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Archives of Applied Science Research, 2010, 2 (4): 191-198

(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-508X
CODEN (USA) AASRC9

Methanol extract of *Phyllanthus niruri* attenuates chlorpyrifos-evoked erythrocyte fragility and lipoperoxidative changes in wistar rats

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Abstract

Experiments were conducted with the aim of evaluating the ameliorative effect of methanolic extract of Phyllanthus niruri (MEPN) on chlorpyrifos (CPF)-evoked erythrocyte fragility and lipoperoxidative changes in Wistar rats. Thirty five rats divided into 5 groups of 7 animals in each group served as subjects for this study. Rats in group I were given only soya oil (2 ml/kg) while those in group II were administered with CPF only (10.6 mg/kg ~1/8th LD_{50} determined in a previous study). Rats in group III were given MEPN only (500 mg/kg). Rats in group IV were pretreated with MEPN (250 mg/kg) and then dosed with CPF (10.6 mg/kg), 30 minutes later. Rats in group V were pretreated with MEPN (500 mg/kg) and then CPF (10.6 mg/kg), 30 minutes later. The different regimens were orally administered once daily for a period of 28 days. At the end of the study, the rats were sacrificed and blood samples collected were evaluated for osmotic fragility, while the erythrocyte packets prepared from another batch of blood samples were examined for malonaldehyde (MDA) concentration. The results showed that rats exposed to CPF only demonstrated a significant increase in erythrocyte fragility and MDA concentration compared to the other groups. This study indicated that MEPN protected rats from CPF-induced increased erythrocyte fragility and lipoperoxidative changes, probably as a result of its flavonoid contents, which has been shown to have antioxidant activity.

Key words: Organophosphate, Chlorpyrifos, *Phyllanthus niruri*, Extract, lipoperoxidation, erythrocyte fragility.

INTRODUCTION

Organophosphate (OP) compounds are one of the most widely used insecticides in agriculture and public health, accounting for 50% of the global insecticidal use [1]. The nearly ubiquitous

nature of OP in terms of accessibility and extensive usage has led to increasing concern about its toxicity. OP toxicity is an important public health problem especially in developing countries, which paradoxically account for ¼ of the global use [2]. The propensity of OP insecticides to cause toxicity at exposure that goes undetected due to the absence of overt systemic signs has heightened the public health concern, especially in occupationally exposed individuals. The mechanism of toxicity of OP is related to acetylcholinesterase inhibition resulting in accumulation of acetylcholine in the cholinergic receptors of the peripheral and central nervous systems. However, oxidative stress is becoming an increasingly important molecular mechanism in OP-induced toxicity [3-5]. Oxidative stress results from the generation of free radicals and alteration in free radical scavenging system.

Chlorpyrifos (CPF) is a phosphorothionate chlorinated broad spectrum OP insecticidal compound that has widespread domestic, agriculture and public health applications. Although, the United States Environmental Protection Agency in 2000 restricted some of its domestic uses, CPF still remains one of most widely used OP insecticides. Anemia has been observed following chronic exposure to CPF in rats [6]. Although the molecular mechanism of the anemia has not been elucidated, earlier studies have shown that CPF causes lipoperoxidative changes in the erythrocyte membrane [3,5,7]. Oxidative stress is one of the complex factors that determine the integrity of the erythrocytes [8]. The erythrocyte osmotic fragility is frequently used as a measure of the tensile strength of the red blood cells [9]. We have earlier shown that vitamin C ameliorated lipoperoxidative changes and increased erythrocyte fragility induced by repeated CPF exposure [5].

