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ARTICLES

Full Length Research Paper

Phenytoin: Is it genotoxic in isolated cultured human lymphocytes without metabolic activation by S9?

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There are many conflicting reports around the phenytoin (PHT)-induced genotoxic effect especially in the *in-vitro* studies. PHT was claimed to cause genotoxic effect by the oxidative stress of its metabolic intermediates. However, by reviewing the distribution and activity of the enzymes responsible for PHT metabolism, we found that PHT is rarely metabolized by human lymphocytes. So that, we will use isolated cultured human lymphocytes to determine which is genotoxic, PHT itself or its metabolites? PHT 60 µg/ml were added to lymphocytes before and after metabolic activation by S9. Also, this study will investigate the possible antioxidant genoprotective effects of Thymoquinone (TQ) 1 µM and Curcumin (CMN) 15 µM on the chromosomal injury induced by PHT or its metabolites. After the end of culture period, the effects of PHT on the lymphocytes were investigated by measuring levels of chromosomal aberrations (CAs); mitotic index (MI); reduced glutathione (GSH); malondialdehyde (MDA); and 8-hydroxydeoxyguanosine (8-OH-dG). Only PHT after metabolic activation caused oxidative genotoxic effects which were significantly ameliorated by TQ more than CMN. Hence, the present study is the first to record that PHT without metabolic activation in isolated human lymphocytes from non epileptic donors cause dose dependant direct toxic effect rather than genotoxic effect.

Key words: Phenytoin, thymoquinone, curcumin, genotoxic

INTRODUCTION

Genotoxins are compounds causing chemical or physical alterations in DNA structure leading to inaccurate replication of that region of the genome (Bajpayee et al., 2005). Approximately 30% of all marketed drugs, exhibit genotoxic effect when tested by the standard genetic toxicology tests (Snyder, 2009).

Phenytoin (PHT), the well known antiepileptic drug has been suspected for teratogenic and mutagenic effects during pregnancy (Kaul et al., 2001). However, there are many conflicting reports observed around its genotoxic effect especially in the *in-vitro* studies (NTP, 1993; IARC, 1996; Snyder and Greenb, 2001; Snyder, 2009). PHT was

claimed to cause genotoxic effect by the oxidative stress of its metabolic intermediates. However, by reviewing the distribution and activity of the enzymes responsible for PHT metabolism, we found that PHT is rarely metabolized by human lymphocytes (Basta-Kaim et al., 2008).

For testing indirect chemical mutagens, human lymphocyte was exposed directly to an Ames-type microsomal (S9) activation system (Sbrana et al., 1984).

Many naturally occurring compounds have been reported to have anti-mutagenic activities (El Hamss et al., 2003). Curcumin (CMN), the active constituent in the rhizomes of Curcuma longa, is a nutriceutical compound with antioxidant (Iqbal et al., 2009) and antimutagenic effects (Corona-Rivera et al., 2007). In addition, Thymoquinone (TQ), is another known antioxidant (Badary et al., 2003) have been reported to exert antimutagenic activity in few studies (Badary et al., 2007; Abou Gabal et al., 2007). However, neither TQ nor CMN were tried yet to protect against PHT-induced genotoxicity in isolated human lymphocytes.

The aim of the present study is to use isolated cultured human lymphocytes to determine which genotoxic, PHT itself is or its metabolites after metabolic activation by S9? Also, this study will investigate the possible antioxidant genoprotective effects of Thymoquinone and Curcumin on the chromosomal injury induced by either PHT or its metabolites.

MATERIALS AND METHODS

Human blood samples

10 ml fresh venous blood samples were taken from 30 adult donors after consent. All donors were of either sexes between the ages of 20-45 years, apparently healthy, non-smoking, non-alcoholic and they did not take any medications recently. The donors were obtained from the blood-banking Center of Mansoura University Hospital, Mansoura Faculty of Medicine, Egypt. All blood samples were taken on heparin to prevent clotting.

Chemicals

All chemicals and reagents used in this study were of the highest analytical grade from Sigma-Aldrich (St. Louis, MO, USA). Phenytoin was purchased as sodium salt ≥ 99%, 25 g soluble in water.

Isolation and culture of human lymphocytes

Lymphocytes were isolated from whole blood samples and cultured as described by Durante et al. (1998) with minor modification. All blood samples were collected in an isolation tube for blood cells. The sample was centrifuged at 1600 g (2900 rpm) for 20 min, and the layer of mononuclear cells and platelets was collected by a pipette and transferred to 10 ml centrifuge tube. PRMI 1640 medium was added up to 10 ml. and the sample was centrifuged at 390 g (1500 rpm) for 10 min. After the removal of the supernatant, the cell pellet was re-suspended in 10 ml RPMI 1640 medium at a density of 1.0×10^6 cells/ml. Isolated lymphocytes from each blood sample were cultured in 10 ml RPMI 1640 culture medium for a total period of 72 h at 37°C in the dark in a 5% CO_2 humidified atmosphere (Watson, 1992).

Plan of the study and grouping of isolated lymphocytes

Isolated lymphocytes from each blood samples were divided randomly into 5 groups, each of 6 samples: the 1st group was none treated; the 2nd treated with PHT 60 ug/ml (Ponnala et al., 2009); the 3rd treated with PHT 60 ug/ml plus S9 (Sbrana et al., 1984, Ponnala et al., 2009); the 4th treated with PHT 60 ug/ml (Ponnala et al., 2009) + S9 + TQ (Khader et al., 2009); the 5th treated with PHT 60 ug/ml + S9 + CMN (Siddique et al, 2010). The potential genotoxic drugs were added twice, at 24 and 48 h from the start of culture period and after stimulation of mitotic division with phytohaemagglutinin that was added at the start of culture period to induce mitosis within 24 h according to standard protocol of Poddar et al. (2004). The genoprotective drugs were added as a prophylactic therapy 2 h prior to addition of the genotoxic drug.

In a pilot study we tried to increase PHT dose above 60 ug/ml (90 & 120), but these doses were very toxic and fatal to isolated lymphocytes as indicated by MI, so that we did not include these fatal doses in this study.

Evaluation of the drug effects

To investigate the chromosomal effect induced by PHT before and after metabolic activation cells were harvested at the end of the culture period (72 h) for screening CAs following the standard protocol (Carrano and Natarajan 1988) in order to avoid heterogenecity of cycle stage of the treated cells and to score only the first division mitotic cells. Colcemid 0.1 ml was added to stop mitosis and prevent spindle formation and was left 1.5 h. The isolated lymphocytes after recovery from the incubator were investigated for chromosomal aberrations (CAs), mitotic index (MI), 8-hydroxy-2'-deoxyguanosine (8-OH-dG), reduced glutathione (GSH) and malondialdehyde (MDA).

Assay of chromosomal aberrations (Karyotype) in isolated lymphocytes using Gimsa stain

It was done according to the protocol of Poddar et al. (2004). Cells were stained using 10% Giemsa for 12 minutes immersed in distilled water for washing and air-dried. Analysis of cytogenetic data was performed using light microscopy. Slides were scored blind and individual aberrations were recorded. Fifty metaphases were examined for each sample in the different groups (300 metaphase for each group), searching for any chromosomal anomalies either structurally or numerically.

Determination of mitotic index (MI) as a measure of cytotoxicity

The mitotic index (MI) was used as indicators of adequate cell proliferation. Its inhibition could be considered as cellular death, or delay in the cell proliferation kinetics (Eroğlu, 2011). The mitotic index evaluates the cytotoxicity of chemical agents (Calderón-Ezquerro et al., 2007). MI, is easily assessed when CAs are performed. The number of lymphocytes in metaphase was counted in 2000 lymphocytes per sample to determine the mitotic index (Kannan et al., 2006).

