The protective effect of piperine on oxidative stress and hepatic damage induced by diisononyl phthalate in rat

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ABSTRACT
Diisononyl phthalate (DINP) is a commonly used polyvinyl chloride product in industrial and consumer products with the ability to induce severe hepatotoxicity via oxidative stress. Piperine (PIPE) is an alkaloid from black pepper with proven antioxidant and anti-inflammatory activities. This study investigated whether piperine could promote hepatoprotection against DINP-induced hepatotoxicity in rats. Thirty-two rats were grouped into four treatment groups (n = 8/group): Control, 200 mg/kg/day DINP, 200 mg/kg/d DINP + 60 mg/kg/d PIPE and 60 mg/kg/d PIPE. Oral exposure to DINP induced oxidative damage in the liver via elevated nitric oxide, malondialdehyde, protein carbonyls, as well as depletion of hepatic antioxidants (reduced glutathione, superoxide dismutase, and catalase). These contributed to an increase in plasma levels of liver biomarkers (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin) and hepatic histopathological alterations. Our data suggest that PIPE alleviated the oxidative stress, depleted antioxidants, and elevated hepatic function biomarkers induced by DINP.

Introduction
Diisononyl phthalate (DINP), bis(7-methylloctyl) benzene-1,2-dicarboxylate (Figure 1a), is the diisononyl ester of benzene-1,2-dicarboxylic acid used as a general-purpose plasticizer for polyvinyl chloride (PVC) [1]. Phthalates are present in flexible paints, personal care products, plastics, toys, food packages, PVC flooring and many other products that form part of our daily lives [2]. Besides exposure at the industrial processing level, humans can also be exposed through interaction with phthalate-containing materials. Phthalates, including DINP, are not chemically bound to the PVC molecules in plastic materials, and therefore are easily released, leading to human exposure from products containing them [3]. Following exposure, DINP is rapidly distributed mainly to the liver and kidneys within 1 hour. It is metabolized into hydrolytic monoesters (primary metabolites), which may undergo oxidative metabolism to hydroxy-, oxo-, and carboxy-containing secondary metabolites [4,5]. DINP exposure has been reported to cause depletion of hepatic antioxidants, oxidative damage and hepatotoxicity among other organ toxicities [6].

Piperine, (2E,4E)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpent-2,4-dien-1-one (Figure 1b), is the alkaloid responsible for the pungency of black pepper and long pepper and has been widely applied in traditional medicine [7,8]. PIPE is an effective antioxidant and anti-inflammatory compound with numerous other physiological activities reported in vitro and in vivo [7–11]. Considering the phytotherapeutic potentials...
offered by PIPE, it is thought that its use in the treatment of DINP-induced hepatotoxicity could yield some potential benefits in DINP exposure. There is currently little or no report on the potential effects of PIPE on DINP-induced toxicity and oxidative stress. Therefore, the present study was designed to evaluate the protective effects of PIPE on DINP-induced oxidative liver damage in rats.

**Materials and methods**

**Chemicals and assay kits**

Diisononyl phthalate is a product of Relonchem Ltd., Gorsey lane, Widnes, Chesire, UK. Guanidine hydrochloride, glutathione and piperine were purchased from AK Scientific Inc., Union City, CA. Tween 80, p-nitrophenyl phosphate, 1-chloro,2,4-dinitrobenzene, 5,5-dithio-bis-2-nitrobenzoic acid, thiobarbituric acid, epinephrine, trichloroacetic acid, 2,4-dinitrophenyl hydrazine were purchased from Merck, Darmstadt, Germany. Hydrogen peroxide (H₂O₂) and ammonium molybdate were products of BDH Poole, London. Other chemicals and reagents used were of analytical grade. Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin were products of Fortress Diagnostic Ltd., Antrim, UK.

**Experimental animals**

Thirty-two (32) male albino rats weighing between 180 and 200 g were used in this study. The animals were acclimatized to laboratory conditions for 3 weeks at the animal breeding unit, Department of Chemical Sciences, Ajayi Crowther University, Oyo. The rats were contained in plastic cages and provided food and water *ad libitum*. They were housed at normal conditions of temperature and humidity and fed with commercial rat diet (Ladokun Feeds, Nigeria Ltd., Ibadan, Nigeria). The handling of the experimental animals is consistent with international principles on the care and use of experimental animals [12]. Ethical approval (FNS/ERC/2019/0006) was provided by the Faculty of Natural Sciences Ethical Review Committee, Ajayi Crowther University, Oyo.

**Dosage and experimental design**

The dosage of DINP (200 mg/kg/d) was chosen based on the previous report of hepatotoxicity resulting from this dose [6]. Dosage for PIPE (60 mg/kg bw) was selected based on its safety and efficacy in earlier studies [13,14]. DINP was prepared in Tween-80 (1:1 v/v) and diluted with

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Figure 1. Chemical structures of diisononyl phthalate (a) and piperine (b).
physiological saline. Piperine was dissolved in a small quantity of Tween-80 and made up to appropriate volume with sterile saline. Thirty-two rats were randomly divided into four groups of eight rats each as follows: I – control (Tween/saline), II – PIPE (60 mg/kg/bw/d PIPE administered orally, p.o.), III – DINP (200 mg/kg bw/d DINP p.o.), and IV – PIPE + DINP (60 mg/kg bw/d PIPE + 200 mg/kg/d DINP) (Figure 2). PIPE administration lasted for 21 days while co-administration with DINP began on day 8 (lasting for the subsequent 14 days). DINP was administered 1 h after the administration of PIPE each day.

