi *et al*., 2016; Liu *et al*., 2020). In addition, it has been demonstrated that PCA exhibits chemo preventive properties by impeding the carcinogenic effects of diverse chemicals in various tissues, including the liver (Gani *et al*., 2019; Punvittayagul *et al*., 2022), colon (Tanaka *et al*., 1993b; Tanaka *et al*., 1995; Farombi *et al*., 2016; Crespo *et al*., 2017), oral cavity (Babich *et al*., 2002), stomach tissue (Hu *et al*., 2020), and the bladder (Hirose *et al*., 1995).

The present study was undertaken to examine the modulatory effects of PCA on DMN-induced hepatic injury, oxidative stress, inflammation, and activities of selected phase I xenobiotic metabolizing enzymes involved in DMN metabolism.

MATERIALS AND METHODS

Materials: Dimethyl nitrosamine (≥ 98%) and protocatechuic acid (≥ 98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alanine amino transferase, asparatate amino transferase, bilirubin, alkaline phosphate and gamma glutamyl transferase assay kits obtained from Randox incorporated, UK. Rabbit polyclonal anti-iNOS antibody from Santa Cruz Biotechnology Inc. USA. Conjugated secondary antibodies obtained from Vector Labs, USA.
All other reagents used were of analytical grade and manufactured by Sigma-Aldrich (St. Louis, MO, USA) and British Drug Houses (Dorset, Poole, UK).

Animals: Sixty (60) male Wistar rats (150-220 g) were obtained from the Central Animal House of the College of Medicine, University of Ibadan and were housed at room temperature under a 12 h light/dark cycle in well-ventilated, plastic cages in the Animal House, Department of Biochemistry. They were acclimatized for a period of 2 weeks and provided with free access to rat pellets and water for the duration of the experiment.

Animal Treatment: Animals were randomly divided into six groups which were treated as follows: one group received only normal saline intraperitoneally (i.p.) for 7 days while two others were given only 50 mg/kg bw and 100 mg/kg bw PCA in normal saline orally (p.o.) respectively also for 7 days. Two groups were administered 50 mg/kg bw and 100 mg/kg bw PCA in normal saline orally (p.o.) respectively for 6 days together with a single i.p. injection of 20 mg/kg bw DMN on day 6. The last group received only 20 mg/kg bw DMN i.p. on day 6 of treatment. All animals were observed twice daily for any abnormal behavior and mortality. All the groups were sacrificed 48 hours after DMN administration.

Collection and Preparation of Serum and Tissues for Biochemical Analyses: The animals were humanely euthanized through cervical dislocation. Blood was obtained via cardiac puncture using a sterile needle and syringe, and then it was collected into clean, dry centrifuge tubes. The blood was left to coagulate by standing for 30 minutes. The blood samples were then centrifuged for 10 mins at 3000 g using a bench centrifuge. The clear supernatant (serum) was collected and stored in the refrigerator at 4Oc. The liver samples were quickly removed, rinsed in ice-cold 1.15% KCl, blotted, and weighed. The liver samples were then minced and homogenized in 4 volumes of ice-cold 0.1 M phosphate buffer, Ph 7.4. The homogenates were centrifuged at 10,000 g for 15 minutes at 4Oc and the supernatants were divided into aliquots and frozen until required for the enzyme assays.

Preparation of Samples used for Immunohistochemistry: Briefly, liver sections were submerged in a 4% phosphate buffer formalin solution, dehydrated through a series of alcohol gradients, and subsequently embedded in paraffin. Thin sections were then prepared and affixed onto glass slides treated with saline.

Preparation of Samples used for Histology: Liver samples were fixed in 10% formaldehyde, dehydrated in graded alcohol, and subsequently embedded in paraffin. Fine sections were obtained, mounted on glass slides, and stained with haematoxylin and eosin for light microscopic analyses.

Evaluation of biomarkers of hepatic injury: Serum alanine transaminase (ALT) and Serum aspartate aminotransferase (AST) activities were determined following the principle described by Reitman and Frankel (Reitman and Frankel, 1957). Alkaline phosphatase (ALP) was determined by the method of Englehardt (Englehardt, 1970). Gamma-glutamyl transferase (γ-GT) was determined using colorimetric method according to Szasz (Szasz, 1969).

Evaluation of biomarkers of oxidative stress: Hydrogen peroxide concentration was determined based on the method of Wolff (Wolff, 1994). Lipid peroxidation (LPO) was evaluated by measuring the level of thiobarbituric acid reactive substances (TBARS) by the method of Varshney and Kale (Varshney and Kale, 1990). The level of reduced glutathione (GSH) was estimated following the method of Beutler (Beutler, 1963).

Evaluation of Antioxidant Enzyme Activity: Catalase activity was determined according to Sinha (Sinha, 1972). SOD activity was assessed by the method of Misra and Fridovich (Misra and Fridovich, 1972). Glutathione peroxidase was assayed according to Rotruck *et al*. (Rotruck *et al*., 1973). Glutathione S-transferase activity was determined according to Habig *et al*. (Habig *et al*., 1974).