Measurement of intracellular reduced glutathione (GSH)

Intracellular reduced GSH in the isolated lymphocytes was
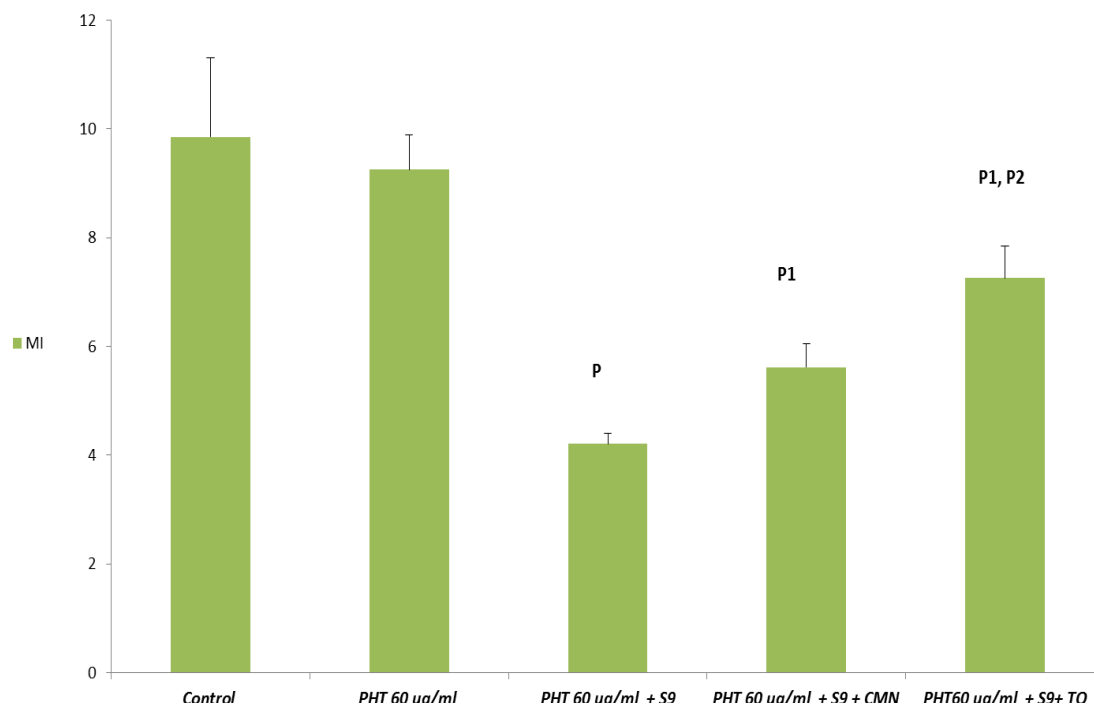
Figure 1. *In vitro* effects of PHT 60 μg/ml alone; PHT + s9 combined with either TQ 1 μM or CNM 15 μM on mitotic index (MI) in isolated human lymphocytes after 72 h of incubation (one way ANOVA, mean ± SD). Each group consists of 6 samples, 50 metaphases were examined for each sample = 300 metaphase for each group, c SD = standard deviation, d MI was obtained for each sample by counting metaphases in 2000 cells. P = significant difference when compared with control normal group, P1 = significant difference when compared with PHT + S9 group, P2 = significant when compared with PHT 60 + S9 + CMN

extracted according to the method described by Anderson, (1985), and then reduced GSH was measured according to the method described by Beulter et al. (1963) employing colorimetric method using spectrophotometer determination method (JENWAY 6405, spectrophotometer).

Measurement of malondialdehyde level (MDA)

Lipid peroxidation products (MDA) were released from isolated lymphocytes by sonication according to the method described by Stacey and Klaassen (1981). Then MDA was measured by thiobarbituric acid (TBA) test according to the method described by Draper and Hadley (1990), employing colorimetric method using spectrophotometer determination method (JENWAY 6405, spectrophotometer).

Measurement of 8-hydroxy-2-deoxy Guanosine (8-OH-dG)

The 8-OH-dG was assayed using Cayman 8-hydroxy-2-deoxy Guanosine enzyme-linked immunosorbent assay (elisa = EIA) Kit (Cayman Chemical's ACE™, USA). Cayman's 8-OH-dG EIA is a competitive assay that can be used for the quantification of 8-OH-dG in urine, cell culture, plasma, and other sample matrices. This assay is based on the competition between 8-OH-dG and 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for a limited amount of 8-OH-dG Monoclonal Antibody (Pradelles et al., 1985; Maclouf et al., 1987).

Statistical analysis of the data

All statistical calculations of the data were performed with SPSS® version 21. Multiple comparisons of the data for each biochemical parameter were performed using one-way analysis of variance (ANOVA) followed by post-Hoc test for comparing the different groups with each other. P-value of ≤ 0.05 was considered significant.

RESULTS

The assessment of the types of chromosomal aberrations (CAs) in the isolated cultured human lymphocytes in all groups of this study caused only structural CAs and no numerical CAs were found.

In vitro **effects of Phenytoin (PHT) 60 μg/ml:** There were insignificant changes in the CAs, MI, MDA, GSH, or 8-OH-dG levels when compared to the non-treated normal group (Figures 1, 2, 3, 4, 5).

In vitro **effects of Phenytoin (PHT) 60 μg/ml plus S9:** They caused significant increase in the structural CAs when compared to control normal group (Figure 2). They caused also cytotoxicity and decrease in lymphocyte proliferation indicated by significant decrease in the MI

Figure 2. *In vitro* effects of PHT 60 µg/ml alone; PHT + s9 and PHT + s9 combined with either TQ 1 µM or CMN 15 µM on structural chromosomal aberrations (CAs) in isolated human lymphocytes after 72 h of incubation (one way ANOVA, mean ± SD). P = significant difference when compared with control normal group, P1 = significant difference when compared with PHT + S9 group, P2 = significant when compared with PHT 60 + S9 + CMN

when compared to control normal group (Figure 1). In addition, there was significant increase in the MDA level, 8-OH-dG level and significant decrease in the GSH level when compared to control group (Figures 1, 2, 3, 4, 5).

The effects of combined use of PHT +S9 with Thymoquinone (TQ) or Curcumin (CMN): The combined use of PHT +S9 with TQ 1 µM, or CMN 15 µM caused significant decrease in the structural CAs when compared to PHT +S9 treated group (Figure 2). It caused also improvement in lymphocyte proliferation indicated by significant increase in the MI when compared to PHT + S9 group (Figure 1). In addition, there was significant decrease in the MDA and 8-OH-dG levels together with significant increase in the GSH level when compared to PHT + S9 group alone (Figures 3, 4, 5). However, the protective effects of PHT + S9 with TQ treated group on CAs, MI, MDA, GSH, and 8-OH-dG were more significant than the protective effects of PHT + S9 with CMN (Figures 1, 2, 3, 4, 5). This means that PHT + S9 - induced toxic changes were significantly ameliorated by TQ more than CMN.

DISCUSSION

In vitro effects of phenytoin (PHT) and PHT + S9 on the isolated cultured human lymphocytes

To the best of our knowledge, this study was the first to

record that PHT is only genotoxic in isolated human lymphocytes after its metabolic activation by S9, and without this activation genotoxicity doesn't occur. This result was collaborated by other studies that reported PHT genotoxic effect after metabolic activation in the presence of an exogenous metabolic activation system (S9) in bacteria (Sezzano et al., 1982) and Chinese hamster ovary cells (Galloway et al., 1987). In addition this result was consistent with some other studies that hold PHT without metabolic activation is not genotoxic. Witczak et al. (2008) assessed the potential genotoxic effect of PHT therapy in pregnancy on DNA of umbilical cord blood lymphocytes using Micronucleus (MN) assay. They did not show any significant differences between the MN rates of PHT-treated patients and controls, indicating a lack of genotoxicity of the PHT. In addition, Schaumann et al. (1990), tested the potential genotoxic effect of PHT using sister chromatid exchange (SCE) assay in isolated cultured lymphocytes from adult epileptic patients treated with PHT. He did not show any significant differences between the SCE rates of PHT-treated patients and controls, indicating a lack of mutagenicity of the PHT. Also, the negative tests for PHT genotoxicity were observed in germ cells of male Drosophila melunogaster (Woodruff et al., 1985), many strains of *Salmonella typhimurium* (Leonard et al., 1984), and cultured Chinese hamster ovary cells (Kindig et al., 1992). Large body of evidence supports this notation.

