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). Combretum 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 (LD50)

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 (PERT 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 Tecco 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 x g 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 Tecco 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 dibutylphthalate, 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 and treatments with EECZ showed 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 antiarrhythmic 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 chloride 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 α-quinones which amplify ROS and oxidative damage to proteins and DNA bases to form the highly mutagenic lesion 8-oxodeoxyguanosine (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 concentration of creatinine 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.
REFERENCES


Ogbonna et al. 881