**Sample collection**

After 21 days, blood sample was collected via the retro-orbital vein into heparinized tubes for preparation of plasma. Thereafter, all the rats were sacrificed and the liver was collected from each animal for histopathological examination and preparation of liver homogenate. Sections of the hepatic tissues were fixed in 10% neutral buffered formalin for the preparation of histopathological slides.

**Preparation of plasma and liver homogenate**

Blood samples were centrifuged at 894 × g for 5 min, and the clear supernatant (plasma) was collected in a sample tube for plasma-related biochemical assays. Approximately 0.2 g of hepatic tissues were homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.4) to give a 10 w/v homogenate. Homogenates were subjected to centrifugation at 10000 × g for 10 min at 4°C. Supernatants were collected for biochemical analyses.

**Hepatic oxidative stress biomarkers**

Nitric oxide (NO) level was determined by the procedure of Green et al. [15]. Level of lipid peroxidation (LPO) was evaluated by measuring the concentration of malondialdehyde (MDA) in the liver following the method of Varshney and Kale [16]. Hepatic levels of protein carbonyls were determined according to the method of Reznick and Packer [17].

![Figure 2. Experimental protocol. mg/kg/d: milligram per kilogram per day, DINP: diisononyl phthalate, PIPE: piperine.](image-url)
**Hepatic antioxidant markers**

Reduced glutathione (GSH) level was determined according to the method of Jollow et al. [18]. Superoxide dismutase (SOD) activity was determined in the liver based on the procedure of Sun and Zigman [19]. The method described by Hadwan and Abed [20] was used to determine the activity of catalase (CAT) in the liver.

**Hepatic function biomarkers**

Activities of ALT, AST and total bilirubin level were determined using assay kits (Fortress, Antrim, UK), according to the manufacturer’s protocol. Alkaline phosphatase (ALP) activity was determined by the method of Wright et al. [21].

**Histopathology**

The fixed samples were embedded in paraffin and thin section (10-μm) was stained with hematoxylin and eosin according to standard protocols [22] and observed using a light microscope (Olympus, Tokyo, Japan). Tissue sections were examined qualitatively by a pathologists in a blinded fashion.

**Statistical analyses**

Results were expressed as mean ± SD. Data were subjected to analysis of variance and complemented with tukey’s test to determine the significance of the differences between groups ($P < 0.05$). Data analysis and graphical construction was done using GraphPad Prism v 6.0.1 (GraphPad Software, La Jolla, CA).

**Results**

**Piperine alleviates DINP-induced oxidative stress**

The effects of administration of DINP and PIPE on oxidative stress markers are presented in Figure 3. DINP caused a significant increase in hepatic level of NO when compared with the control. Hepatic content of MDA and protein carbonyls also increased in a similar pattern. However, administration of PIPE alongside

**Figure 3.** Effects of piperine co-administered with DINP on hepatic biomarkers of oxidative stress in rats: (a) nitric oxide concentration, (b) MDA concentration and (c) protein carbonyls concentration. *Significant difference compared with the control group; #significant difference compared with the DINP group ($P < 0.05$). DINP-diisononyl phthalate, PIPE-piperine, NO-nitric oxide, MDA-malondialdehyde.
DINP significantly protected against elevated levels of NO and oxidative damage products MDA and protein carbonyls.

**Piperine alleviates DINP-induced alteration in hepatic antioxidants**

Figure 4 presents the effects of DINP and PIPE on hepatic GSH concentration and activities of SOD and CAT. Hepatic GSH level decreased significantly in response to DINP exposure, when compared with the control. DINP treatment also caused a significant decrease in the activities of hepatic SOD and CAT. Co-administration of DINP and PIPE significantly protected against the decrease in hepatic antioxidant levels caused by DINP treatment.

**Piperine alleviates DINP-induced alterations in plasma markers of hepatotoxicity**

The protective effects of PIPE on DINP-induced alteration in plasma markers of hepatotoxicity are shown in Figure 5. There was a significant increase in the levels of hepatic function biomarkers (ALT, AST, ALP and total bilirubin) in the plasma of rats in response to 200 mg/kg bw/d DINP exposure (Figure 5) when compared to control rats. Co-treatment with 60 mg/kg bw/d PIPE ameliorated the DINP-induced alterations in hepatic function biomarkers.