But in contrary to our study, there were other studies that reported PHT genotoxicity without metabolic
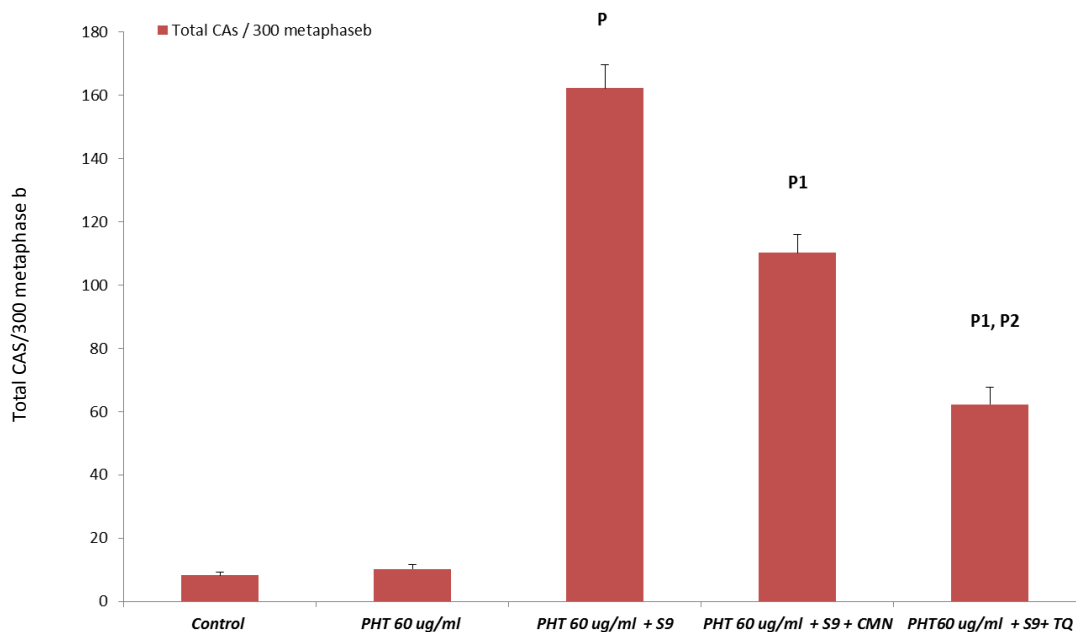
Figure 3. *In vitro* effects of PHT 60 µg/ml alone; PHT + s9 and PHT + s9 combined with either TQ 1 µM or CMN 15 µM on malondialdehyde (MDA) in isolated human lymphocytes after 72 h of incubation (one way ANOVA, mean ± SD). P = significant difference when compared with control normal group, P1 = significant difference when compared with PHT + S9 group, P2 = significant when compared with PHT 60 + S9 + CMN
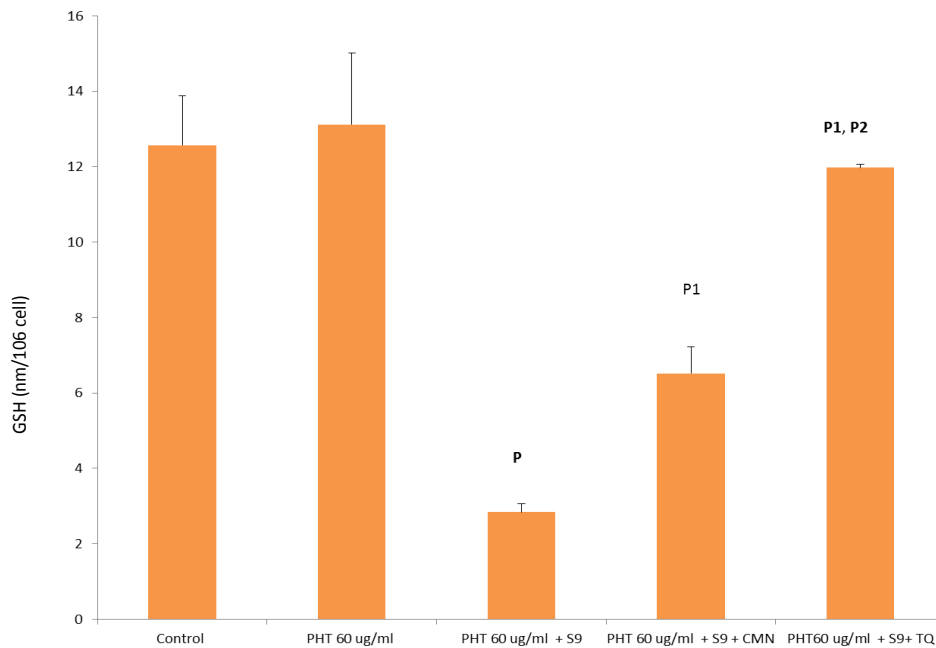
Figure 4. *In vitro* effects of PHT 60 µg/ml alone; PHT + s9 and PHT + s9 combined with either TQ 1 µM or CMN 15 µM on glutathione (GSH) in isolated human lymphocytes after 72 h of incubation (one way ANOVA, mean ± SD). P = significant difference when compared with control normal group, P1 = significant difference when compared with PHT + S9 group, P2 = significant when compared with PHT 60 + S9 + CMN

activation in Chinese hamster ovary cell (Winn et al., 2003), rodents (Kim et al., 1997), and some isolated human cells (Ponnala et al., 2009; Al-Jassabi and Azirun, 2010). This debate around the ability of PHT to induce
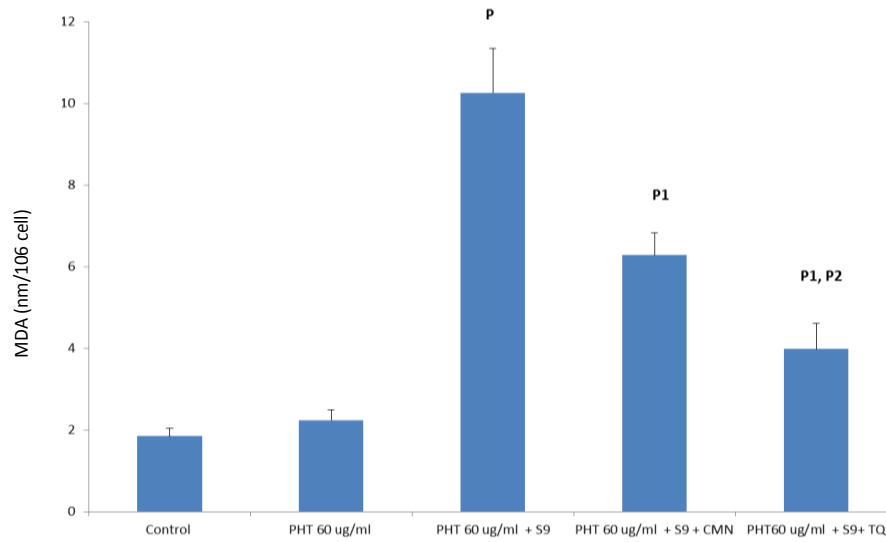
Figure 5. *In vitro* effects of PHT 60 µg/ml alone; PHT + s9 and PHT + s9 combined with either TQ 1 µM or CMN 15 µM on 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in isolated human lymphocytes after 72 h of incubation (one way ANOVA, mean ± SD). P = significant difference when compared with control normal group, P1 = significant difference when compared with PHT + S9 group, P2 = significant when compared with PHT 60 + S9 + CMN.
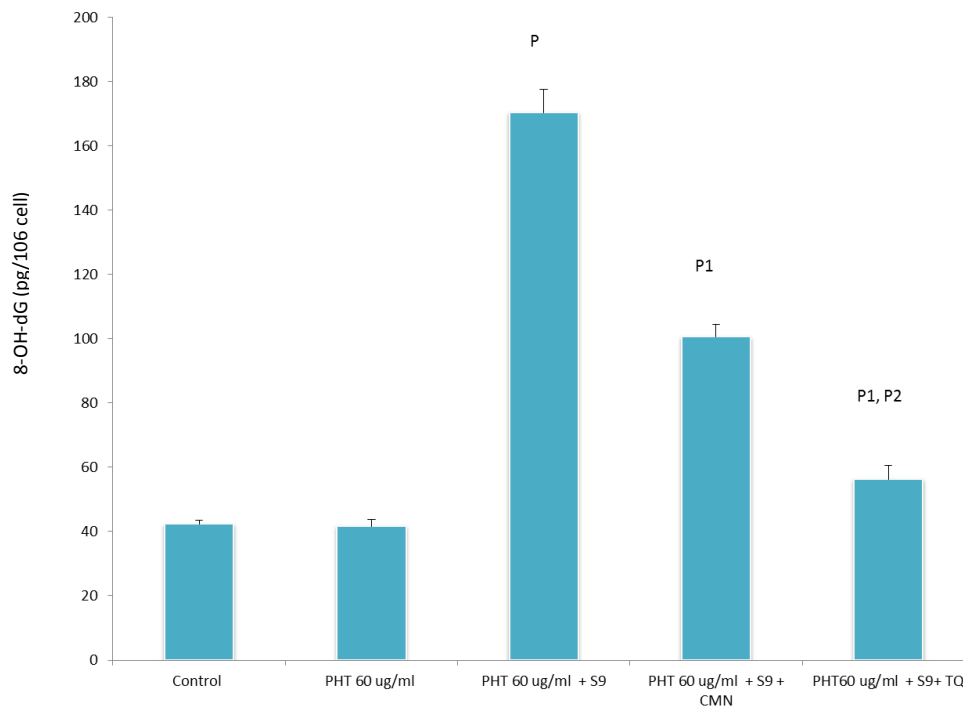
genotoxic effects was not observed only by our group, but was also observed in many other reports (NTP, 1993; IARC, 1996; Snyder and Greenb, 2001; Snyder, 2009).

