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FULL ARTICLE

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Pterocarpus mildbraedii (Harms) extract resolves propanil-induced hepatic injury via repression of inflammatory stress responses in Wistar rats

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Abstract

Pterocarpus mildbraedii (PME) is a green leafy vegetable from the Papilionaceae family. This study evaluated the anti-inflammatory activity of PME in Wistar rats exposed to experimental hepatotoxicity using propanil (PRP), a post-emergent herbicide. Animals were grouped as control, PRP, PME, and PME + PRP. After 7 days, the levels of stress-activated protein kinases/c-Jun N-terminal kinase (SAPK/JNK), p38 mitogen-activated protein kinase (p38 MAPK), and signal transducer and activator of transcription (STAT-3) were measured in rat liver. Furthermore, myeloperoxidase (MPO) and nitric oxide (NO) levels, as well as protein expressions of nuclear factor-κB (NF-κB p65), inducible nitric oxide synthase (iNOS), and cyclo-oxygenase-2 (COX-2) were determined. Compared with PRP-treated rats, PME significantly reduced the hepatic MPO and NO levels. PME also diminished NFκB, iNOS, and COX-2 protein expressions in PRP-treated rats. This study showed that *Pterocarpus mildbraedii* leaves produce active principles with relevant anti-inflammatory potential.

Practical applications

Previous studies have shown that bioactive principles contained in medicinal plants can offer protection against chemically induced inflammation. *Pterocarpus mildbraedii* leaves, with rich content of polyphenols, flavonoids, and essential fatty acids, could be exploited as a therapeutic agent against pesticide-induced oxidative stress and inflammation. This current study has also shown that the potential of PME as a functional food is boosted by the presence of α-linolenic acid, an omega-3-fatty acid known to possess anti-inflammatory activity. Here, we elucidated the cellular mechanisms of the anti-inflammatory action of PME.

KEYWORDS

anti-inflammation, Propanil, *Pterocarpus mildbraedii*, rats, transcription factors

1 | INTRODUCTION

The role of pesticides in the etiology of liver ailments has been well-documented (Karami-Mohajeri et al., 2017; Manfo et al., 2020). For example, in animal studies, rats exposed to the copper-based fungicides, fipronil, and paraquat dichloride presented with oxidative damage and liver injury (Gad El-Hak & Mobarak, 2018; Kartheek & David, 2018; Ujowundu et al., 2018). Other pesticides, such as imidacloprid (Arfat et al., 2014) and Fenitrothion (Ehsan et al., 2014), caused a hepatic injury in mice and fish models of animal experimentation, respectively. Propanil (PRP), a post-emergent pesticide, is initially metabolized to 3, 4-dichloroaniline (DCA), and later oxidized to

3, 4-dichlorophenylhydroxylamine (McMillan et al., 1991; Otuechere et al., 2012). In an elegant review by Salazar and co-workers (2008), propanil was implicated as the cause of spleen enlargement and degeneration of the thymus. The study further highlighted the role of the herbicide in depleting lymphoid cells' development in the thymus and bone marrow. In a recent study, different pharmacokinetic profiles were observed in C57Bl/6 mice exposed to 3′, 4′-dichloropropionanilide alone (low dose) and a combination of 3′, 4′-dichloropropionanilide, and 3′, 4′-dichloropropionaniline (high dose). Hence, there is a need to re-evaluate the toxicity of propanil and its potential to elicit adverse outcomes in humans (Schafer et al., 2018). Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are important enzymes involved in inflammatory processes. While the former is a rate-limiting enzyme in the biosynthesis of prostaglandins, the latter is implicated in the excessive production of nitric oxide following the onset of inflammation (Attiq et al., 2018). In abnormal situations, nitric oxide acts as a pro-inflammatory mediator. Therefore, the abolition of the activities of COX-2 and iNOS could be a potential therapeutic target(s) (Ahmad et al., 2020; Tudor et al., 2020). The transcription factor, NF-κB, governs variegated forms of innate and adaptive immune functions. Apart from the induction of the expression of several pro-inflammatory genes, NF-κB is also associated with the regulation of the inflammasome. Another transcriptional factor that participates in the inflammatory process is STAT3. It has been suggested that these transcription factors play critical roles in inflammatory responses in hepatic tissues (Smale & Natoli, 2014). It has been reported that NF-κB shares a common inflammatory signaling pathway with Mitogen-activated protein kinases (MAPKs), with their co-activation leading to an inflammatory response via the release of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α (Xiao et al., 2020). Suppression of NF-κB and COX-2 expressions could block the activation of downstream cytokines, and thus, afford protection against propanil-induced hepatotoxicity (Wali, Rashid, et al., 2020).