Evaluation of biomarkers of inflammation: Tumor necrosis factor (TNF)-α and interleukin-1-Beta (IL-1β) were analysed using specific enzyme-linked immunosorbent assay (ELISA) kits (Bioo Scientific Corporation®, USA) according to the manufacturer's instructions. The cytokines present in a serum sample conjugates with an immobilized IL-Iβ antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase and TMB substrate to the well results in an antibody–antibody coloured complex. The intensity of colour developed at 450 nm is directly proportional to the concentration of IL-Iβ (measured in pg/mL) in the sample. Immunochemical analyses of iNOS expression were carried out according to the method previously described by Farombi *et al*. (Farombi *et al*., 2009).

Evaluation of Phase 1 Metabolizing Enzymes: Hepatic aniline hydroxylase activity was evaluated by the method of Schenkman *et al*. (Schenkman *et al*., 1967) based on the measurement of the quantity of p-aminophenol formed during the hydroxylation of aniline hydrochloride. Aminopyrine-n-demethylase activity was determined by the method of Holtzmann *et al*. (Holzmann *et al*., 1968). Hepatic p-nitroanisole-o-demethylase activity was estimated by the method of Netter and Seidel (Netter and Seidel, 1964).

Statistical Analysis: Data are expressed as mean ± SD and analyzed with Microsoft Excel and SPSS statistical packages. Statistical analyses were performed by Student t-test and one-way analyses of variance (ANOVA). P value of less than 0.05 was considered statistically significant.

RESULTS

Evaluation of Biomarkers of Hepatic Damage and Oxidative Stress: In summary, Table 1 shows a significant increase in the levels of serum ALT and AST with DMN treatment when compared with the control. Pre-treatment with PCA at both doses caused a significant reduction in the levels of these liver function enzymes. Also, DMN significantly increased the level of serum ALP, and γ-GT was increased by 37.50% when compared with the control. Again, pre-treatment with PCA, reduced ALP by 29.93% at 100 mg/kg and γ-GT was not affected by PCA at 100 mg/kg.

Figures 1, 2, and 3 showed that DMN caused a significant increase ($p<0.05$) in LPO, reduction in GSH, and 10.64% increase in H2O2 concentration. While PCA at both doses prevented DMN-induced LPO. PCA at 50 mg/kg increased GSH concentration by 2.58% and H2O2 generation was lowered by 11.80%. However, PCA at 100 mg/kg produced a significant ($p<0.05$) elevation in GSH level and reduction in H2O2 concentration.

Evaluation of Antioxidant Enzyme Activity and Biomarkers of Inflammation: Table 2 indicates that DMN caused a significant reduction ($p<0.05$) in the activities of CAT and SOD, while PCA at both doses induced CAT and SOD activities significantly. The table also shows that DMN caused the induction of GPX and GST activities. GPX was induced by about 4% and GST by about 10%, but PCA at 100 mg/kg reversed both activities significantly.
DMN significantly increased the serum levels of IL-1β and TNF-α while pre-treatment with PCA significantly reversed this observed elevation in serum levels of these cytokines as presented in Table 3. Furthermore, Table 3 indicates that DMN caused a significant induction in the activity of MPO, and PCA significantly inhibited the MPO activity at 100 mg/kg and by about 6% at 50mg/kg**.**

Evaluation of Phase 1 Drug Metabolizing Enzymes: Table 4 shows that DMN caused a significant increase in the activities of aniline hydroxylase and p-nitroanisole-O-demethylase, and a significant decrease in the activity of aminopyrine-N-demethylase. PCA at 50 mg/kg significantly inhibited the activity of aniline hydroxylase, reduced the activity of p-nitroanisole-O-demethylase by about 30%, and reduced the activities of aniline hydroxylase and p-nitroanisole-O-demethylase by about 12% and 8% respectively at 100 mg/kg. Both doses of PCA significantly induced the activity of aminopyrine-N-demethylase

Table 1:
Chemo protective effects of PCA on serum biomarkers of DMN-induced hepatic injury in rats

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	γ-GT (U/L)
Normal Saline (I.P.)	3.2±0.87	29.83±4.16	4.91 ± 0.53	1.93 ± 0.55
50mg/kg PCA	3.93±1.45	29.67±7.75	11.04 ± 4.87	1.29 ± 0.59
100mg/kg PCA	2.65±0.41	30.88±3.15	7.97 ± 1.06	1.93 ± 0.55
20mg/kg DMN	38.95±4.07*	66.88±3.54*	58.65 ± 14.46*	3.09 ± 1.02 (37.50%) ****
50mg/kg PCA + 20 mg/kg DMN	28.87±3.89**	51.33±6.01**	26.45 ± 3.48**	1.93 ± 0.32**
100mg/kg PCA + 20mg/kg DMN	25.2±2.09**	55.13±4.61**	41.09 ± 9.22 (29.93%) ***	3.22 ± 0.89 (4.17%) ***

* indicates $p < 0.05$ when compared with the Normal Saline
** indicates $p < 0.05$ when compared with the DMN group.
*** indicates percentage change when compared with the DMN group.
**** indicates percentage change when compared with the Normal Saline.

