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Protective Effect of the Methanolic Leaf Extract of *Persea americana* (Avocado) Against Paracetamol-Induced Acute Hepatotoxicity in Rats

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Abstract: Paracetamol (PCM) is an analgesic, antipyretic drug available as an over the counter (OTC) medication which causes hepatotoxicity at high doses. The effect of *Persea americana* (PA) (200 and 400 mg kg^{-1} body weight, administered for 8 days) on paracetamol-induced acute hepatic damage was studied by investigating the effects on liver function, Glutathione-S-transferase (GST), reduced glutathione (GSH) and antioxidant enzymes - Superoxide dismutase (SOD) and catalase (CAT). Acute hepatotoxicity was induced by administering 2 g kg^{-1} body weight of PCM orally on the eight day. All rats were sacrificed 7 h after the administration of PCM. The results show that PCM at a dose of 2 g kg^{-1} body weight induced acute hepatotoxicity 7 h after oral administration as evident by the increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. This was also associated with depletion of hepatic GSH, decrease in GST activity and decrease in the activities of antioxidant enzymes (SOD and CAT). The methanol leaf extract of PA dose-dependently protected against acute hepatotoxicity induced by PCM by increasing the activity of the antioxidant enzymes and preventing GSH depletion. The results indicate that the extract protects against PCM-induced hepatotoxicity presumably via antioxidant action.

Key words: Paracetamol, acute hepatotoxicity, *Persea americana*, oxidative stress

INTRODUCTION

Many therapeutic agents have injurious effects on the liver and impair liver function leading to liver damage (Rang *et al*., 1995; Lee, 1993) and it is also a major reason for the removal of new drugs from clinical development and widespread use (Maria and Victorino, 1998). Paracetamol (PCM) is one of the most commonly used non - narcotic analgesic, antipyretic agents and main concern over it is that of hepatic toxicity resulting from accidental or deliberate over-dosage. Massive overdosage of PCM is associated clinically with severe central lobular hepatic necrosis and death from liver failure (Nelson, 1990; Vermeulen *et al*., 1992). With therapeutic doses, side effects are few and uncommon, though allergic skin reactions sometimes occur. In some sensitive patients however, liver damage can occur at recommended dosage (Kurtovic and Riordan, 2003). There have been many efforts to prevent PCM-induced hepatotoxicity either by interference with biochemical processes involved in the generation of its toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), (Devalia *et al*., 1982), or by modification of the structure of PCM (Van de Straat *et al*., 1987).

Herbs have become attractive as food that confer a health benefit and as a source of material for the development of drugs (Lee *et al*., 2001). The avocado, *Persea americana* (PA), family Lauracea, is a cultivated species which has been domesticated so far, back in antiquity and has undergone such drastic transformation under prehistoric human selection that its ancestry is unknown. The plant contains amongst many others alkaloids, saponins, flavonoids, steroids, carbohydrates, fatty acids, flavonols, essential oils and flavones (NAPRALERT, Chicago). Ethnomedical uses of the plant include as an emmenagogue (hot water extract, decoction), for asthma (bark), used for cough, fever, kidney and liver troubles, for diabetes, as food, for skin blemishes, as an abortifacient, for female disorders etc. Persin, isolated from avocado leaves has been shown to possess antifungal properties (Oelrichs *et al*., 1995). The avocado and other fruits have been reported to slow liver

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damage caused by galactosamine in rat (Kawagishi *et al*., 2001). Kim *et al*. (2000) also isolated compounds from avocado which showed marked inhibitory activities towards superoxide (O_2^-) and nitric oxide (NO) generation in cell culture systems.

The present study aimed at investigating the possible modulatory effect of the methanolic extract of avocado leaf against liver damage and oxidative stress induced by acute PCM intoxication in rat. Liver damage was evaluated by measuring the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, albumin level and total protein. The activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), glutathione-S-transferase (GST) and hepatic glutathione (GSH) level were analyzed as the determinant of oxidative stress. Present results show that the methanolic leaf extract of PA protected against hepatotoxicity and oxidative stress induced by PCM.

MATERIALS AND METHODS

Chemicals: Paracetamol (PCM), 1-Chloro-2,4-dinitrobenzene (CDNB) (Sigma USA), 5',5'-dithiobis-2-nitrbenzoic acid (DTNB), GSH, metaphosphoric acid and tris buffer were purchased from MRS Scientific Ltd. UK, adrenaline and thiobarbituric acid (TBA) from Aldrich Sigma, AST and ALT Kits were obtained from Randox Laboratories, UK. Other chemicals include methanol (BDH), trichloroacetic acid (TCA), glacial acetic acid, potassium chloride (KCl), hydrogen peroxide (H_2O_2), ethylenediamine tetra acetic acid (EDTA), potassium dichromate ($K_2Cr_2O_7$), sodium chloride (NaCl), sodium dihydrogen phosphate ($NaHPO_4$), disodium hydrogen phosphate (Na_2HPO_4), sodium carbonate (Na_2CO_3) and were all of analytical grade.