Natural products possessing antioxidant and inflammatory properties have been shown to confer protection against various disorders. For example, D-Limonene, extracted from citrus plants, has reportedly shown an antifibrotic effect in a carbon tetrachloride model of hepatotoxicity (Ahmad et al., 2018). Similarly, zingerone, a pharmacologically active compound from dry ginger, was found to have mitigated oxidative damage in the hepato-renal system following the intoxication of rats with lead (Amin et al., 2020). A flavonoid-rich fraction of Bergamot juice also alleviated gingival inflammation via mechanisms such as reduction of nuclear NF-κB translocation and myeloperoxidase activity (Gugliandolo et al., 2019). On the other hand, *Pterocarpus mildbraedii* is used traditionally to treat headaches, pains, fever, and convulsions, and other respiratory disorders. The leaves from *Pterocarpus mildbraedii* have also been reported to possess anti-inflammatory and antibacterial properties (Omale & Ugwu, 2011). Further, *Pterocarpus mildbraedii* fractions have been reported to possess some antioxidant activity, suggesting its usefulness in the treatment of diseases, especially liver-borne diseases (Hamzah et al., 2018). In another experiment, the dichloromethane extract of the leaves elicited free radical scavenging activity and showed potent cytotoxicity against five different cancer cell lines (Iweala et al., 2015). The purpose of this work, therefore, is to determine the anti-inflammatory potential of PME in the liver of rats exposed to propanil.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Propanil was purchased from Harvest Field Industries in Lagos, Nigeria. COX-2, iNOS, and NF-κB antibodies were purchased from Abcam (UK). Phospho-p38MAPK (catalog #4631), phospho-SAPK/JNK (catalog #7272), and phospho-STAT3 (catalog #9139), ELISA kits were obtained from Cell Signaling Technology, USA. Other chemicals used were of analytical grade.

2.2 | Extract preparation and phytochemical investigations

Leaves of *Pterocarpus mildbraedii* were sourced from a local market in Lagos State, Nigeria. They were taxonomically identified at the Department of Botany Herbarium, University of Lagos, Nigeria, and assigned Voucher number LUT/5913. 235.7 g of dry plant material was extracted with two solvents, dichloromethane, and methanol (1:1), using a soxhlet extractor. Preliminary phytochemical analyses were performed using GCMS-QP 2010 ULTRA. Gas chromatograph equipped with a fused silica capillary column Rxi-1MS 30 m × 0.25 mm × 0.25 μm (Restek) on ultrapure helium gas at 3.0 ml/min and coupled to a mass detector (mass spectrometer). The injector and interface were operated at 250°C. The oven temperature was raised from 100°C (3 min) to 250°C at a heating rate of 7°C/min, and then, held isothermally at that temperature. A 1 μl sample solution was injected in a split mode (ratio 1:10). The GC-MS interface was at a temperature of 260°C. The MS was operated at an ionization voltage of 70 eV (electron impact) over a mass acquisition range. The constituents of the sample were identified based on their linear retention indices and comparing their MS spectral with data obtained from the National Institute of Standard and Technology. The relative proportions of the constituents were percentages from the GC peak areas.

2.3 | Animals and study design

Twenty-four (24) male albino rats (140–160 g) were purchased from the primate colony of the Department of Veterinary Anatomy, University of Ibadan. Rats were randomly grouped into four ($n = 6$) and accustomed to the new environment for two weeks before treatment with PRP and PME. Animals were given standard rat chow (Ladokun Feeds Ltd, Ibadan, Nigeria). They also had unlimited access to drinking water.

The rats were subjected to natural photoperiod of 12 hr light and 12 hr dark per day. The study was conducted after due approval by the Department of Biochemistry, University of Ibadan Ethical Committee, with the study code as SI. 68021. Additionally, the National Institute of Health protocol on the care and use of laboratory animals was adopted.

Group 1: Olive oil (2 ml/kg).

Group 2: PRP (200 mg/kg) dissolved in olive oil.

Group 3: PME (200 mg/kg).

Group 4: PRP (200 mg/kg) + PME (200 mg/kg).

Dose selection and duration of the study was based on the effective pharmacological data from pilot and previous studies in our laboratory (Otuechere et al., 2012, 2019; Otuechere, Avwioroko, et al., 2020). All animals were sacrificed 24 hr following the last treatment by dislocating the cervical vertebrae.