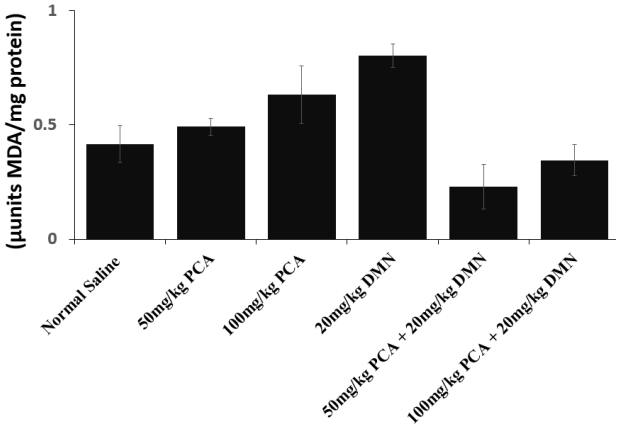

Figure 1
Chemo preventive effects of PCA on DMN-induced LPO
** indicates p < 0.05 when compared with Normal Saline (Control)*
*** indicates p < 0.05 when compared with the DMN alone group.*

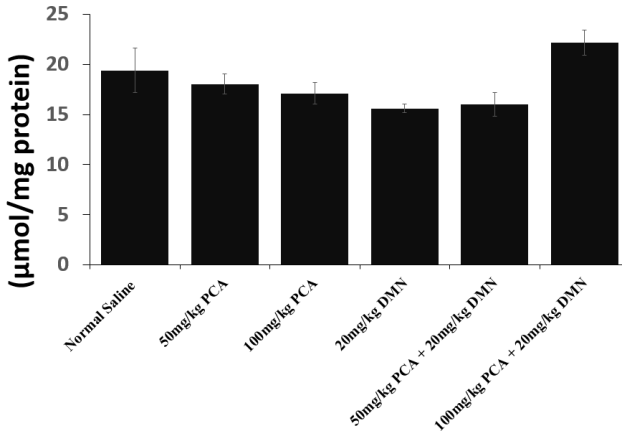

Figure 2
Chemo preventive effects of PCA on DMN-induced GSH
** indicatesp< 0.05 when compared with Normal Saline (Control)*
*** indicates p < 0.05 when compared with the DMN alone group*

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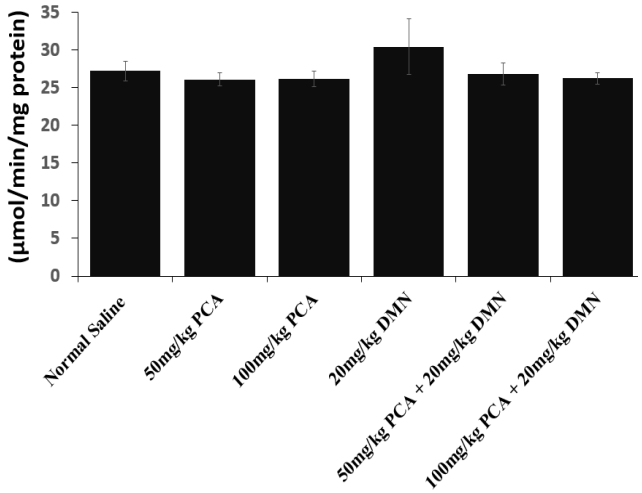

Figure 3
Chemo preventive effects of PCA on DMN-induced H_2O_2 generation
** indicates p< 0.05 when compared with Normal Saline (Control)*
*** indicates p < 0.05 when compared with the DMN alone group*

DISCUSSION

There has been considerable interest in N-nitrosamines because of the known carcinogenicity and mutagenicity of these compounds. N–Nitrosamines are long-established environmental/food contaminants (Poste *et al*., 2014; Park *et al*., 2015; Chen *et al*., 2018; Mazari *et al*., 2019; Suvorov *et al*., 2023). Several experimental studies have reported the hazards of nitrates and nitrites in food as precursors of carcinogenic nitrosamines such as DMN (Magee, 1971; Wolff and Wasserman, 1972; Sebranek and Cassens, 1973; Scanlan and Issenberg, 1975).