Plant material: PA leaves were collected in Sagamu, Ogun State, South-West of Nigeria. The herbarium specimen was validated at the Forestry Research Institute of Nigeria (FRIN), Nigeria, where voucher specimen with the herbarium number FHI06584 was deposited. The leaves were washed with water, shade dried and then oven dried at 40°C. The dried leaves were later pulverized. The powdered leaves were extracted in methanol using the soxhlet apparatus. The solid extract was obtained by evaporating the methanol and preserved in a refrigerator until ready for use. The dose of the extract administered was given on a kg body weight basis.

Animals and experimental design: Albino rats of both sexes and of the Wistar strain, weighing 220-350 g were obtained from the Department of Biochemistry, University of Ibadan, Nigeria. The rats were acclimatized for 21 days before the start of the experiment, during which they were allowed free access to standard pelleted chow and drinking water in an environment with 12 h dark/12 h light cycle. Animals were randomly assigned into five groups of 4-6 rats per group. Group 1 served as control and rats were given normal saline 1mL kg^{-1}. The animals in group 2 were treated with a single dose of PCM (2 g kg^{-1} p.o.) on the 8th day of the experiment. Group 3 was treated with a single dose of the extract of PA (400 mg kg^{-1}) daily for 8 days. Rats in groups 4 and 5 were treated daily with 200 mg kg^{-1} and 400 mg kg^{-1}, p.o. of PA respectively. On the 8th day, rats were treated with PCM (2 g kg^{-1}, p. o). Animals were deprived of food 7-11 h prior to PCM administration.

Preparation of samples for enzyme assays: Rats were scarified by stunning 7 h after treatment with paracetamol and blood removed by cardiac puncture to determine serum activities of AST and ALT and for determination of serum albumin and total protein levels. After bleeding, the livers were carefully removed, trimmed of extraneous tissue and rinsed in ice-cold 1.15% KCl. These were then blotted dry, weighed and homogenized in four volumes of ice - cold phosphate buffer (100 mM, pH 7.4). The homogenates were centrifuged first at 6000 rpm for 6 min to remove nuclear debris after which the obtained supernatants were centrifuged at 10,000 rpm for 20 min to obtain the post-mitochondrial supernatant (PMS) which was used for enzyme assays.

Biochemical evaluation: Hepatic function was evaluated by quantifying the activities of AST and ALT, levels of albumin and total protein in serum. Serum AST and ALT activities were measured following the principle described by Reitman and Frankel (1957) by monitoring the concentration of hydrazone formed with 2,4-dinitrophenyl-hydrazine, using standard kits according to manufacturers instructions. Serum albumin and total protein levels were determined using standard laboratory methods. Reduced glutathione (GSH) was estimated following the procedure of Beutler *et al.* (1963) and GST activity by the method of Habig *et al.* (1994). The activity of SOD was determined based on the ability of this enzyme to inhibit the spontaneous oxidation of adrenaline to adrenochrome as described by Misra and Fridovich (1972) and CAT activity assessed according to the method of Singha (1972), based on the ability of CAT to induce the disappearance of H_2O_2, which was followed spectrophotometrically.

Statistical analysis: Data were expressed as mean±SEM (standard error of mean) and were analyzed using student's t-test statistics by comparing control with PCM - or PA-treated group and PCM - treated group with PCM + PA treated groups. All statistical significances are expressed at the level of $p \leq 0.05$.

RESULTS

Assessment of liver function: Table 1 shows the effect of a single oral dose a of PCM (2 g kg^{-1} body) 7 h after administration in rats and the effect of pretreatment with the methanolic leaf extract of PA before induction of toxicity on the eight day. PCM induced acute liver damage as indicated by the elevation of serum levels of AST and ALT. Mild decreases were observed in serum concentrations of albumin and total protein. The decrease in serum ALT activity was moderate and statistically non-significant when compared with the control (Table 1). Pretreatment with PA dose-dependently protected against acute PCM toxicity in rats. Effects were however not statistically significant, when compared with PCM-treated rats. Treatment with PA alone on the other hand significantly reduced serum AST ($p<0.05$), caused mild decrease in ALT and did not cause any significant change in serum levels of albumin and total protein in rats when compared with the control.