2.4 | Determinations of myeloperoxidase activity and nitric oxide level

Portions of the liver from each experimental group ($n = 6$) were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) and processed for the measurement of myeloperoxidase (MPO) and nitric oxide (NO) levels. MPO activity was determined by measuring the hydrogen peroxide-dependent oxidation of o-dianisidine (Bradley et al., 1982), while NO level was determined by the Griess reaction as previously reported (Otuechere, Adewuyi, et al., 2020).

2.5 | Determination of the levels of inflammatory transcription factors

Liver sample (100 mg), from each experimental group ($n = 6$), were homogenized in 1 ml lysis buffer and the lysates were used to determine phospho- p38MAPK, phospho- SAPK/JNK, and phospho-STAT3 levels using specific ELISA kits (Cell Signaling Technology, USA).

2.6 | Immunohistochemistry of inflammatory proteins

The representative liver sections from each group (three rats per group) were fixed in formal saline. Subsequently, they were incubated overnight in a humidified chamber using the primary rabbit monoclonal antibodies: NF-κB (p65), COX-2, and iNOS. Further incubations with biotin-labeled secondary antibody and horseradish peroxidase-conjugated streptavidin followed. 3, 3'-diaminobenzidine tetrahydrochloride was used for staining.

2.7 | Determination of total protein

Total protein levels were determined by a previously described method (Gornall et al., 1949).

2.8 | Statistical analysis

Data are presented as Mean ± *SEM*. Comparisons between the groups were determined by one-way analysis of variance and Tukey's post hoc test (Graph Pad Prism, version 6). $p < .05$ was considered as significant.

3 | RESULTS

3.1 | Phytochemical investigations of dichloromethane: methanol extract

GC analysis of the chloroform extract from *Pterocarpus mildbraedii* identified nine major compounds, mostly fatty acids, with chain lengths between C-12 and C-20. The major constituents were C16:0 (hexadecanoic acid) and C18:0 (octadecanoic acid), at 22.65% and 13.05%, respectively. The total polyunsaturated fatty acid was 8.34%, while the saturated fatty acids and monounsaturated fatty acids were 39.81% and 7.60%, respectively (Table 1). Figure 1 represents the chromatograms of the compounds and their retention times.

3.2 | Liver/body weight ratio

The administration of PRP resulted in a significant elevation in liver weight to body weight ratio (Table 2). A nonsignificant reduction in the liver weight to body weight ratio was observed in the groups treated with PME compared with the PRP group.

3.3 | Assessment of inflammation biomarkers

A significant increase in NO and MPO levels was observed in the liver samples of rats after 7 days of exposure to PRP when compared with the control group (Figure 2). In contrast, a significant reduction of these inflammatory markers was found in the tissues of animals treated with PRP in combination with PME.

TABLE 1 Chemical composition of dichloromethane: methanol extract from *Pterocarpus mildbraedii* subjected to GC analysis

Peak #	Compounds	RT (min)	% Yield
1	Dodecanoic acid (12:0)	2.1	0.89
2	Tetradecanoic acid (14:0)	3.0	0.73
3	Hexadecanoic acid (16:0)	4.5	22.65
4	Palmitoleic acid (16:1)	5.0	0.94
5	Octadecanoic acid (18:0)	6.0	13.04
6	Oleic acid (18:1 n9)	6.5	6.66
7	Linoleic acid (18:2 n6)	7.4	1.49
8	α-linolenic acid (18:3:3)	8.0	6.85
9	Eicosanoic acid (20:0)	7.7	2.50
10	Others	–	44.25