Table 2:
Chemo preventive effects of PCA on DMN-induced reduction antioxidant enzyme activities

Groups	CAT (µmol H2O2 consumed/min/mg protein)	SOD (units/mg protein)	GPX (µmol GSH consumed/mg protein)	GST (µmol/min/mg protein)
Normal Saline (I.P.)	510.71 ± 28.55	10.10 ± 0.59	156.71 ± 12.52	0.19 ± 0.03
50mg/kg PCA	481.84 ± 9.45	10.68 ± 0.49	151.89 ± 1.30	0.20 ± 0.02
100mg/kg PCA	468.78 ± 16.86	10.41 ± 0.82	145.85 ± 6.30	0.21 ± 0.02
20mg/kg DMN	379.41 ± 12.42*	8.84 ± 0.44*	122.61 ± 4.54*	0.10 ± 0.01*
50mg/kg PCA + 20mg/kg DMN	422.96 ± 24.98**	9.91 ± 0.66**	127.14 ± 5.63	0.11 ± 0.01
100mg/kg PCA + 20mg/kg DMN	542.01 ± 34.94**	12.41 ± 1.07**	177.53 ± 11.69**	0.16 ± 0.03**

* indicates p < 0.05 when compared with the Normal Saline ** indicates p < 0.05 when compared with the DMN alone group.

Table 3:
Chemo preventive effects of PCA on biomarkers of DMN-induced inflammation in rats

Groups	IL-1β (pg/ml)	TNF-α (pg/ml)	MPO (µmol H_2O_2 split/min/mg protein)
Normal Saline (I.P.)	550.61 ± 7.35	137.64 ± 38.05	1.37 ± 0.13
50mg/kg PCA	423.20 ± 94.22	289.95 ± 125.49	1.35 ± 0.21
100mg/kg PCA	408.05 ± 28.87	122.14 ± 9.93	1.34 ± 0.13
20mg/kg DMN	1685.30 ± 13.15*	699.35 ± 139.58*	2.48 ± 0.24*
50mg/kg PCA + 20 mg/kg DMN	672.74 ± 12.41**	246.23 ± 54.63**	2.32 ± 0.37 (6.48%) ***
100mg/kg PCA + 20mg/kg DMN	592.58 ±100.96**	181.74 ± 70.51**	2.18 ± 0.19**

* indicates p < 0.05 when compared with the Normal Saline ** indicates p < 0.05 when compared with the DMN group.
*** indicates percentage change when compared with the DMN group. **** indicates percentage change when compared with the Normal Saline

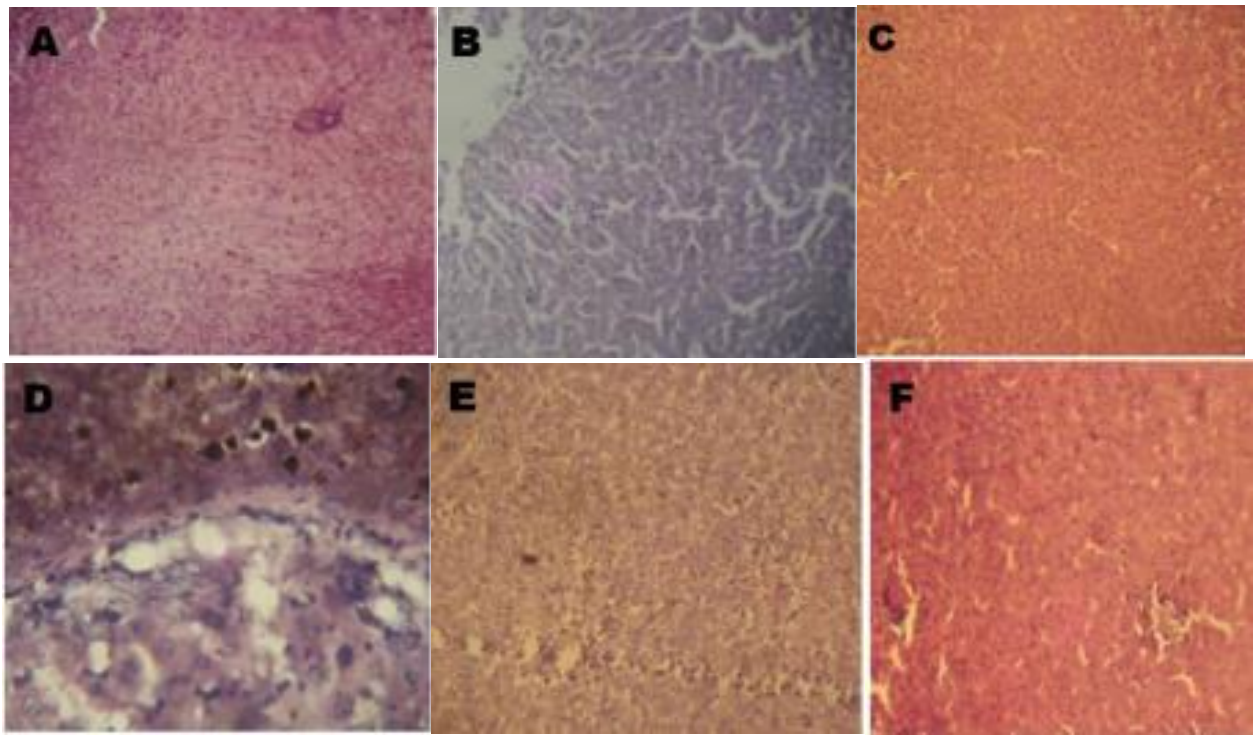

Plate 1A-F
(Histopathology): (A) Control Normal Saline – No visible lesions. (B) PCA (50mg/kg) – No visible lesions. (C) PCA (100mg/kg) – No visible lesions. (D) DMN Only – There is a severe portal congestion. There is also a severe diffuse vacuolar degeneration and necrosis of hepatocytes, with diffuse cellular infiltration by mononuclear cells. (E) PCA (50mg/kg) and DMN – There is a mild diffuse vacuolar degeneration and necrosis of hepatocytes, with diffuse cellular infiltration by mononuclear cells. (F) PCA (100mg/kg) and DMN – No visible lesions. *Magnification X100*