This debate may be solved if we can understand the mechanism of PHT induced-genotoxic effects. Most of the genotoxic effects of PHT were due to PHT metabolic intermediates not the PHT itself, mainly the para hydroxyphenyl phenyl hydantion (p-HPPH) metabolite (Kaul et al., 2001). These metabolic intermediates induce production of ROS leading to exhaustion of the cellular antioxidant systems (Jacobsen et al., 2008) with subsequent oxidation of DNA, proteins, and lipids (Zegura et al., 2004). This toxic effect will lead to oxidative DNA base modification with DNA strand breaks, lipid peroxidation and decreased GSH-mediated cytoprotection (Al-Jassabi and Azirun, 2010). Our study supports this explanation because when we used PHT without metabolic activation by S9, its genotoxic effect was insignificant. However, when S9 was added to PHT, its genotoxic effect was significant indicating that PHT genotoxic effect could be achieved only after metabolic activation to reactive metabolic intermediates. This conclusion may be true if we were able to prove that the isolated lymphocytes do not contain any of the enzymes responsible for PHT metabolism.

There are three main metabolic pathways for the conversion of PHT to reactive metabolic intermediates. The first include the bioactivation of about 80% of PHT to para hydroxyphenyl phenyl hydantion (p-HPPH) (Soga et al., 2004), a process catalyzed mainly by the CYP2C9 and to much lesser by CYP2C18 and CYP2C19 (Al-Jassabi and Azirun, 2010). The second pathway includes the hydroperoxidase component of Prostaglandin endoperoxide synthetase pathway (Parman et al., 1998). Last pathway included the bioactivation of PHT to reactive free radical intermediates through the myeloperoxidase enzyme commonly present in leukocytes (Mays et al., 1995). However, by reviewing the distribution and activity of the enzymes responsible for PHT metabolism, we found that the CYP450s present in lymphocytes are mainly CYP1A1, CYP1B1, CYP 2E1 and CYP3A4 (Anzenbacher and Anzenbacherová, 2001) while those included in PHT metabolism are CYP2C9, CYP2C18 and CYP2C19 (Al-Jassabi and Azirun, 2010). The prostaglandin endoperoxide synthetase was found in relatively high concentrations in lymphocytes (Dailey and Imming, 1999), but this enzyme can not metabolize PHT without metabolic activation through addition of high amounts of arachidonic acid (Kubow and Wells, 1989). The last enzyme myeloperoxidase was found to be distributed unequally between leucocytes where it is excess in neutrophils and very little in lymphocytes (Tay

et al., 1998). These facts about the metabolism of PHT in isolated human lymphocytes support the results of our study and previous studies with similar results and give an alert for contrary studies to reconsider their results specially when used PHT in isolated non liver cells without metabolic activation.

Poojan et al. (2015) hold that curcumin combined with selenite in utero may prevent the disruption of Skin Stem Cell through different mechanisms including de novo GSH biosynthesis. In the same direction, Sankar et al. (2014) support protective effects of curcumin on genotoxic effects exerted by Arsenic in bone marrow cells through attenuation of its drawbacks on chromosomal aberrations, micronuclei formation and DNA damage. Tawfik et al. (2013) studied protective effects of curcumin on irradiated mice and found that it had significant radio-protective and radio-recovery activities.

Malhotra et al. (2012) reported that combined treatment with curcumin and resveratrol stimulate apoptosis and hence, they modulates mitotic injury in benzo(a)pyrene - treated mice. Curcumin normalize mRNA expression levels and ameliorate progression of diabetic nephropathy (Ibrahim et al., 2016). Nicotinamide phosphoribosyl transferase and sirtuin proteins play crucial roles in threshold of cell death modulation and curcumin can increase their levels, so it can be potentially used to reduce chemotherapy-induced nephrotoxicity (Ugur et al., 2015).

Badary et al. (2007) studied daily intake of TQ to rats after and before benzo(a)pyrene exposure, that significantly reduced the frequencies of CAs and damaged cells. Aboul-Ela (2002) also found that TQ can protect against chromosomal aberrations in mouse cells infected with schistosomiasis. El-Sheikh et al. (2016) found that TQ can reverse intestinal microscopic changes induced by methotrexate and improve oxidative/ nitrosative stress, inflammatory and apoptotic markers in intestine. Gökce et al. (2016) stated that TQ improved decreased levels of oxidative products Like MDA and proinflammatory cytokines, and reduce motor neuron apoptosis. Hepatic level of MDA in mice exposed to Aflatoxin B1, was reduced by TQ pre-treatment (Daba et al., 1998).

In conclusion, TQ more than CMN can significantly ameliorate oxidative genotoxic effects exerted by PHT. PHT without metabolic activation in isolated human lymphocytes from non-epileptic donors cause dose dependant direct toxic effect rather than genotoxic effect.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biochemical and histological evaluation of benzo[a]pyrene induced nephrotoxicity and therapeutic potentials of *Combretum zenkeri* leaf extract

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This study determined the therapeutic effect of ethanol leaf extract of *Combretum zenkeri* on kidney function of rats exposed to benzo[a]pyrene. Male Wistar albino rats were treated with ethanol leaf extract of *C. zenkeri* (400 mg/kg) in the presence or absence of BaP (200 mg/kg) for four weeks. The blood obtained at the end of the treatment was processed into serum and used for biochemical analyses and kidney tissues for histology. The serum creatinine, urea, sodium ion and potassium ion concentrations of the group exposed to benzo[a]pyrene without treatment with *C. zenkeri* extracts were significantly higher ($p<0.05$) as compared to the normal control group. The kidney histology indicated severely damaged glomeruli. The groups treated with the leaf extract pre- or post- benzo[a]pyrene exposure showed significant reduction ($p<0.05$) in serum concentration of urea, creatinine and sodium ion, while the pre-treated group showed no significant reduction ($p<0.05$) in serum potassium ion concentration. The biochemical and histological studies indicated that, *C. zenkeri* can ameliorate benzo[a]pyrene induced oxidative kidney damage.

Key words: *Combretum zenkeri,* benzo[a]pyrene, PAHs, oxidative damage, kidney functions.

INTRODUCTION

Benzo[*a*]pyrene, a polycyclic aromatic hydrocarbon (PAHs) is an ubiquitous environmental pollutant and an established mutagen and carcinogen (ATSDR, 1996; IARC, 2010). Polycyclic aromatic hydrocarbons are ubiquitous and persistent as a result of natural and human activities. Human activities that release PAH into the environment are rampant in Nigeria, especially in the Niger-delta. The Niger-delta region of Nigeria is known for petroleum pollution resulting from crude oil exploration, exploitation and gas flaring (Ujowundu et al., 2013). Gas flaring is the unscientific elimination by

combustion, of excess hydrocarbons accumulated in oil/gas flow station, releasing green house gases, soot, heavy metals and hydrocarbons (PAHs) (Coker, 2007; Ujowundu et al., 2013, 2014a). The exposure of Niger-delta populations to avalanche of pollutants has caused several health challenges, of which etiologies of most cannot be ascertained. Also, cancer prevalence is shown to increase (Ana et al., 2009; Ana and Sridhar, 2009).

The most common mechanism of carcinogenesis induced by PAHs is DNA damage through the formation of adducts. Human activities such as coke manufacturing

or asphalt production, power generation, refuse incineration, are sources of PAH emissions (Jongeneelen, 2001). Also, PAHs emission from motor vehicles and motorcycles are in the increase due to increased use of vehicles with old combustion technology and per capita increase in vehicle ownership. Furthermore, the consumption of charcoal broiled or smoked meats, fish, grains, and vegetable fats and oils (Yu, 2005; Ujowundu et al., 2014b, 2016), are sources of xenobiotic (especially PAHs) exposure..