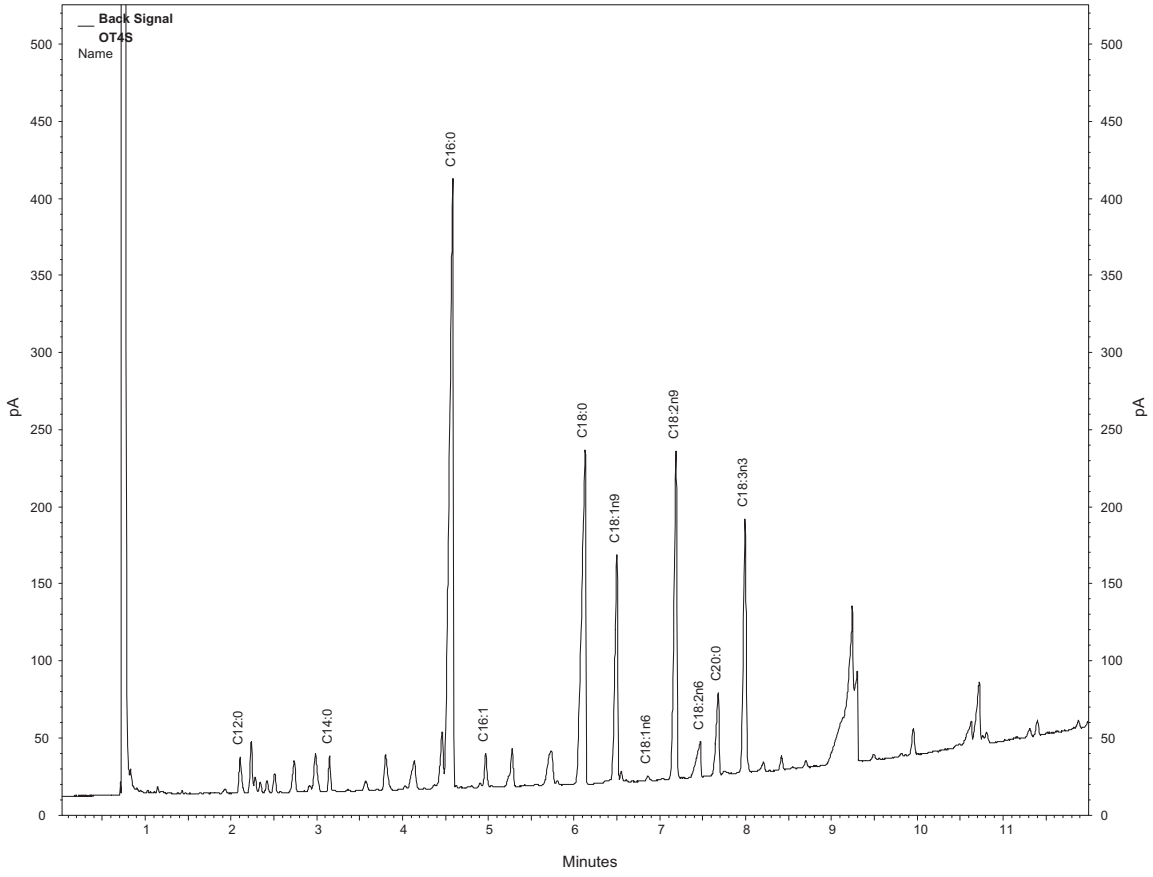

FIGURE 1 GC-MS chromatograms of identified compounds in dichloromethane: methanol *Pterocarpus mildbraedii* (PME) extract

TABLE 2 Effect of PME on body weight, absolute liver weight and liver weight to body weight ratio in rats treated with PRP

Treatment	Body weight (g)	Liver weight (g)	Liver weight: Body weight	Change in L:B ratio (%)
Control	147.80 ± 8.05	4.47 ± 0.46	0.030 ± 0.003	0
PRP	132.70 ± 6.52	5.37 ± 0.21	0.041 ± 0.002[a]	36
PME	154.30 ± 9.67	4.70 ± 0.29	0.031 ± 0.002	3
PRP + PME	161.10 ± 8.65	5.82 ± 0.41	0.036 ± 0.003	20

Note: Data expressed as Mean ± *SEM* ($n = 6$).

Abbreviations: L:B, liver weight to body weight ratio; PME, *Pterocarpus mildbraedii* extract; PRP, propanil.

[a]Significantly different from control ($p < .05$).

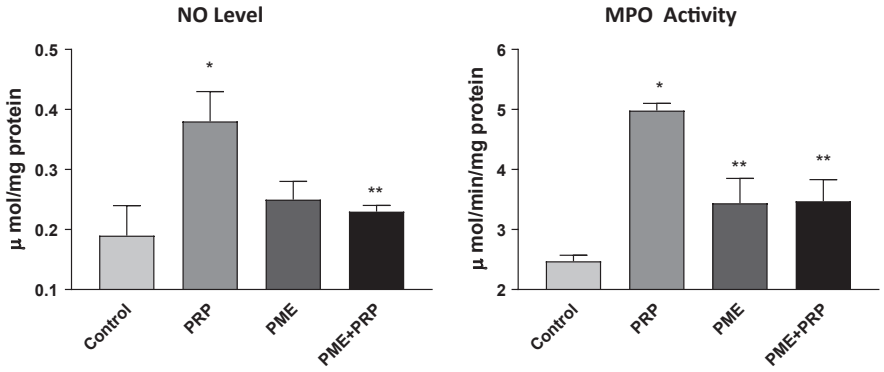

FIGURE 2 Effect of *Pterocarpus mildbraedii* (PME) extracts on levels of Nitric Oxide (NO) and Myeloperoxidase (MPO) in the liver of propanil (PRP)-exposed rats. The data are expressed as mean ± *SEM*; $n = 6$. *Values differ significantly from control ($p < .01$). **Values differ significantly from PRP ($p < .05$)