Table 1: Effect of methanolic leaf extract *Persea americana* (PA) on paracetamol-induced acute hepatic toxicity in rats

Treatment groups	AST (u L^{-1})	ALT (u L^{-1})	Albumin (g/100 mL)	Total protein (g/100 mL)
Control (Saline, 1 mL kg^{-1})	44.0±3.0	25.5±0.5	5.0±0.4	8.8±0.1
PCM (2 g kg^{-1})	91.0±2.0*	31.5±11.5	4.7±0.1	8.6±0.3
PA (400 mg kg^{-1})	27.7±0.7**	18.8±3.3	4.6±0.2	8.4±0.3
PA (200 mg kg^{-1}) + PCM (2 g kg^{-1})	65.7±13.7	23.3±2.8	4.8±0.1	8.3±0.2
PA (400 mg kg^{-1}) + PCM (2 g kg^{-1})	58.5±17.5	24.3±5.1	5.0±0.2	9.1±0.6

Enzymes and proteins measured in serum. PCM (Paracetamol), AST (Aspartate aminotransferase), ALT (Alanine aminotransferase). Values are expressed as mean±SEM (standard error of mean). u L^{-1} (units/litre). *$p<0.005$ and **$p<0.01$ when compared with control

Table 2: Modulation of acute paracetamol (PCM) toxicity in rat liver post-mitochondrial fraction (PMF) by methanolic leaf extract of *Persea americana* (PA)

Treatment groups	GSH (µg mg^{-1} protein)	GST (mmol min^{-1} mg^{-1} protein)	Total protein (g/100 mL)
Control (Saline, 1 mL kg^{-1})	15.2±4.8	0.61±0.15	2.1±0.6
PCM (2 g kg^{-1})	7.6±0.7	0.58+0.08	2.7±0.3
PA (400 mg kg^{-1})	12.7±1.2	0.68+0.11	2.1±0.2
PA (200 mg kg^{-1}) + PCM (2 g kg^{-1})	15.6±3.8	0.79±0.36	1.8±0.3
PA (400 mg kg^{-1}) + PCM (2 g kg^{-1})	11.6±1.2*	0.65±0.34	2.1±0.1

Values are expressed as mean±SEM (standard error of mean). GSH (reduced glutathione), GST (glutathione-S-transferase). *$p<0.05$ when compared with Paracetamol (PCM)-treated group

Table 3: Effect of methanolic leaf extract of *Persea americana* (PA) on the activity of antioxidant enzymes in post-mitochondrial fraction of rat liver in Paracetamol (PCM)-induced acute hepatotoxicity

Treatment groups	SOD (units/mg protein)	CAT (µg H_2O_2 consumed/ min/mg protein)
Control (Saline, 1 mL kg^{-1})	0.24±0.04	0.11±0.04
PCM (2 g kg^{-1})	0.22±0.06	0.06±0.02
PA (400 mg kg^{-1})	0.30±0.07	0.10±0.03
PA (200 mg kg^{-1}) + PCM (2 g kg^{-1})	0.31±0.04	0.19±0.01*
PA (400 mg kg^{-1}) + PCM (2 g kg^{-1})	0.71±0.29	0.21±0.03*

SOD (Superoxide dismutase), CAT (Catalase). Values are expressed as mean±SEM (standard error of mean). *$p<0.01$ when compared with Paracetamol (PCM)-treated group

Effects of treatments on hepatic antioxidants and glutathione-s-transferase activity: Results presented in Table 2 and 3 show the effects of PCM and PA on antioxidant enzymes (SOD and CAT), GST activities and GSH level. Acute PCM intoxication produced mild to moderate and non-significant decreases in SOD, CAT and GST activities and caused depletion in hepatic GSH level when compared with the control. The methanolic leaf extract of PA increased the activities of these enzymes and prevent hepatic depletion of GSH in the PCM - treated rats. The effect on the activity of antioxidant enzymes was dose dependent and significant for CAT ($p<0.05$) when compared with the PCM - only treated rats. The protective effect against GSH depletion and GST activity at the higher dose (400 mg kg^{-1}) however did not appear to be greater than the effect produced by the lower dose (200 mg kg^{-1}), although the protective effect against GSH depletion was significant ($p<0.05$) when compared with the PCM only treated rats (Table 2).

DISCUSSION

Liver diseases remain one of the serious health problems worldwide. The liver is of paramount importance in drug metabolism and hepatocytes are exposed to high concentrations of nascent metabolites, which are formed by cytochrome P450 dependent drug oxidation. The occurrence of hepatic damage is a common reason for the

abandonment of potential new drugs during toxicity testing. Understanding the mechanisms of drug-induced hepatotoxicity and search for compounds that could modulate the disease process and offer protective effects become very necessary. Extensive knowledge of the molecular mechanisms of PCM-induced cytotoxicity makes PCM an interesting and useful model toxin to study effects and mechanisms of cytoprotective agents (Vermeulen *et al*., 1992).