Kidneys (and liver) are organs selectively targeted and injured by xenobiotics. The kidneys receive large amount of xenobiotic containing blood per unit time; it is a repertoire of xenobiotic metabolising enzymes (cytochrome P450s). Compounds metabolised by these enzymes may interact with and damage kidney cells. The excretory functions of kidney can up-concentrate xenobiotics or their metabolites. The kidneys, liver and lungs are involved in the metabolism of benzo[a]pyrene. Benzo[a]pyrene (BaP) undergo metabolic activation by CYP1A1 to diol-epoxides which form adducts with DNA or induce oxidative stress that provokes mutations. Benzo[a]pyrene derivatives have the potential to undergo redox-cycling, inducing production of superoxide radicals which are converted to hydroxyl radicals by the Haber-Weiss reaction (Chatgilialoglu and O'Neill, 2001; Briede, et al., 2004). Chronic exposure to PAHs (especially BaP) is related to a high rate of mutagenesis which may induce DNA damage. Cancer of the bladder, lung, kidney, liver and breast is associated with chronic exposure of PAH (Shen et al., 2006; Karami et al., 2011).

The use of fruits, leaves, roots and stems of plants for medicinal purposes lies on the ability of its chemical constituents to illicit biochemical and physiological actions in living systems (Sofowora, 1982, Malikharjuna et al., 2007). Chemical constituents of plants have chemoprotective potentials on vital organs such as liver, kidneys, ovaries, testes etc. of biological models (Ujowundu et al., 2011, 2014c). *Combrtum zenkeri* leaves contain appreciable amount of flavonoid, alkaloid, saponin, tannin, vitamins A and C and important minerals such as manganese, zinc and selenium (Ujowundu et al., 2010). Flavonoids possess wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic, as well as ability to modify the gene expression. Flavonoids have free radical scavenging and *in vivo* biological activities (Ujowundu et al., 2010b). Saponins have hypocholesterolemic and anticarcinogenic effects (Ujowundu et al., 2010b). Ascorbic acid, iron, selenium, zinc and manganese improves the immune functions as antioxidants (Talwar et al., 1989; Zhao, 2007). Earlier study on the root of *C. zenkeri* showed its frequent use in the management of cancer (Sowemimo et al., 2009).

The aim of this study was to determine the therapeutic effect of ethanol extract of *C. zenkeri* leaf on benzo[a]pyrene induced nephrotoxicity in rats, using biochemical and histological evidences.

MATERIALS AND METHODS

Chemicals

This study used benzo[a]pyrene (purity ≥ 96% high-performance liquid chromatography), CAS Number 50-32-8, B-1760 Lot Number SLBC6864V obtained from Sigma-Aldrich Co, St Louis, MO USA. Other chemicals and reagents used were of analytical grade.

Collections and processing of plant sample

Fresh leaves of *C. zenkeri* were harvested from a farm at Ihiagwa in Owerri-West LGA, Imo-State Nigeria. The leaf sample was identified by a plant taxonomist, Dr. F. N. Mbagwu of Imo State University, Owerri, Nigeria. The leaf sample was deposited at Imo State University herbarium with voucher number IMSU/0124. The leaves were shredded from the stalk, rinsed with clean water and air-dried for 15 days. The dried leaves were pulverized and stored in an air-tight glass jar at room temperature.

Preparation of ethanol extract of *C. zenkeri*

To obtain the ethanol leaf extract of *C. zenkeri* (EECZ), 450 g of the pulverized sample of *C. zenkeri* were soaked in 1200 ml of absolute ethanol (Handa et al., 2008), and this was agitated by shaking for four (4) days. Thereafter, the setup was filtered and the extract concentrated *in vacuo,* with rotary evaporator (NYC). The extract was stored at 4°C in a refrigerator until used.

Acute toxicity study (LD$_{50}$)

Acute toxicity study on ethanol leaf extract of *C. zenkeri* followed the Lorke (1983) method as described by Chinedu et al. (2013). Sixteen (16) male mice with a mean weight of 25±4 g were obtained from a laboratory animal farm- Animal Friend Company, Owerri. In the first phase of the study, the animals were administered the ethanol leaf extract of *C. zenkeri* at doses of 10, 100 and 1000 mg/kg. No mortality was observed amongst the treated mice. In the second phase, the animals were administered higher doses, 1600, 2900 and 5000 mg/kg of the extract. They were observed for 24 h for any behavioural change, as well as mortality. There was no observable behavioural change or mortality. These results informed the use of 400 mg/kg body weight of the extract for the study.

Experimental design

Adult male Wistar albino rats weighing 80-100 g were purchased from a laboratory animal farm belonging to Animal Friend Co. The ethics committee of the Department of Biochemistry, Federal University of Technology Owerri, Nigeria approved (FUTO/BCH/EC/2013/10) this study and it adhered to the guideline for the care and use of laboratory animals (NIH, 1985). The animals were acclimatized for 7 days in metal cages in light/dark photoperiod (12 h light : 12 h darkness) at room temperature (21-25°C), and were allowed free access to rat pellets and portable water *ad libitum.* After this period, they were divided into five groups of six rats each, and treated as follows:

Group I (Control): Received rat pellets and portable water only.

Group II (Benzo[a]pyrene (BaP) group): Were administered

(intra-peritoneally) 200 mg/kg bw of BaP dissolved in olive oil (200 µl) without treatment with ethanol extract of *C. zenkeri* (EECZ).

Group III (PST group): They were administered (intra-peritoneally) 200 mg/kg bw of BaP (dissolved in 200 µl olive oil) for two weeks, and later treated (orally) with 400 mg/kg bw of (EECZ) (dissolved in 200 µl olive oil) at alternate days for two weeks.

Group IV (PRT group): They were initially treated (orally) with 400 mg/kg bw (EECZ) at alternate days for two weeks before intra-peritoneal administration of 200 mg/kg bw BaP at alternate days for two weeks.

Group V (plant extract only). The animals in this group were administered 400 mg/kg bw of (EECZ) at alternate days throughout the duration of the study. Animals in all the groups were allowed rat's pellets and water *ad libitum.*

At the end of the treatment period (28 days), the animals were fasted for 24 h and sacrificed following mild ether anaesthetics. Blood was obtained by cardiac puncture in plain tubes for serum processing. The kidney tissues were excised and stored in freshly prepared formal saline, for histological studies.

Biochemical studies

Serum sodium was determined by the colorimetric method of Maruna (1958) as outlined in *Teco* diagnostic test kit. Briefly, test tubes were labeled blank, standard and sample. Filtrate reagent (1.0 ml) was added into all the tubes, 50 µl standard reagent and 50 µl serum were added to standard and sample tubes respectively. Distilled water was added to blank tube and all tubes were mixed and left to stand for 3 min. The tubes were centrifuge at 1500 xg for 10 min and supernatant obtained was used for colour development. Test tubes were labeled as above and 1.0 ml acid reagent was added to all tubes. Then, 50 µl of supernatant were added respectively to the corresponding tubes and appropriately mixed. Finally, 50 µl of colour reagent were added to all tubes, mixed and absorbance taken at 550 nm.

Serum potassium was determined by the method of Terri and Sesin (1958) as outlined in *Teco* diagnostic test kit. Briefly, test tubes were labeled standard, blank and sample. One milliliter (1.0 ml) of potassium reagent was added to all test tubes and 10 µl of standard and serum samples were added to standard and sample tubes respectively. Then 10 µl of distilled water was added to blank tube, mixed appropriately and left to stand for 3 min. Finally, absorbances of the tubes were taken at 500 nm using the blank to zero.

Serum chloride was determined by the method described by Tietz (1999). Briefly, test tubes were labeled, blank, standard and sample. One milliliter (1.0 ml) of chloride reagent was added to all test tubes. Then, 10 µl of samples (standard and serum) were added to the standard and sample test tubes, respectively. Also, 10 µl distilled water was added to the blank tube, and setups were mixed appropriately and left to stand for 3 min. Finally, absorbances of the tubes were taken at 480 nm using the blank to zero.

Serum creatinine was determined by Jaffe alkaline picrate method as described by Tietz (1999). Briefly, test tubes were labeled standard and sample. Then, 0.2 ml of creatinine standard and serum were added to the standard and sample tubes, respectively. Furthermore, 2 ml of working reagent was added to all tubes, mixed appropriately and allowed to stand. Absorbance was taken after 30 and 120 s.

Serum urea concentration was determined as described by Tietz (1995). Briefly, test tubes were labeled standard, blank and sample. To the sample and standard tubes, 0.01 ml of serum and standard reagent were respectively, added. Then, 1.0 ml of working reagent was added to all tubes and the contents of tubes were each mixed appropriately and incubated at 37°C for 3 min. Finally, 0.02 ml of

reagent 2 was added to all tubes, mixed appropriately and incubated at 37°C for 5 min and absorbance taken at 578 nm.