**Piperine alleviates DINP-induced alteration in hepatic histological structure**

Figure 6 shows the representative photomicrograph (100 and x400) of hematoxylin and eosin-stained liver sections of rats in the control group, 60 mg/kg PIPE group, 200 mg/kg DINP group and 200 mg/kg DINP plus 60 mg/kg PIPE group. Images in the control group show that normal liver, the central vein and sinusoids appear normal and the hepatocytes show normal morphology. In the 200 mg/kg DINP-treated rats, there is moderate portal infiltration and the sinusoids show mild dilation with moderate infiltration by inflammatory cells. Images in the co-administered group (DINP plus PIPE) as well as those administered PIPE only show normal liver, the central venules appear normal, the sinusoids appear normal without infiltration of inflammatory cells and the hepatocytes show normal morphology.

![Figure 4](image_url) **Figure 4.** Effects of piperine co-administered with DINP on hepatic antioxidants in rats: (a) GSH concentration, (b) SOD activity and (c) CAT activity. *Significant difference compared with the control group; #significant difference compared with the DINP group (P < 0.05). DINP-diisononyl phthalate, PIPE-piperine, GSH-reduced glutathione, SOD-superoxide dismutase, CAT-catalase.
Exposure to phthalates is currently a source of concern worldwide, considering the industrial importance of phthalates as well as their potential health risks [23,24]. DINP is one of the most commonly applied phthalates in wide varieties of plastic products and other household materials [25]. Studies have demonstrated the ability of DINP to induce hepatotoxicity via oxidative stress, inflammation and depletion of the hepatic antioxidant system [6,26,27]. Piperine (PIPE), an alkaloid from black pepper, has demonstrated huge potentials in the treatment of chronic diseases, mostly attributed to its antioxidant and anti-inflammatory activities [28]. This study was designed to investigate the protective effects of PIPE in DINP-induced hepatotoxicity in the rat model.

Our data show that DINP administration caused a significant increase in the hepatic levels of oxidative stress indicators: NO, MDA and protein carbonyls. NO is known to play a significant role in NO-based cell signaling [29]. However, high level of NO production with limited post-production scavenging mechanism can lead to a number of physiological disorders due to compromised NO
functionality [30]. MDA is an established biomarker of oxidative stress. Free radicals’ attack on polyunsaturated fatty acids (PUFA) initiate the lipid peroxidation process, creating peroxidation products, one of which is MDA [31,32]. An increased level of free radicals is associated with an elevated production of MDA [33]. High hepatic MDA content has been reported to be associated with DINP toxicity at a dose of 200 mg/kg. Protein carbonyls are products of protein carboxylation, a type of protein oxidation promoted by reactive oxygen species [34]. Oxidative decomposition of PUFAs initiates chain reactions that lead to the formation of a variety of carbonyl species, the most reactive and cytotoxic being 4-hydroxy-trans-2-nonenal and acrolein [34,35]. The group co-administered with PIPE show a significant decrease in the levels of these oxidative stress biomarkers. These decrease may be attributed to anti-radical and antioxidant properties of PIPE as illustrated in Figure 7. Moreover, several studies have shown that PIPE is able to protect against high level of NO, MDA and protein carbonyls [36,37].

DINP also caused a significant decrease in hepatic GSH level and activities of SOD and CAT. The depletion of hepatic GSH level observed in this study is similar to that reported earlier by Ma et al. [6] for 200 mg/kg DINP. GSH is a major cellular thiol involved in redox regulation. It fulfills several roles such as free radicals scavenging, thiol-disulfide recycling and in hepatotoxicity [38,39,40]. SOD is involved in the dismutation of superoxide anion to H₂O₂ and O₂ while CAT detoxifies the H₂O₂ produced to H₂O and O₂ [41,42]. Previous reports have shown that PIPE is capable of protecting against oxidative damage in vivo, via improving physiological antioxidant system [43,44]. This is achieved by boosting the level of GSH and activities of SOD and CAT.

Data from this study revealed significantly high plasma activities of ALT, AST, ALP and bilirubin concentration in DINP-treated rats. These are established biomarkers of hepatotoxicity: ALT and AST are indicators of hepatocellular injury while ALP and bilirubin are indicators of hepatobiliary damage [45]. Histopathological studies also support this observation with the visible presence of inflammatory cells and dilation of sinusoids in the group administered with DINP. However, the administration of 60 mg/kg PIPE protected the hepatocytes against DINP-induced hepatotoxicity. Earlier studies have reported the hepatoprotective effects of PIPE on hepatotoxicity induced by other types of hepatotoxicants [46,47].

![Figure 7](image_url)

**Figure 7.** Protective mechanism of PIPE against DINP-induced toxicity. DINP-diisononyl phthalate, PIPE-piperine, GSH-reduced glutathione, ROS-reactive oxygen species, MDA-malondialdehyde, ALT, alanine aminotransferase, AST, aspartate aminotransferase, NF-kB-nuclear factor kappa-light-chain-enhancer of activated B cells.
Conclusions

Data from this study show that 200 mg/kg DINP induced hepatotoxicity characterized by oxidative damage, depleted antioxidant defense and elevated plasma liver function indices. Administration of piperine at a dose of 60 mg/kg effectively protected against DINP-induced hepatotoxicity in rat.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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