3.4 | Evaluation of inflammatory transcription factors and proteins

The effect of *Pterocarpus mildbraedii* (PME) extract on the levels of phospho-p38MAPK, phospho-SAPK/JNK, and phospho-STAT3 in PRP-exposed rats is shown in Figure 3. PRP decreased the levels of phospho-p38MAPK and phospho-STAT 3, while the levels of phospho- SAPK/JNK were increased, although these changes were not significantly different from the control groups. Treatment with PME alone significantly reduced phospho-p38MAPK and phospho-STAT3 levels, but significantly increased phospho-SAPK/JNK levels. Furthermore, the treatment of rats with PME + PRP significantly

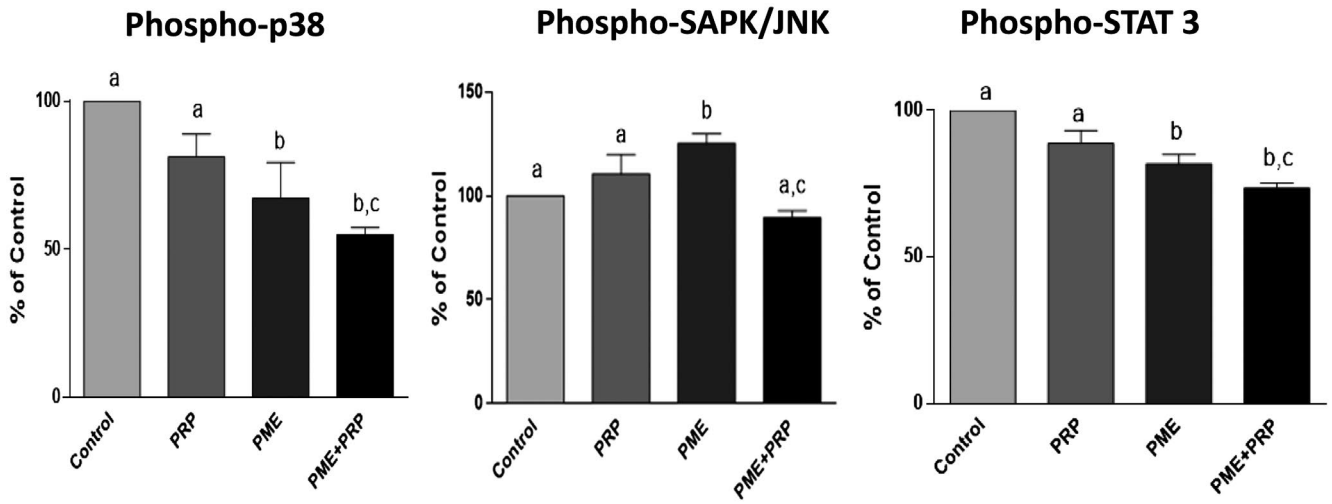

FIGURE 3 Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylation of p38 mitogen-activated protein kinase (phospho-p38 MAPK), stress-activated protein kinases/c-Jun N-terminal kinase (phospho-SAPK/JNK), and signal transducer and activator of transcription (phospho-STAT3) in propanil (PRP)-exposed rats. Results are expressed as a percentage of relative levels of phosphoproteins and are normalized to the control (100%). Values are means with their standard errors of technical duplicates. [a,b,c]mean values with different letters are significantly different