Histopathology of kidney

The method as described by Okoro (2002) was used with minor modifications for the kidney histological studies. The kidneys sample were fixed with formal saline and subjected to dehydration, clearing (dealcoholisation), infiltration and embedding processes. The tissues were dehydrated by passing through ascending grades of alcohol (30, 50, 70, 90% and absolute alcohol) for 1, 2 and 3 h each. After dehydration, alcohol was removed (clearing) from the tissues by immersing them in xylene for 3 h. Tissues were transferred from the clearing agent to a bath of molten paraffin wax in the embedding oven. The clearing agent was eliminated from the tissue by diffusion into the surrounding wax. The excess wax was removed by trimming to ensure uniform sections and prevent the block from cracking. The tissues were sectioned with a rotary microtome and the paraffin sections were stained to permit differentiation of various structural compounds. The sections were cleared in xylene and mounted with dibutylpthalate, polystyrene and xylene (DPX).

Statistical analysis

Results obtained were analysed by one-way analysis of variance (ANOVA) using SPSS 17.0 software. Multiple comparisons for the ANOVA were done using least significant difference (LSD) to determine the statistical significance at $P < 0.05$.

RESULTS

Figure 1 shows the effect of BaP intoxication and EECZ treatment on serum sodium ion concentration respectively. The result shows BaP and EECZ groups had the highest and lowest sodium ion concentration. The sodium ion in BaP group showed significantly higher ($p < 0.05$) concentration as compared to other groups.

Figure 2 presents the effect of BaP intoxication and EECZ treatments on serum potassium ion concentration. The group exposed to BaP only, shows a significantly ($p<0.05$) higher potassium ion concentration than normal control and other treated groups except pre-treated (Prt) group. The post-treated group shows significant ($p<0.05$) reduction as compared to the EECZ and NC groups.

Figure 3 presents the effect of intoxication with BaP and treatments with EECZ on serum chloride concentration. The group exposed to BaP only shows a significantly ($p<0.05$) low serum chloride as compared to other groups. In comparison, the pre- and post-treated groups showed no significant ($p>0.05$) difference in chloride ion concentration, but significantly ($p<0.05$) lower than that of the normal control group. The EECZ group maintained a concentration not significantly ($p>0.05$) different from NC group.

The effect of exposure to BaP and EECZ treatments on serum urea concentration is shown in Figure 4. Rats exposed to BaP only showed significantly ($p<0.05$) higher serum urea concentration as compared to other groups. However, EECZ group showed the least urea
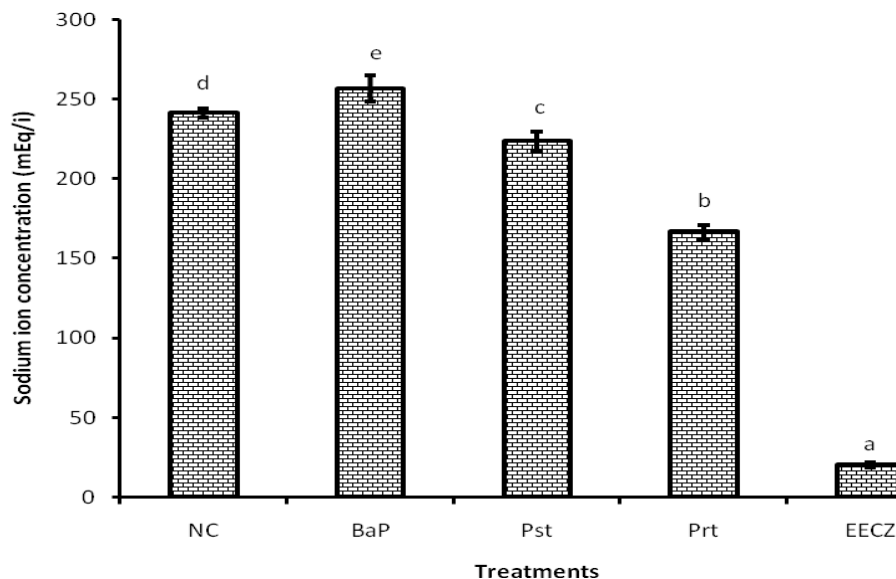
Figure 1. Effect of EECZ on serum sodium ion concentration of rats intoxicated with BaP. Bars with different letters are statistically significant (p<0.05).
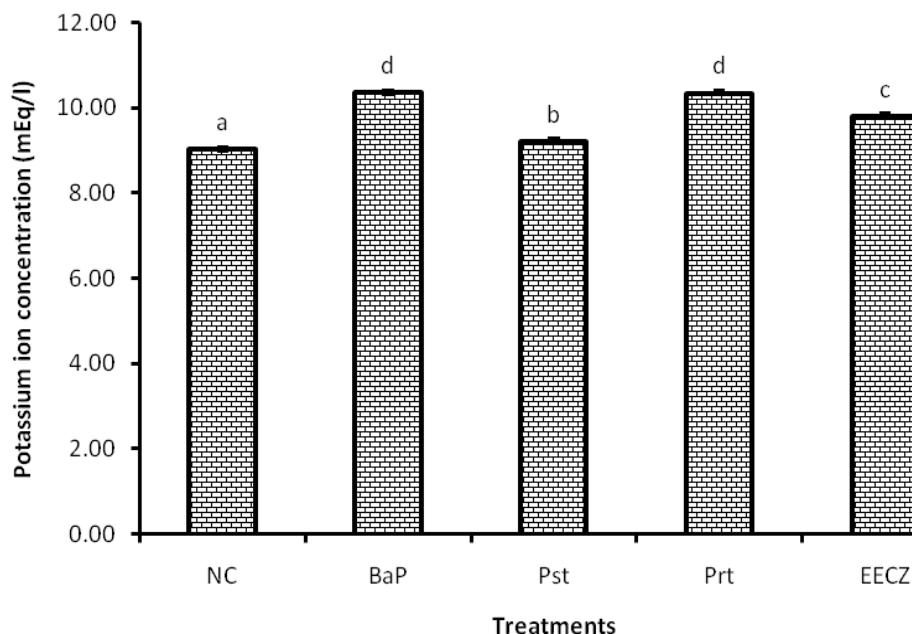

Figure 2. Effect of EECZ on serum potassium ion concentration of rats intoxicated with BaP. Bars with different letters are statistically significant (p<0.05).

concentration when compared with others. Also, the post- and pre-treated groups showed a significant (p<0.05) reduction in urea concentration.

Figure 5 presents the effect of exposure to BaP and treatments with EECZ on serum creatinine concentration. The BaP-group shows a significantly (p<0.05) higher serum creatinine concentration as compared to other groups. Also, significant decreases were observed in the groups treated with EECZ after intoxication when compared with the group exposed to BaP only. The rats treated with the EECZ only, showed a creatinine concentration significantly (p<0.05) below the normal control. Treatment with EECZ reduced the concentration significantly (p<0.05).

Figure 3. Effect of EECZ on serum chloride ion concentration of rats intoxicated with BaP. Bars with different letters are statistically significant (p<0.05).