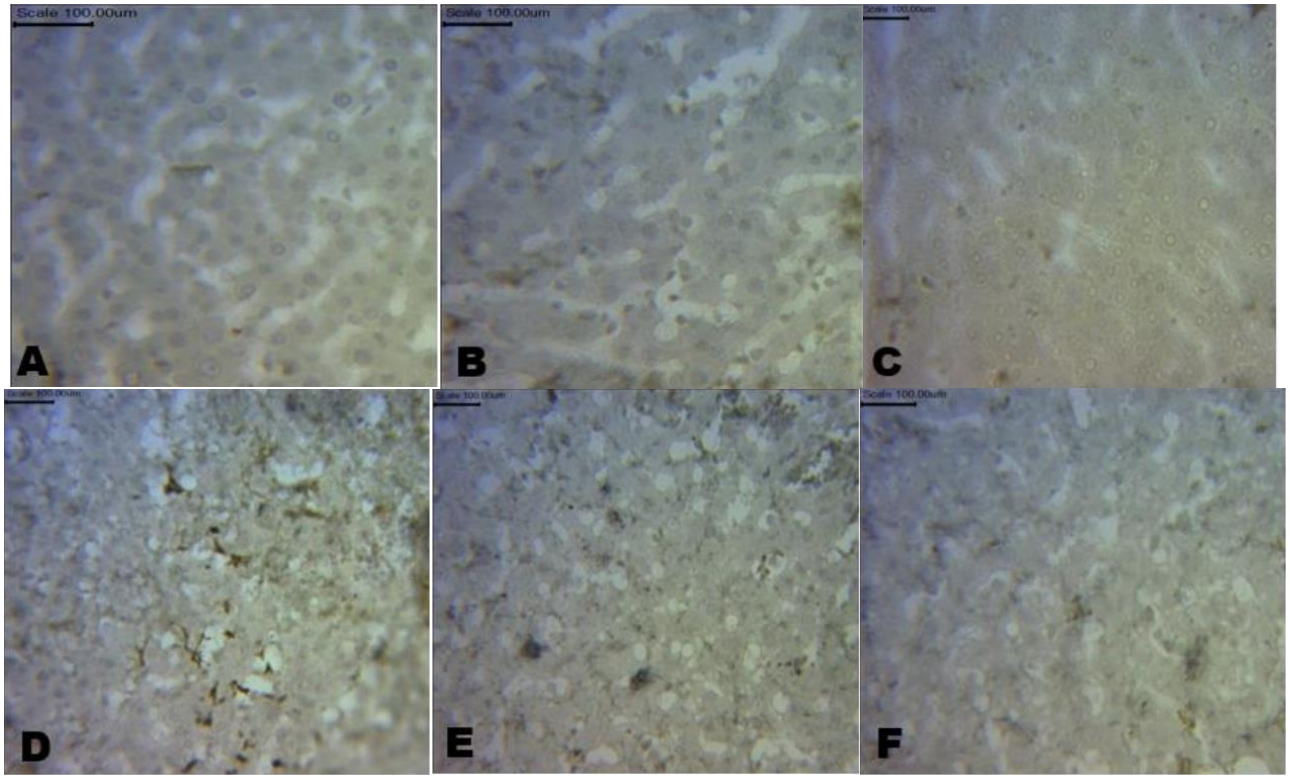

Plate 2A-F
(Immunohistochemistry results of iNOS expression): (A) Control Normal Saline. (B) PCA (50mg/kg). (C) PCA (100mg/kg). (D) DMN Only. (E) PCA (50mg/kg) and DMN. (F) PCA (100mg/kg) and DMN. iNOS expression is depicted by brown staining; the intensity of which determines the extent of enzyme expression. Results indicate that the DMN only group showed increased expression of iNOS compared to control while PCA was able to diminish this expression.