Phyllanthus niruri is an annual erect glabrous herb that is found in tropical and subtropical regions of the world [10-12]. It has been used in herbal medicine worldwide for centuries where it grows [13] for the treatment of various ailments including diabetes, malaria, colic, fever, jaundice, kidney and gall bladder stones, tuberculosis, bacterial infections such as cystitis, prostatitis, gonorrhea, urinary tract infections and viral infections [14]. The plant is a rich source of phytochemicals such as alkaloids, astragalin, brevifolin, carboxylic acids, corilagin, geraniin, hypophyllanthin, lignans, methyl salicylate, terpenes, tanins, saponins and flavonoids, such as quercetin, quercetol, quercetrin and rutin [15-17]. The flavonoid contents of the plant are suggested to be responsible for its antioxidants properties [18], and may have been responsible for most of its medicinal uses. The physiological effects of flavonoids are particularly significant in those pathologies where the oxidative stress hypothesis is accepted and supported by experimental data [19]. Flavonoids, which are polyphenolic compounds found in plants have been shown to be capable of scavenging superoxide anions [20] and hydroxyl radicals [21], in addition to its efficient iron chelating activity [22]. Polyphenolic compounds are capable of elevating the redox and antioxidant level [23]. In red blood cells, polyphenols enhance cell resistance to oxidative insult [24]. Therefore, the aim of the present study was to evaluate the attenuative effect of methanolic extract of *P. niruri* (MEPN) on erythrocyte fragility and lipoperoxidative changes evoked by subchronic CPF exposure in Wistar rats.

MATERIALS AND METHODS

Experimental animals

Thirty five male Wistar rats (12-14 weeks old) weighing 115-126g used for this study were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. The animals were housed in metal cages and fed on pellets made from grower's mash, maize bran and groundnut cake in the ratio of 4:2:1, and water was provided *ad libitum*.

Plant Collection, Identification and Preparations

Phyllanthus niruri was collected from Kano, Nigeria. It was taxonomically identified and authenticated in the Herbarium of the Department of Biological Science, Ahmadu Bello University, Zaria, with voucher No 2522. The plant was air dried under shade and then subjected to powder using mortar and pestle. The powdered material was extracted with methanol at a ratio of 1:5 w/v in a glass funnel for 24 hours, with periodic shaking to enhance the extraction process. Thereafter, the solution was filtered and the filtrate concentrated *in-vacuo* using the rotary evaporator coupled to a thermocirculator. The residue labeled methanolic extract of *Phyllanthus niruri* (MEPN) was further air-dried to a constant weight and then preserved in a refrigerator at 4°C.

Chemical acquisition and preparations

Commercial grade CPF (20% EC) marketed as Termicot® (Sabero Organics, Gujarat limited, India) was prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution.

Dosing schedules

The rats were weighed and then assigned at random into 5 groups of 7 rats in each group. Rats in group I served as the control group (labeled S/oil) and were given only soya oil at the dose of 2ml/kg while those in group II (labeled CPF) were administered with CPF only at a dose of 10.6 mg/kg (~1/8th LD_{50} of 85 mg/kg determined in a previous study). Rats in group III were dosed with the extract only (500 mg/kg, randomly chosen following inability to determine the LD_{50}). Rats in group IV (labeled 250 MEPN+CPF) were pretreated with MEPN (250 mg/kg) and then dosed with CPF (10.6 mg/kg), 30 minutes later. Rats in group V (labeled 500MEPN+CPF) were pretreated with MEPN (500 mg/kg) and then CPF (10.6 mg/kg), 30 minutes later. The different regimens were orally administered once daily for a period of 28 days. At the end of the study period, the rats were sacrificed by severing the jugular vein after light ether anesthesia and blood was collected into heparinized sample bottles. The study was carried out according to the specification of the Ahmadu Bello University Animal Research Committee and the Helsinki Declaration.