Figure 4. Effect of EECZ on serum urea concentration of rats intoxicated with BaP. Bars with different letters are statistically significant (p<0.05).
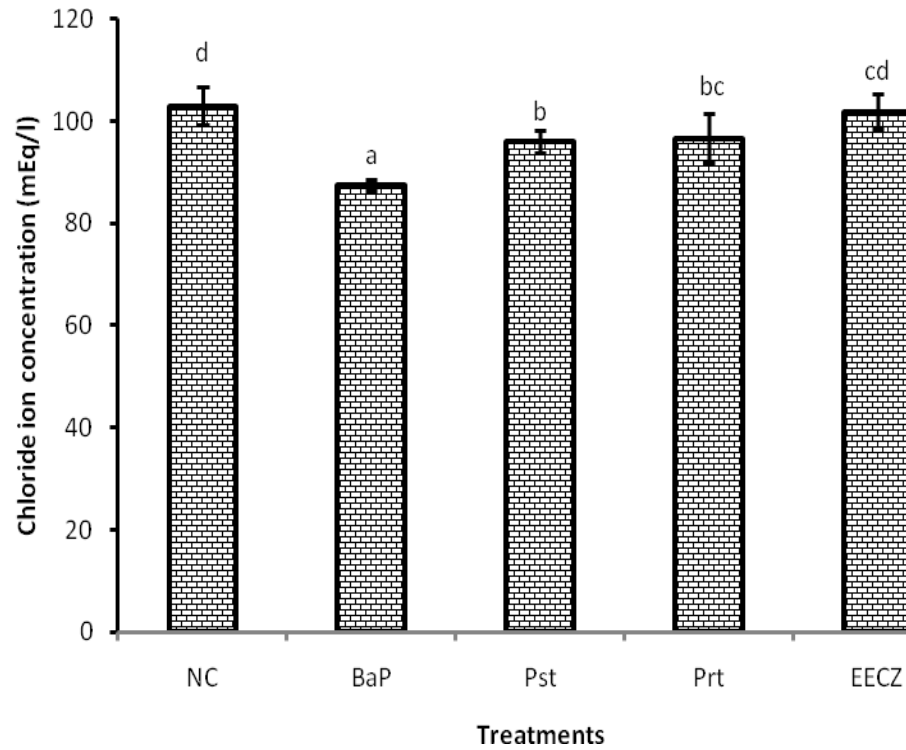
Figure 5. Effect of EECZ on serum creatinine concentration of rats intoxicated with BaP. Bars with different letters are statistically significant ($p < 0.05$).
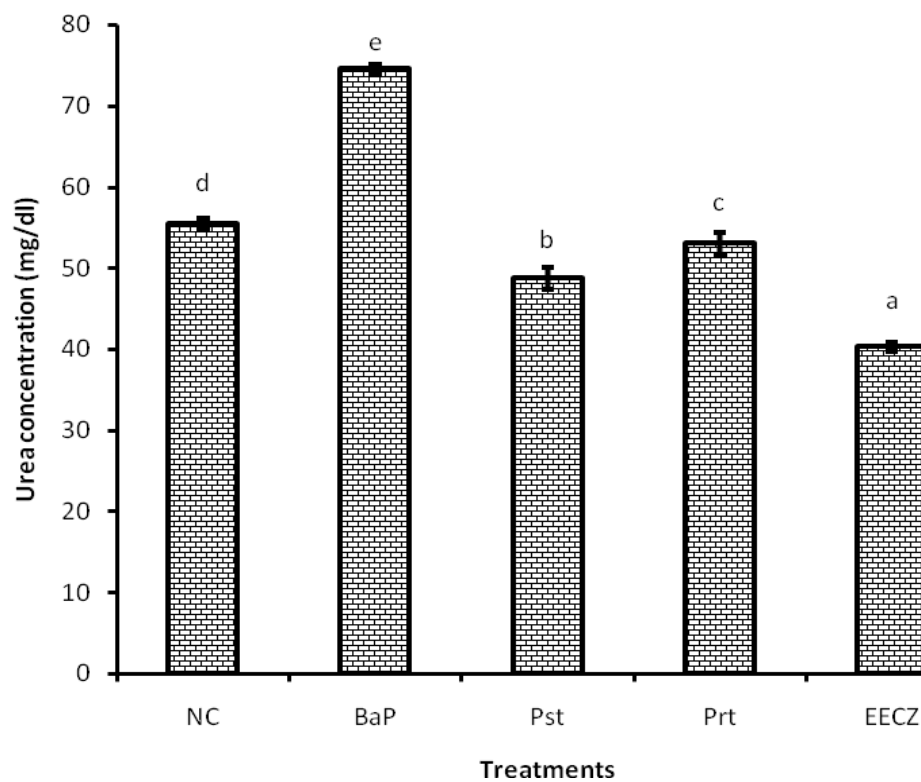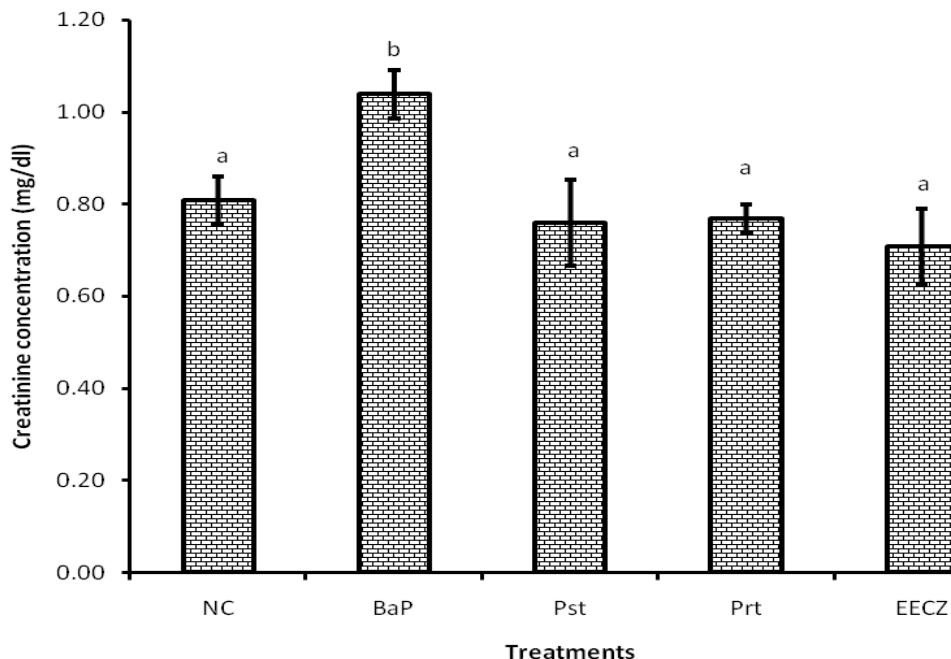
Figure 6 shows the light micrograph of HE-stained sections of formal-saline fixed kidneys of rats intoxicated with BaP and treated with EECZ. (a) represents section of normal control group, showing normal tissue architecture with the glomeruli (G) appearance; (b) represents the BaP group, showing glomeruli (G) and glomeruli (C) congested by clotted blood-cells with virtually solid appearance indicating severely degenerated glomeruli; (c) represents the post-treated group, showing slightly congested glomeruli (G) as the blood cell congestion lessens; (d) represents the pre-treated group, showing normal architecture with appearance of the glomeruli G; (e) represents the EECZ group, showing normal architecture with appearance of the glomeruli (G) blood vessels not filled with clotted blood.

DISCUSSION

The kidney functions by filtering harmful substances from the blood making it to be unusually susceptible to damage and dysfunction by regular exposure to xenobiotics. Toxic injury to the kidney can be acute or chronic and can lead to end-stage renal failure or cancer. Benzo[a]pyrene is a representative polycyclic aromatic hydrocarbon (PAH) (Zhang, 2012), and a well-known mutagen and carcinogen (Wu et al., 2007; Uno and Makishima, 2009). The kidneys function to excrete waste in urine and regulate water, electrolyte, and acid-base content of the blood. Renal dysfunction or diminished function of the kidney may be acute and temporary or may progress to complete loss of kidney functions.

The potential of benzo[a]pyrene radicals to undergo redox-cycling, inducing production of superoxide radicals and hydroxyl radicals may have caused the oxidative damage to the kidneys, observed in Figure 6 (plate b) as congested and severely degenerated glomeruli with virtually solid appearance. The glomeruli damage could cause hypernatremia resulting in significantly increased serum sodium concentration as observed in BaP group.

Elevated sodium is usually associated with dehydration, which occurs due to decreased water level (Kandaswamy et al., 2013). Similarly, the observed significant decrease in serum chloride in toxicant exposed animals when compared with the control is indicative of renal toxicity and pathology. This in part is in agreement with studies on renal toxicity (Kluwe, 1981; Orisakwe et al., 2004; Kandaswamy et al., 2013). The concentration of sodium and chloride ions in the groups treated with EECZ indicates that the extracts may be an effective regulator of these electrolytes in toxic conditions. *C. zenkeri* contains appreciable amount of alkaloid (Ujowundu et al., 2010a), and the alkaloid sparteine is a known sodium channel blocker and an antiarrythemic agent (Korper et al., 1998).