Table 4:
Chemo preventive effects of PCA on DMN-induced liver injury on Hepatic Phase I Drug Metabolizing Enzymes

Groups	ANILINE HYDROXYLASE (µmol p-aminophenol formed/mg protein/hr.)	P-NITRO ANISOLE-O-DEMETHYLASE (nmol p-nitro phenol formed/mg protein/hr.)	AMINOPYRINE-N-DEMTHYLASE (nmol HCHO formed/ mg protein/hr.)
Normal Saline (I.P.)	0.38 ± 0.03	2.54 ± 0.30	61.98 ± 11.74
50mg/kg PCA	0.38 ± 0.02	2.46 ± 0.30	80.60 ± 6.27
100mg/kg PCA	0.36 ± 0.02	1.76 ± 0.19	53.25 ± 6.56
20mg/kg DMN	0.59 ± 0.01*	4.12 ± 1.21*	36.40 ± 5.46*
50mg/kg PCA + 20mg/kg DMN	0.34 ± 0.04**	2.88 ± 0.42	54.25 ± 3.53**
100mg/kg PCA + 20mg/kg DMN	0.52 ± 0.08	3.79 ± 0.55	53.73 ± 4.65**

* indicates $p < 0.05$ when compared with the Normal Saline

** indicates $p < 0.05$ when compared with the DMN group.

Protocatechuic acid (PCA), also known as 3, 4-dihydroxybenzoic acid, is a potent antioxidant Thapa *et al*., 2023). It is a phenolic acid belonging to the broad class of polyphenols. It is widely available in oil, vegetables, fruits, and tea (Khan *et al*., 2015; Singh *et al*., 2017). It is mainly found in Roselle (Hibiscus sabdariffa) which is a species of hibiscus found in Old World tropics (Ali *et al*., 2005). PCA is a strong antioxidant that has been reported to show therapeutic potential as an anti-carcinogenic agent (Ali *et al*., 2005; Kakkar and Bais, 2014).

In the present study, we investigated the chemo-preventive effect, and possible mechanisms of chemoprevention of PCA on DMN-induced hepatotoxicity, oxidative stress, and inflammation. Our result, exemplified by the significantly elevated levels of serum AST, ALT, γ-GT, and ALP after the male rats were challenged with DMN; is in agreement with previous research reports that DMN is a potent hepatotoxin (George *et al*., 2001; Andrzejewski *et al*., 2005; Farombi *et al*., 2009; Choi *et al*., 2016; Rani *et al*., 2018).

Alanine aminotransferase (ALT) is an enzyme present in the hepatocytes. When the cell is damaged, this enzyme leaks into the blood. The serum level of ALT significantly increases during acute liver damage. ALT is considered a more dependable indicator of liver health and integrity in comparison to Aspartate Aminotransferase (AST). (Kaplan, 2002; Ojiako and Nwanjo, 2006). AST is also found in red blood cells, cardiac muscle, and skeletal muscle, making it less specific to liver damage as compared to ALT. It is also associated with liver parenchymal cells, and it requires acute liver damage to elevate blood AST levels (Moss *et al*., 1999; Vozarova *et al*., 2002). As a liver specific enzyme, ALT only significantly increases in the serum during hepatobiliary disease (Kaplan, 2002). Hence, the observed elevated serum ALT level in this present study is an indication that DMN caused hepatic injury. However, pre-treatment with PCA significantly lowered this rise in serum ALT level; with the 100 mg/kg treated group showing a greater decrease. Similarly, PCA pre-treated groups also showed a reduction in AST levels when compared with the DMN-treated group. Elevated levels of Alanine and Aspartate Aminotransferases are indicative of liver damage (Giboney, 2005; Chapman and Hostutler, 2013).

Alkaline Phosphatase (ALP) is an enzyme present in the cells lining the biliary ducts of the liver (Sharma *et al*., 2014; Poupon, 2015). The increased serum level of ALP in the DMN alone group observed in this present study may be attributed to damage in the structural integrity of hepatic cells causing the release of ALP into the circulation (McComb *et al*., 2013). However, in the groups pre-treated with 50 mg/kg PCA, we observed a statistically significant decrease in ALP levels; while the group that was pre-treated with 100 mg/kg PCA showed about a 30% decrease in ALP levels compared with the control group.

Gamma Glutamyl Transpeptidase (γ-GT or GGT) is relatively liver-specific and serves as a more sensitive indicator for cholestatic damage when compared to ALP. Elevated levels of γ-GT may occur even in cases of minor, sub-clinical liver dysfunction (Gowda *et al*., 2009; Anand and Mallick, 2019). The 37.50% elevation in γ-GT (Table 1) in the DMN-only treated rats when compared with the normal control may reflect the progress of carcinogenesis since the animals were sacrificed just 24 hours after treatment with 20 mg/kg DMN. However, the 50 mg/kg PCA pre-treatment caused a significant reduction in the γ-GT level. Unpredictably, 100 mg/kg PCA pretreatment could not reverse the elevated γ-GT caused by DMN. This might be due to the induction of γ-GT synthesis in the presence of chemical carcinogens (Vanisree and Shyamala, 1999; Lee *et al*., 2004).

To further validate damage to the liver, histopathology of the liver tissue was carried out. The results of the histopathological examination showed severe portal congestion, severe diffuse vacuolar degeneration, and necrosis of hepatocytes with diffuse cellular infiltration by mononuclear cells in the group that received DMN alone (PLATE D). In contrast, no lesion was observed in the control groups (PLATE A). However, PCA administration at both doses (PLATES E and F) reversed this hepatic degeneration and necrosis.