Evaluation of in vitro erythrocyte osmotic fragility

In vitro erythrocyte osmotic fragility was then evaluated in all the rats in each group using the method described by Faulkner and King [25] as modified by Oyewale [26] using different concentrations of sodium chloride (pH 7.4) from 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/L. Briefly, freshly obtained whole blood from each rat was pipetted into the test tubes containing varying concentrations of NaCl and then followed by careful mixing and incubation for 30 minutes at

room temperature, 26-28˚C. The test tubes were then centrifuged at 2000 x g for 10 minutes using a centrifuge model IEC HN-SII (Damon/IEC Division, UK). The supernatant was transferred into a glass cuvette and the absorbance of the supernatant measured colorimetrically with Spectronic 20 (Bausch and Lomb, USA) at wavelength of 540 nm. The percent hemolysis for each sample was then calculated using the following formula:

$$\% \text{ hemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}} \times 100$$

Evaluation of erythrocyte malonaldehyde concentration

2.5 ml of heparinized blood samples obtained from each animal was centrifuged at 3000 x g and the plasma discarded. Erythrocyte packets were prepared by washing erythrocytes three times in cold isotonic saline (0.9% w/v). The washed erythrocytes were used to analyse for malondialdehyde (MDA) concentrations using the double heating method of Draper and Hadley [27] as modified by Altuntas *et al*. [28]. The principle of the method was based on spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 2.5 ml of 100 g/L trichloroacetic acid solution was added to 0.5 ml of erythrocytes in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 x g for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g/L TBA in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance measured using a UV spectrophotometer (Jenway, 6405 model, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex, 1.56×10^5 cm^{-1} M^{-1}, and expressed in nanomoles per gram of hemoglobin. The hemoglobin concentration was determined on the packed erythrocytes using the method of Dacie and Lewis [29].

Statistical analysis

Values obtained as mean±SEM were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, using GraphPad Prism version 4.0 for windows from GraphPad Software, San Diego, California, USA. Values of $p < 0.05$ were considered significant.

RESULTS

***In vitro* erythrocyte osmotic fragility**

The effect of treatments on erythrocyte fragility is shown in Figure 1. The percentage erythrocyte fragility was significantly higher ($p < 0.05$) in the CPF group compared to the other groups at 0.9% NaCl concentration. At 0.7% NaCl concentration, the percentage erythrocyte fragility in the CPF group was significantly higher ($P < 0.01$) compared to the other groups. The value obtained at 0.5% NaCl concentration was higher in the CPF group compared to S/oil ($p < 0.05$), MEPN ($p<0.01$), 250MEPN+CPF ($p<0.05$) and 500MEPN+CPF ($p<0.05$).

Erythrocyte malonaldehyde concentration

The MDA concentration in the CPF group was significantly higher ($p<0.01$) compared to the other groups. The MDA level in the MEPN group was significantly lower compared to S/oil ($p<0.05$), 250MEPN+CPF ($p<0.01$) and 500MEPN+CPF ($p<0.01$). No significant change

(p>0.05) in MDA level in the 250MEPN+CPF group compared to 500MEPN+CPF group (Figure 2).