The action of antidiuretic hormone (ADH) (synthesized by pituitary gland) on kidney ensures that the right amount of sodium is maintained in the blood by controlling the amount of water removed through the
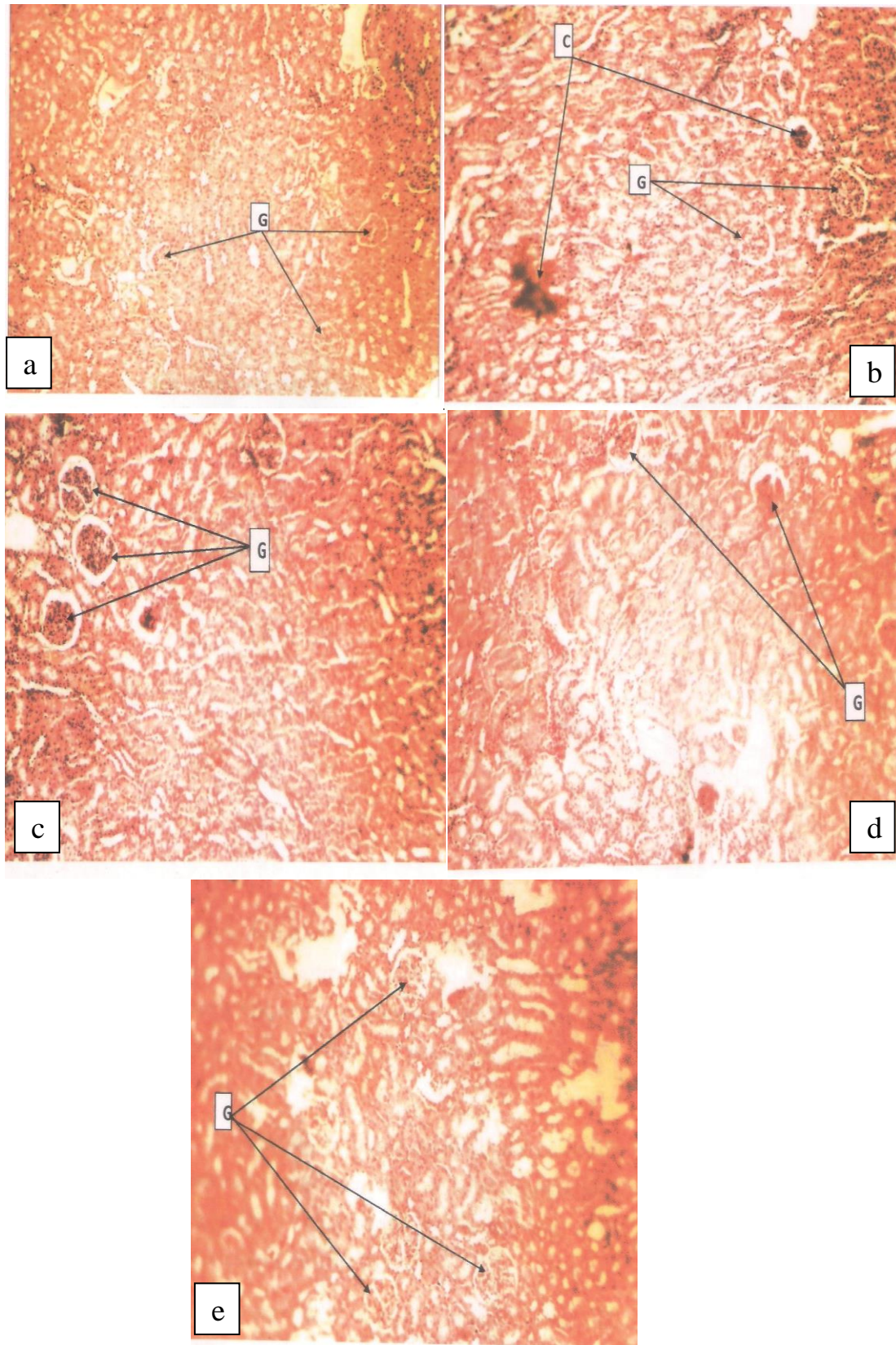
Figure 6. Light micrograph of HE-stained sections of formal-saline fixed kidneys of rats intoxicated with BaP and treated with EECZ. (a) Normal control; (b) BaP group; (c) post-treated group; (d) pre-treated group; (e) EECZ group. G = glomeruli appearance.

kidney tubules. The expression of ADH, a peptide hormone may be reduced by BaP induced oxidative damage of cell renal cell membranes, genetic materials, hypothalamus or the peptide themselves. Oxidative damage to kidney tissues could also reduce the tubules' sensitivity to the hormone. This could have caused the increase in serum sodium concentration in the BaP group as water reabsorption may have reduced. Water reabsorption through the aquaporins in the apical membrane of the tubule takes chlorine back into circulation. Damage to this membrane due to BaP-derived intermediates may have caused the low concentration of serum chloride in the BaP group. Decline in renal functions in the rats exposed to BaP may have caused the increased serum potassium concentration observed in the BaP and Prt groups. This indicates poor clearance of potassium by the kidney tubules and could be linked to oxidative damage by BaP metabolites. Cytochrome P450 enzymes, epoxide hydrolase and aldo-keto reductases, oxidize PAHs (e.g BaP) to reactive and redox-active o-quinones which amplify ROS and oxidative damage to proteins and DNA bases to form the highly mutagenic lesion 8-oxo-deoxyguanosine (Guengerich, 2000; Quinn and Penning, 2006; Quinn et al., 2008). The groups treated with EECZ, post- and pre-exposure to BaP demonstrated significant reduction in sodium and potassium ion concentration. These may be attributed to the chemical constituents of EECZ.

The increase in serum creatinine concentration in the BaP-group indicates retention of creatinine in the blood. This could imply gradual degeneration of the kidney due to exposure to reactive metabolites of benzo[a]pyrene which readily elicits free radicals. This agrees with the study by Cosan et al. (2008), where serum creatinine concentration in rats administered benzo[a]pyrene increased significantly. However, groups treated with EECZ, pre- and post, demonstrated significant reduction in serum creatinine. Creatinine levels remained positively associated with continuous adducts formation stating that a measure of impaired renal function, increasing blood creatinine, within the normal range, is associated with the detection of pro-carcinogenic DNA damage in the blood (Madsen et al., 2005). This implies that decreased kidney function, even at levels previously considered normal, is associated with lowered levels of detoxification and/or excretion (Madsen et al., 2005). Elevation of creatinine in the blood can indicate kidney function impairment (Reddy et al., 2012). This implies decreases in glomerular filtration rate (GFR) which is an indication of impaired glomerular function. Significant damage to glomeruli is clearly shown (C) in the light micrograph tissue section of BaP group. The glomeruli indicated blood congestions and severe degeneration, with solid appearance. However, treatment with EECZ (micrograph sections c and d) in Pst and Prt group shows gradual restoration of glomeruli structures. Renal impairment could also

result from cellular membrane damage of the renal tissues due to the oxidative intermediates produced from benzo[a]pyrene biotransformation.

Valentovic et al. (2006) in their study stated that no change in blood urea nitrogen (BUN) was observed following 5 weeks of BaP (10 mg/kg) treatment in the normoglycemic animals. However, the results of this study showed significant increase in serum urea following benzo[a]pyrene exposure. Serum urea accumulates when the rate of urea production exceeds the rate of clearance signalling renal dysfunction and/or disease (Reddy et al., 2012). Serum urea concentration may have increased due to increased breakdown of proteins and enzymes by the presence of reactive oxygen species. Secondly, reduced kidney activity means much of the urea will be held back or accumulated. If serum urea and creatinine elevation is an index of nephrotoxicity (Palani et al., 2009), their significant reduction ($p<0.05$) by ethanol leaf extracts of *C. zenkeri* indicates relief from nephrotoxic effect of benzo[a]pyrene.

Biochemical examination is usually supported by histopathology. Histological observations of kidney sections shows that exposure to benzo[a]pyrene, may cause distortions and damage to the kidney tissues when compared with the normal control groups. This could pose serious oxidative risk to health. This is due to the inability of the damaged kidney tissues to perform in the clearance of the reactive substances from the body. Renal tissues' injury was attributed in this study to the toxic effects of benzo[a]pyrene exposure. The distortions in the glomeruli architecture could have given rise to increased serum creatinine and urea concentration in rats exposed to BaP only. However, degenerative manifestations were halted in kidney sections of Pst and Prt treated groups, showing great potentials for functional restoration (Cosan et al., 2008). The restoration of the tissue structures in the groups treated with EECZ indicates protective effect in benzo[a]pyrene induced toxicity.

Conclusion

This study indicates the protective and therapeutic effect of *C. zenkeri* against benzo[a]pyrene-induced oxidative damage and degenerative glomeruli. It effectively inhibited the benzo[a]pyrene induced nephrotoxicity in rats. This is most likely due to its high content of flavonoids, alkaloids, saponins, tannins and vitamins A and C. Further studies will however involve a more precise identification of the lead component responsible for the protective effect and a study of the molecular mechanism behind it.

Conflict of Interests

The authors have not declared any conflict of interests.

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