Furthermore, our results indicate increased H2O2 production and LPO in the group treated with DMN alone. Studies have shown that DMN mediates oxidative stress through the generation of reactive oxygen species (ROS), resulting in the alteration of the antioxidant defense system and attendant cellular injury (George *et al*., 2001; Zhang *et al*., 2016). Also, experimental studies suggest that when biological systems are subjected to oxidative stress, oxygen radicals like superoxide anion (.O2-), hydroxyl radical (.OH), and peroxyl radicals (˙OOH) are generated. These ROS oxidize cellular thiols, damaging protein structure and function, and abstracting hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Halliwell and Gutteridge, 2015). This may explain the observed elevation of H2O2, and the statistically significant increase in LPO of the DMN alone group compared to the control. However, pretreatment with 50 mg/kg and 100 mg/kg PCA significantly reduces LPO.

Furthermore, our results demonstrated significant elevation of lipid peroxidation (LPO), lowered levels of reduced glutathione (GSH), lowered activity of catalase (CAT), decreased activity of superoxide dismutase (SOD), reduced activity of glutathione peroxidase (GPX), and decreased activity of glutathione S-transferase (GST) in the rats challenged with DMN when compared with the normal control. Under normal physiological conditions, a variety of antioxidant systems which include redox molecules like GSH; antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) are employed by the mammalian system to eliminate cellular ROS, and thus offer protection against the harmful effect of ROS. During the oxidative assault of the cell, cellular ROS generation is exaggerated, and/or the antioxidant defense system is impeded resulting in an imbalance in the rate of generation versus the removal of cellular ROS (Halliwell and Gutteridge, 2015). The observed decrease in the investigated components of the antioxidant system in this present study can be attributed to the finding that ROS-generating xenobiotics like DMN can inhibit the activity of the antioxidant system (Liu, 2004; Hsu *et al*., 2008; Halliwell and Gutteridge, 2015; Rojas and Buitrago, 2019). It is worthy of note that our results show a correlation between the activity of CAT (an enzyme responsible for the removal of H2O2 by reduction to molecular oxygen and water) and the levels of H2O2. This is evidenced by the significantly lowered CAT activity, and increased levels of H2O2 and LPO in the DMN alone group. In the present study, we demonstrated that pretreatment with PCA prevented DMN-induced reduction of antioxidant enzyme activities (Table 2). Our observation aligns with the report of Masella *et al*. (Masella *et al*., 2004), that protocatechuic acid restores glutathione peroxidase activity and reduces glutathione to control levels. Similarly, our data demonstrated that PCA elicited antioxidant effects evidenced by restoration of GSH levels as previously reported by Liu *et al*., and Tsuda *et al*. (Tsuda *et al*., 1999; Liu *et al*., 2002).

In addition, our findings indicated that DMN treatment resulted in a significant elevation in the serum levels of pro-inflammatory cytokines, specifically TNF-α and IL-1β. Furthermore, hepatic myeloperoxidase (MPO) activity and the expression of inducible nitric oxide synthase (iNOS) in the liver were significantly increased. (Plate 2 and Table 3). On the other hand, PCA administration at both 100 mg/kg and 50 mg/kg lowered the serum levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) significantly. Similarly, MPO activity was statistically significantly lowered in the 100 mg/kg PCA pre-treated group, and about 6.5% decrease in the 50 mg/kg PCA pre-treated group when compared to their toxicant alone counterpart.

iNOS expression was increased in the DMN-treated animals and differential lowered expressions were observed in the animals pre-treated with PCA in a dose-dependent manner. Inflammatory cells, such as neutrophils, monocytes, macrophages, dendritic cells, eosinophils, mast cells, and lymphocytes, are typically mobilized in large numbers in response to damage or infection. This inflammatory response could potentially play a role in the initiation and advancement of cancer (Siaga *et al*., 1978; Allavena *et al*., 2008; Fernandes *et al*., 2015). Prostaglandins, cytokines, nuclear factor kappa B (NFκB), chemokines, and angiogenic factors are crucial molecular components that establish a connection between inflammation and genetic alterations. While on the other hand, the free radical species from oxygen (ROS) and nitrogen (RNS) are the key chemical effectors of these cellular insults (Tripathi and Aggarwal, 2006; Mantovani *et al*., 2010). This assertion is evident in the results of our present study wherein the levels of markers of inflammation were elevated significantly in the group challenged with DMN alone.