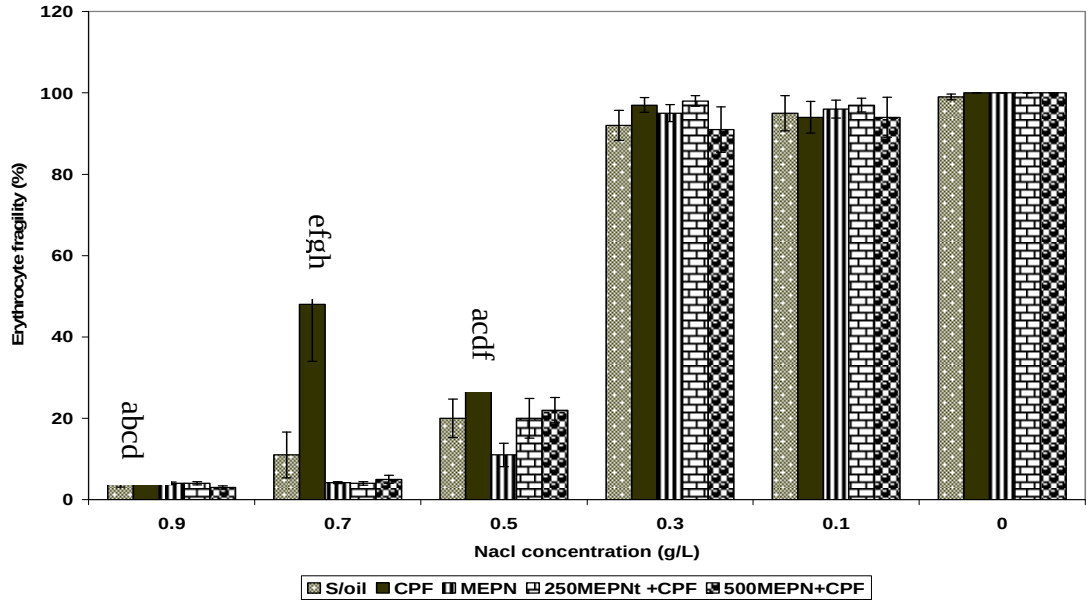

Figure 1: Effect of chlorpyrifos and/or methanolic extract of *Phyllanthus niruri* on erythrocyte osmotic fragility

[a]p<0.05 versus soya oil group; [b]p<0.05 versus extract group; [c]p<0.05 versus 250MEPN+CPF group; [d]p<0.05 versus 500MEPN+CPF group; [e]<0.01 versus soya oil group; [f]p<0.01 versus extract group; [g]p<0.01 versus 250MEPN+CPF group; [h]p<0.01 versus 500MEPN+CPF group. Values are mean ± SEM of 7 animals per group

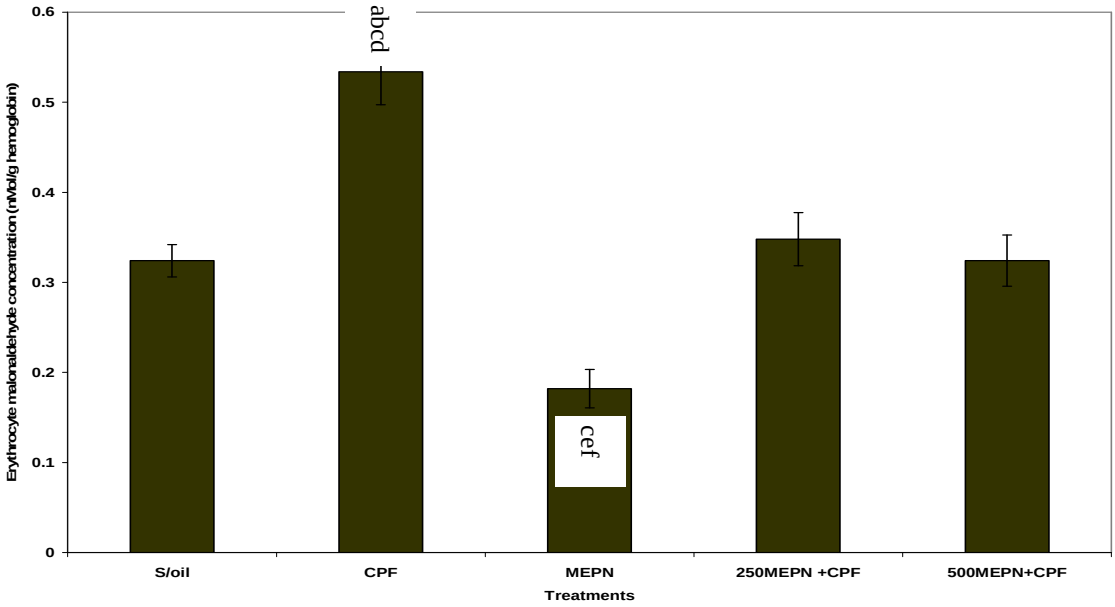

Figure 2: Effect of treatments on erythrocyte malonaldehyde concentration

[a]p<0.01 versus soya oil group; [b]p<0.01 versus MEPN group; [c]p<0.01 versus 250MEPN+CPF group; [d]p<0.01 versus 500MEPN+CPF group; [e]p<0.05 versus soya oil group; [f]p<0.05 versus 500MEPN+CPF group. Values are mean ± SEM of 7 animals per group.