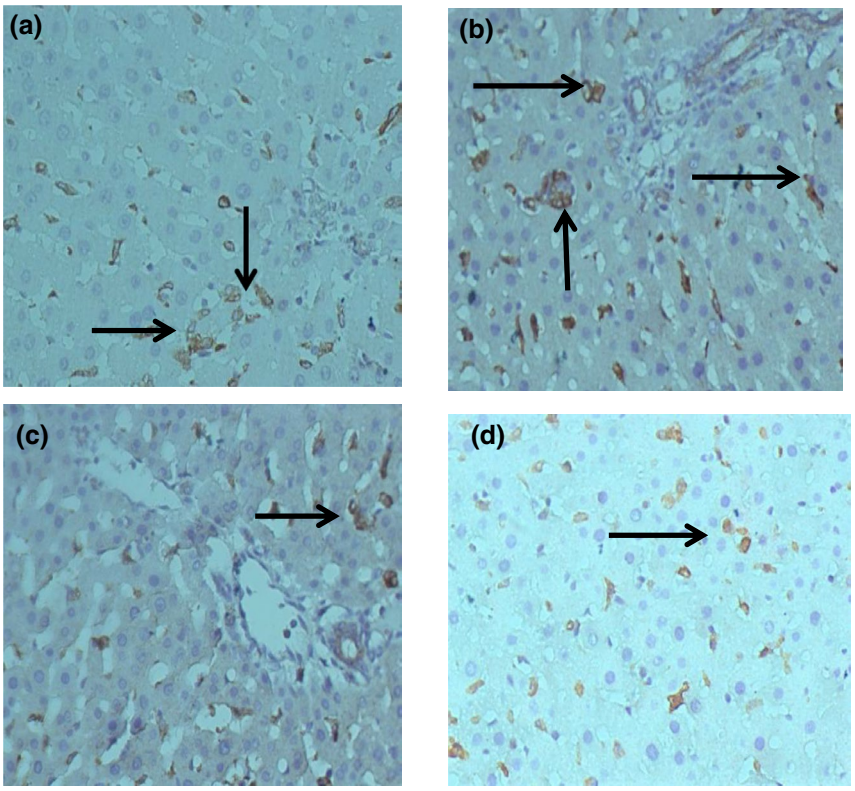

FIGURE 4 Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on cyclo-oxygenase-2 (COX-2) expression in the liver of propanil (PRP)-treated rats (a) Control (b) PRP-induced group (c) PME group, and (d) PME + PRP. A more intense expression of COX-2 was observed in the PRP-induced group when compared with other groups. Original magnification ×400

depleted the phospho-p38MAPK and phospho-STAT 3 levels when compared with the PRP group. The hepatic iNOS, COX-2, and NF-κB expressions were found to increase significantly in the PRP-treated group in comparison with the control group. However, pretreatment with PME significantly diminished the expressions of iNOS, COX-2, and NF-κB as compared to the PRP-treated group (Figures 4–6).

4 | DISCUSSION

This research evaluated the protective potential of *Pterocarpus mildbraedii* extract in male rats exposed to propanil for 7 days. PME protected against PRP-induced increases in inflammatory markers in the liver of rats. Also, PME prevented PRP-induced increase in the liver: body weight ratio in rats after treatment for 7 days.

Higher organisms have evolved several defense mechanisms to protect them from harmful stimuli. One of these mechanisms, the inflammatory response, involves a complex interplay of cellular and molecular processes at the site of infection and injury aimed at restoring homeostasis in the affected tissue. Unfortunately, these immune-mediated responses could be dysregulated, leading to diseased conditions, including liver disorders (Choudhari et al., 2013). NO is the product of Arginine in a reaction catalyzed by the nitric oxide synthases. Interestingly, the constitutively expressed neuronal and endothelial nitric oxide synthases play physiological roles, while the inducible isoform iNOS, under certain deleterious stimuli, produces a pro-inflammatory effect due to increased NO production (Förstermann & Sessa, 2012). The elevated level of NO in rats fed with PRP could lead to the formation of the obnoxious nitrite anion as a result of excess NO reacting with superoxide anion. Similar elevations in NO concentration in rodent models of chemically induced liver injury have been reported (Abolaji et al., 2017; Freitag et al., 2015). Treatment of rats with PME, at the dose of 200 mg/kg, diminished over-production of NO when compared with the PRP group.

Furthermore, results from this study showed that the treatment of rats with PRP produced a significant increase in hepatic MPO activity, suggesting infiltration, and accumulation of polymorphonuclear leukocytes in the tissue. This observation, which supports the inflammatory mechanism in propanil hepatotoxicity, corroborates similar findings from recent studies (Otuechere, Adewuyi, et al., 2020; Owumi & Dim, 2019). PME-induced depletion of MPO activity could increase $NO^{\cdot}$ bioactivity and subsequently retard oxidative stress in the liver. The mitogen-activated protein kinases (MAPKs) are mediators of intracellular signaling in response to several stressors, and inhibitors of MAPK pathways could exhibit anti-inflammatory effects (Xiao et al., 2020). In our study, p38 MAPK activity was non-significantly altered by PRP, an