ROS and RNS are generated in response to pro-inflammatory cytokines, both in phagocytic and non-phagocytic cells, as a result of the activation of protein kinase signaling pathways (Moldogazieva *et al*., 2018). For example, TNF-α enhances the formation of ROS by neutrophils and other cells, while interleukin-1-β (IL-1-β), TNF-α and interferon (IFN)-γ stimulate the expression of inducible nitric oxide synthase (iNOS) in inflammatory and epithelial cells. This may account for the high expression of iNOS observed in this present study. To further substantiate the involvement of these cytokines in tumorigenesis, Moore *et al*. demonstrated that the knockout of the TNF-α gene in experimental animals significantly inhibits the development of skin tumors when exposed to DMBA and phorbol esters (Moore *et al*., 1999). This may explain the involvement of these cytokines in the hepato-pathogenesis observed in this present study. Therefore, from observations in this present study, and reports of previous investigators, these cytokines and proteins may have additive effects in hepatic injury.

To further probe the modulatory effect of PCA on DMN-induced liver injury in the present study, we investigated the roles of phase 1 drug-metabolizing enzymes. We determined the activities of liver microsomal cytochrome p450 enzymes aniline hydroxylase, aminopyrine-N-demethylase, and p-nitroanisole-O-demethylase. Our results showed that there were significant changes in the activities of these enzymes under different experimental conditions (Table 4). Aminopyrine-N-demethylase activity was inhibited in the DMN-only group, an observation which is in consonance with the findings of Kim *et al*., (Kim *et al*., 2009), where DMN caused a decrease in microsomal aminopyrine-N-demethylase activity. Since this enzyme is responsible for DMN metabolism and subsequent clearance, it can be suggested that DMN overwhelmed the enzyme, thereby enhancing the persistence of DMN in the tissue and causing damage. However, it was observed in this present study that pretreatment with PCA at both concentrations prevented DMN-induced loss in the activity of aminopyrine-N-demethylase.

On the other hand, the activities of aniline hydroxylase which is selective for cytochrome p450 2E1 (CYP 2E1) and p-nitroanisole-O-demethylase were significantly induced by DMN while the group treated with PCA at 50 mg/kg prior to DMN administration showed significant reduction of aniline hydroxylase activity when compared with the DMN-only group. This observation supports the report of Baer-Dubowska *et al*. (Baer-Dubowska *et al*., 1998) that protocatechuic acid inhibited the catalytic activity of certain cytochrome p450 enzymes better than chlorogenic acid, and propyl gallate more effectively than dodecyl gallate. Experimental studies have also shown that protocatechuic

acid administered at a dose of 50 mg/kg every 3 days for 2 weeks to rats that were exposed to 3-methylcholantrene on the 12th day of protocatechuic acid treatment resulted in decreases in activities and expression of CYP1A1, CYP1A2, and CYP2E1 (Krajka-Kuźniak *et al*., 2004). It is well-established that CYP2E1 plays a role in activating N-nitrosamines found in tobacco smoke, various compounds in food (Wang *et al*., 2002; Danko and Chaschin, 2005), numerous industrial carcinogens (Nakajima and Aoyama, 2000), as well as several endogenous carcinogens (Bartsch *et al*., 2000). In addition, CYP2E1 possesses the ability to reduce molecular oxygen to highly reactive compounds like superoxide anion radical, singlet oxygen, hydrogen peroxide, and hydroxyl radical even in the absence of substrate which can result in lipid and protein peroxidation, DNA damage, and carcinogenesis (Danko and Chaschin, 2005). Therefore, the inhibition of aniline hydroxylase and p-nitroanisole-O-demethylase by protocatechuic acid as observed in this present study indicates the ability of PCA to offer cytoprotection by preventing bio-activation of DMN.

The chemo-preventive ability of PCA observed in this present study is plausible because other studies have shown that protocatechuic acid (PCA) possesses the dual ability to inhibit the formation and scavenging of free radicals (Kakkar and Bais, 2014; Farombi *et al*., 2016; Hu *et al*., 2020; Thapa *et al*., 2023). The capacity of protocatechuic acid to create complexes with transition metal ions like Cu (II) and Fe (II), or to reduce the activity of enzymes involved in reactions that produce free radicals as by-products, such as xanthine oxidase, is linked to its ability to inhibit the generation of free radicals. The neutralization of free radicals occurs when they react with the hydroxyl groups present in protocatechuic acid. In vitro models have demonstrated that protocatechuic acid effectively prevents oxidative DNA damage and lipid peroxidation (Tanaka *et al*., 2011).

In conclusion, our results indicate that DMN is a potent hepatotoxin as evidenced by the elevated levels of serum liver enzymes, increased lipid peroxidation, and reduction in the antioxidant defense systems of the liver. In addition, we observed increased levels of pro-inflammatory cytokines (IL-1β and TNF-α), and increased expression of inflammatory biomarkers (iNOS and MPO) during DMN administration; indicating the involvement of inflammation in DMN-induced hepatic injury. Similarly, the group of rats challenged with DMN showed induced levels of phase 1 metabolizing enzymes except for aminopyrine-N-demethylase, thereby increasing the bioactivation and cytotoxicity of DMN. PCA however, acted as a potent hepatoprotective and chemo-preventive agent by ameliorating the various adverse hepatoxic conditions induced by DMN.

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