The aim of the present study was to investigate the possible protective action of the methanolic leaf extract of PA against liver damage and oxidative stress induced by acute PCM toxicity in rats. PCM in this study produced hepatic damage in rats following a single oral dose of 2 g kg^{-1} body weight, 7 h after administration as evident by a rise in both aspartate aminotransferase (AST) and alanine aminotransferase (AST) levels in the serum. Clinical observations and experimental studies have shown that subtle membrane changes are sufficient to allow passage of intracellular enzymes to the extracellular space (Friedel *et al*., 1979). A very large concentration gradient between the hepatocyte and the sinusoidal space usually exists for enzymes. Cell damage increases permeability, causing cytosolic isoenzymes to spill into the sinusoids and from there into the peripheral blood (Friedel *et al*., 1979). PCM-induced elevation in serum AST and ALT levels has been attributed to the damaged structural integrity of the liver, because these enzymes are normally located in the cytoplasm of hepatocytes and are released into the circulation after cellular damage (Vermeulen *et al*., 1992). Since the liver is the primary site of the synthesis of plasma proteins, there were also decreases in serum albumin and total protein concentrations. These decreases were expectedly not significant in this acute liver damage because of the liver's reserve capacity and the relatively long half-life of these proteins, except in severe or long - standing hepatic disease where the effect on serum proteins become apparent. The significant rise in serum AST may be attributed to damage to both the liver and other tissues as this enzyme is located both in hepatic and extra hepatic cells.

Result from the present study further provides evidence of induction of oxidative stress 7 h following acute PCM intoxication. There was a rise in GST activity, indicating increased GST - catalyzed conjugation of the PCM toxic metabolite NAPBQI, with GSH and leading to depletion of cellular GSH level as previously reported (Vermeulen *et al*., 1992; Moore *et al*., 1985; Albano *et al*., 1983). The activity of the antioxidant enzymes, SOD and CAT, was also reduced, resulting in decrease in cellular defense against oxidative damage, although not statistically significant. The present result also provides evidence that the methanolic extract of PA could be protective against toxicity and oxidative stress arising from acute PCM intoxication. The mechanism of this protection is probably due to an antioxidant action. CAT, SOD and glutathione peroxidase (GPX) are the primary intracellular defense mechanisms to cope with increased oxidant stress. They eliminate superoxide anion and hydroperoxides that may oxidize cellular substrates and they prevent free radical chain reactions. Although the activities of the antioxidant enzymes measured (SOD and CAT) did not change significantly in normal rats treated with the methanolic leaf extract of PA, the extract however, significantly induced the activity of these enzymes during hepatic damage produced by acute PCM toxicity. SOD is known to catalyze dismutation of the superoxide radical and is the only enzyme known to use a free radical as a substrate. Apart from the hydroxyl radical, H_2O_2, generated by the action of SOD, is highly toxic by itself and can generate hydroxyl radicals by the so-called Fenton reaction, by reacting with ferrous ions and by interaction with superoxide through the Haber-Weiss reaction (Haber and Weiss, 1934). Hydroxyl radicals are highly toxic and induce lipid peroxidation of cell membranes. H_2O_2 is neutralized by the enzymes CAT and GPX. Thus, the free radical scavenging activity of SOD is effective only when it is followed up by increased CAT and/or GPX activity. The increase in the activity of SOD and CAT induced by PA in the PCM-treated rats in this study provides protection against free radical-induced oxidative stress caused by acute PCM toxicity. This effect may also contribute to the preservation of cellular GSH levels in the PCM-treated rats, which further provides cellular defense both as a hydroxyl radical scavenger (Ross, 1988) and also as a detoxifying agent against NAPBQI, the toxic intermediate of PCM, in a GST- catalyzed reaction (Rang *et al*., 1995). This result seems to suggest the presence of compounds in avocado leaf with inhibitory activity towards free radical-generating biochemical pathways similar to those reported previously by Kim *et al*. (2000), who isolated and demonstrated the presence of compounds (perenone A and B) with unique antioxidant properties in avocado fruit. The present result however has to be treated with caution since persin, isolated from avocado leaves was reported to cause necrosis of the acinar epithelium of the lactating mammary gland and the myocardium in mice, though the mechanism of action remain to be resolved (Oelrichs *et al*., 1995). An effective dose-response study may therefore be necessary to identify the dose range at which the leaf extract is beneficial and toxic. It is also important to clearly identify the compounds with this antioxidant and

hepato-protective properties in the leaf extract of this plant. In conclusion, the antioxidant activity exhibited by the methanolic leaf extract of PA (avocado) and its hepato-protective action against acute PCM toxicity make it a potential agent against liver diseases and other pathologies associated with oxidative stress.

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