DISCUSSION

The present study has shown that pretreatment with MEPN ameliorated the CPF-induced erythrocyte fragility and lipoperoxidative changes in Wistar rats. Earlier studies have shown that CPF exposure increases the red cell membrane MDA concentration [3,5,7]. The significantly increased MDA concentration in rats exposed to CPF showed that the insecticide promotes the peroxidation of lipid membranes of the erythrocytes. MDA is a marker of membrane lipid peroxidation resulting from interaction of reactive oxygen species and the cellular membranes [30]. Peroxidation of the polyunsaturated fatty acid (PUFA) components of the erythrocyte cell membranes leads to disruption in the integrity of the membrane lipid bilayer and/or alter the boundary lipid microenvironment of membrane proteins especially those that maintain selective transmembrane ionic and potential gradients [31,32]. Lipid peroxidation products depress the activity of Na-K ATPase [33-35]. By-products of lipid peroxidation causes profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids [36]. This CPF-induced lipoperoxidative alteration in the structural and functional components of the erythrocyte membranes may have caused perturbation in the membrane integrity resulting in increased erythrocyte fragility. This indicates that anemia observed in rats repeatedly exposed to CPF [6] may have been partly due to increased erythrocyte fragility arising from lipoperoxidative damage to the erythrocyte membranes. The RBC is vulnerable to lipoperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reactions and availability of high amount of PUFA, which is susceptible to lipid peroxidation. Inability to repair membrane damage and regenerate, and poor antioxidant enzymes composition of the plasma medium in which they are bathed are some of the other factors that enhance the vulnerability of the RBC to lipid peroxidation [37,38].

The decrease in MDA concentration and erythrocyte fragility in the two groups pretreated with MEPN demonstrated the protective effect of the extract in CPF-induced oxidative changes. No significant difference in MDA concentrations and % erythrocyte fragility was recorded in rats pretreated with 250 mg/kg of MEPN compared to those given 500 mg/kg MEPN. The protective effect of MEPN may have been due to the polyphenolic compounds, flavonoids, contained in the extract [39].

The antioxidant activity of flavonoids is due to their ability to donate a hydrogen atom from an aromatic hydroxyl group to a free radical, and yield a resonance-stabilized phenolic radical [40]. Furthermore, the amphiphilicity of the flavonoids enhances their ability to trap chain-initiating radicals at the interface of the membranes, preventing progression of the radical chain reaction [40, 41]. In addition to radical scavenging properties in aqueous and organic environments, the antioxidant activity of flavonoids is exacerbated by the protection or enhancement of endogenous antioxidants, as well as their ability to chelate transition metal ions, and inhibit the propagation of the lipoxygenase reaction [40,42]. Accumulating evidence suggests that beneficial effects of flavonoids may be mediated by interactions with cellular components of protein kinase and lipid kinase signalling cascades such as phosphoinositide 3-kinase, protein kinase C and MAP kinases [43-46]. The interaction of flavonoids with these signalling pathways leads to the transcriptional modulation of different genes [47,48]. Therefore, the modulation of signalling pathways and

gene expression exerted by flavonoids provides a wide base for the physiological effects ascribed to these compounds. Equally, polyphenolic compounds stimulate detoxifying enzymes [49]. Indeed, these compounds have been shown to have paraoxonase- 1 (PON-I) gene promoter activity [50], resulting in increased expression of PON-I, a high density serum lipoprotein that plays an essential role in the detoxification of organophosphate compounds [4]. The summation of all these properties of flavonoids may have been responsible for its ameliorative effect on increased erythrocyte fragility and oxidative changes observed in rats repeatedly exposed to CPF.

CONCLUSION

The present study has shown that MEPN ameliorated increase in erythrocyte fragility and oxidative changes evoked by subacute CPF administration in Wistar rats. This action by MEPN is probably mediated by the flavonoid contents of the plant partly through its antioxidant effect. This shows that *P. niruri* may be useful in protecting individuals from the adverse effect arising from constant exposure to CPF.

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