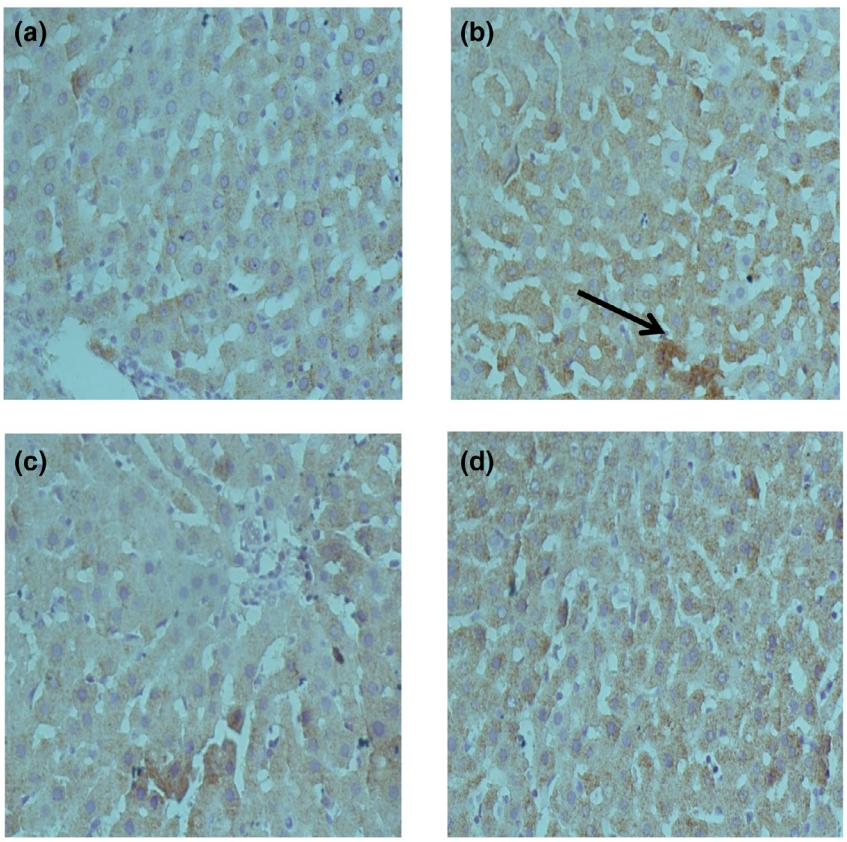

FIGURE 5 Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on inducible nitric oxide synthase (iNOS) expression in the liver of propanil (PRP)-treated rats. (a) Control (b) PRP-induced group, (c) PME group; and (d) PME + PRP. Abnormal expression of iNOS was observed in the PRP-induced group when compared with other groups. Original magnification 400

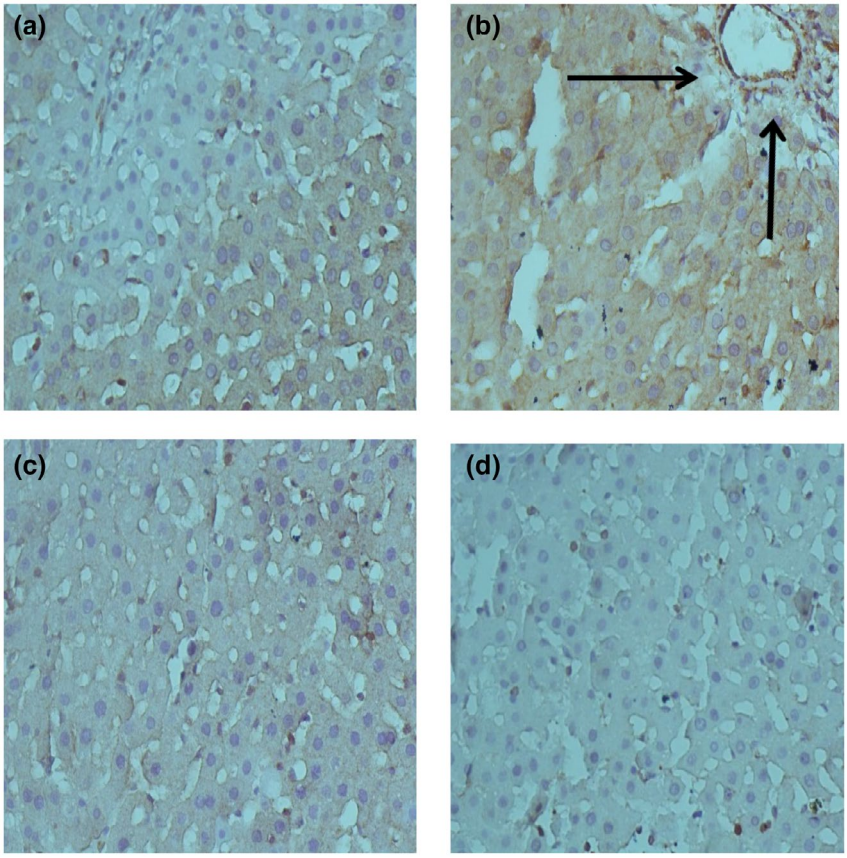

FIGURE 6 Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on nuclear factor-κB (NFκB) expression in the liver of propanil (PRP)-treated rats. (a) Control (b) PRP-induced group, (c) PME group; and (d) PME + PRP. Abnormal expression of NFκB was observed in the PRP-induced group when compared with other groups. Original magnification ×400

indication that PRP had no induction effect on p38 phosphorylation. Similar effects have been shown in the selective activation of the phosphorylation of ERK1/2 and C-Jun, but not p38 MAPK by organochlorine insecticides in HaCat cells (Ledirac et al., 2005). Interestingly, PME alone and in combination with PRP further suppressed p38 MAPK induction, and this is an indication of their anti-inflammatory effects. Previously, sakuranetin, a flavonoid obtained from *Baccharis retusa*, had been shown to inhibit the activation of p38MAPK and STAT-3 in a murine experimental asthma model (Santana et al., 2019). The observed PME- mediated decrease in phosphorylation of STAT-3 seemed to parallel p38 MAPK downregulation, correlating an earlier report that p38 MAPK is an upstream activator of STAT-3 (Cheng et al., 2017). In this study, PME also elicited an increase in p-SAPK/JNK levels. A similar adaptive response has been observed after a single dose administration of *Rhodiola rosea* in male Chinchilla rabbits (Panossian et al., 2007). Nonetheless, the co-administration of PME and PRP significantly inhibited SAPK/JNK phosphorylation.

NF-κB is a versatile transcription factor moderating the activation of several genes involved in the pro-inflammatory responses to stressors such as COX-2 and iNOS. Exposure of rats to PME for 7 consecutive days diminished PRP induced NF-κB expression in the liver of rats. Our findings agree with a similar report on the inhibitory effect of D-limonene on carbon tetrachloride-induced increases in the expression of NF-κB (Ahmad et al., 2018). Another study reported that oligonol, a phenolic product from *Litchi chinensis* Sonn. Extract, ameliorated CCl_4-induced liver injury in rats via the NF-κB pathway (Bak et al., 2016). Possibly, the inhibition of NF-κB activity by PME is a potential therapeutic target for chemical-induced hepatotoxicity. Also, our study revealed increased iNOS and COX-2 protein expressions in the hepatic tissue of rats exposed to PRP. Since iNOS is induced in response to inflammation, this also confirms our observations with NO. The association between COX-2 overexpression and diseases has been well documented. Moreover, hepatic COX-2 overexpression had been shown to induce spontaneous hepatocellular carcinoma formation in mice (Chen et al., 2017). So the increased expression of COX-2, as shown in this study, is evidence of PRP-induced toxicity. However, treatment with PME ameliorated PRP-induced overexpression of these inflammatory proteins. Other research groups have also suggested the involvement of iNOS and COX-2 in the anti-inflammatory mechanisms exerted by bioactive molecules. For instance, the administration of flavonoid-rich extract of orange juice diminished iNOS in the colon of dinitrobenzene sulfonic acid-treated mice (Fusco et al., 2017). Wali, Rashid, et al. (2020) reported that naringenin, at the doses of 50 and 100 mg/kg, decreased the expressions of COX-2 in experimental hepatic inflammation triggered by doxorubicin.

Vegetables, cheap and readily available, had long been identified as sources of biologically active nutraceuticals (Arya et al., 2019). Similar to our findings, the GC-MS analyses of the ethanolic extracts of *Pistia stratiotes and Eichhornia crassipes* leaves revealed hexadecanoic acid, hexadecanoic acid (ethyl ester), palmitic acid (ethyl ester), 9, 12-Octadecadienoic acid, (ethyl ester), Linolenic acid (ethyl ester) as the major constituents (Tyagi & Agarwal, 2017). Furthermore, the hepatoprotective effects of the ethanolic extract of *Vitis vinifera* seeds in diabetic rats have been attributed to the presence of fatty acids such as hexadecanoic, octadecadienoic, and octadecanoic acids (Giribabu et al., 2018). The potential of PME as a functional food is boosted by the presence of α-linolenic acid, an omega-3-fatty acid that has been reported to possess anti-inflammatory activity (Ros et al., 2018; Wen et al., 2019). However, additional characterization studies are necessary to delineate the specific active principles responsible for the pharmacological action of *Pterocarpus mildbraedii*.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the article.

AUTHOR CONTRIBUTION

Chiagoziem A. Otuechere: Formal analysis; Investigation; Project administration; Software; Writing-original draft; Writing-review & editing. **Ebenezer O. Farombi:** Conceptualization; Data curation; Methodology; Supervision; Validation; Visualization; Writing-review & editing.

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