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Protective Effect of Ethanol Leaf Extract of *Combretum Zenkeri* on Liver Functions of Albino Rats Following Benzo(A)Pyrene Exposure

Okwu, G. N.*, Ogbonna, C. U., Ujowundu, C. O., Igwe, K. O., Igwe, C. U. and Emejulu, A. A.
Department of Biochemistry, Federal University of Technology, Owerri, Nigeria.

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Abstract: This study investigated the protective effect of ethanol leaf extract of *Combretum zenkeri* on liver functions of albino rats exposed to Benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) with oxidant, inflammatory and carcinogenic effects. Acute toxicity analysis with the plant extract produced no lethality even at high doses. In this study, male albino rats were divided into 5 groups; the first group served as normal control and were not administered either benzo(a)pyrene or ethanol leaf extract of *C. zenkeri*. The 2nd group was treated intraperitoneally(i.p) with 200 mg/kg benzo(a)pyrene only once and were not administered ethanol leaf extract of *C. zenkeri*. The 3rd group were exposed to 200 mg/kg BaP (i.p) and after two weeks were treated with 400 mg/kg ethanol leaf extract of *C. zenkeri,* while the fourth group were treated with 400 mg/kg ethanol leaf extract of *C. zenkeri* for two weeks before exposure to 200 mg/kg BaP.The fifth group was treated orally with 400mg/kg plant extract only.In each case, *C. zenkeri* treatment was on alternate days and the duration of the studies was for four weeks. Blood samples were used for biochemical analyses and liver tissues for histology.The malondialdehyde, total bilirubin concentrations and ALT and AST activity of groups exposed to benzo(a)pyrene without treatment were significantly higher ($p<0.05$) than those treated with the leaf extract before or after benzo(a)pyrene exposure. The GSH, total protein and albumin concentrations of groups exposed to benzo(a)pyrene without treatment were significantly lower ($p<0.05$) compared to the control animals and those treated with the leaf extract together with benzo(a)pyrene exposure. Histological slides of the liver tissue showed a better outcome in the plant extract treated groups. The results of the study showed that *C. zenkeri* has a potential to ameliorate and repair damage to liver tissues.

Keywords: Combretum zenkeri, benzo(a)pyrene, oxidative damage, liver function.

1. Introduction

Polycyclic (or polynuclear) aromatic hydrocarbons (PAHs) are a large group of hydrocarbons containing two or more benzene rings fused to each other or to other hydrocarbon rings. Benzo(a)pyrene represents one of the most important mutagenic and carcinogenic PAHs (Perera *et al.*, 2005). Fossil fuel combustion by motor vehicles, residential heating units, power plants, and industrial activities are major sources of PAHs (Bostrom *et al.*, 2002). Benzo(*a*)pyrenes (BaPs) are also produced by the combustion of cigarettes, creosote railroad ties, and coke ovens (Izzotti *et al.,*1991; Burchiel *et al.,* 2004). They can also be produced in heat processed foods such as fried chicken, potato chips, smoked dried meat (Aygun and Kabadayi, 2005; Lee and Shim, 2007) and smoked cheese (Anastasio *et al.,* 2004).

Corresponding author:
Gloria Nkiruka Okwu, Ph.D./Associate Professor, Reseasrch Field: Biochemistry,Department of Biochemistry, Federal University of Technology. E-mail: gnokwu@yahoo.com.

Benzo(a)pyrene (3,4-benzopyrene) which is the most common representative of the benzopyrene group is regarded as a potent oncogenic agent (Karkabounas *et al.,* 2002). It is known that even though the body possesses a mechanism that corrects damages and alterations to DNA ultra-structure (Wiseman and Halliwell, 1996), at a certain stage, the mechanism to orchestrate this correction process could be undermined (Wiseman and Halliwell, 1996; Valko *et al.,* 2007).

Chemoprevention is a means of disease control in which disease occurrence is prevented by the administration of one or a combination of several chemical agents. A large number of compounds with great structural diversity, have shown dependable promise in several animal bioassay systems (Al-Fify and Aly, 2010). Some of the compounds, found in some of those plants, which include flavonoids, alkaloids and vitamins have been identified in ethanol leaf extract of *Combretum zenkeri* (Ujowundu *et al.,* 2010). *Combretum zenkeri* (family - Combretaceae) is widely distributed and used along the tropical West Africa from Guinea through Nigeria up to Cameroon. *Combretum zenkeri* leaves are used in Ogun State, Nigeria for the treatment of inflammatory diseases like rheumatoid-arthritis (Gbolade *et al*., 2010). In Sowemimo *et al.,* (2009), it was stated that *Combretum zenkeri* roots were frequent in recipes for the management of cancer from an ethnobotanical survey of traditional medical practitioners in Western Nigeria. The research further reported that *Combretum zenkeri* root showed less than 40% cytotoxic activity at 500µg/ml.

This work seeks to study the therapeutic and protective effects of the ethanol leaf extract of *Combretum zenkeri* against Benzo(a)pyrene induced hepatotoxicity in rats, using both biochemical and histological evidence. Since we are all exposed to Benzo(a)pyrene in car exhaust, refuse burning, cigarette smoking and in the food we eat, knowledge of the efficacious use of *C. Zenkeri* can help ameliorate the toxic effects of such exposure.

2. Materials and Methods

Chemicals

The benzo(a)pyrene (BaP) (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 was used in the present investigation.

Combretum zenkeri Sample Collection and Preparation

The leaves of *C. zenkeri* were collected in the morning (7 am) in December, 2013, from a farm at Ihiagwa in Owerri-West LGA, Imo-State Nigeria. The plant sample was authenticated by a professional taxonomist, Dr. F. N. Mbagwu of Imo State University, Owerri, Nigeria with voucher number IMSU/0124 and thereafter deposited in the Imo State University Herbarium. The fresh leaves were plucked out from the plant stalk, rinsed with clean water and air-dried for 14 days. The dried leaves were pulverized with a mechanical grinder, packaged in air-tight glass jar and stored at room temperature until analysis was carried-out.

Preparation of Ethanol Extract

The pulverized sample was extracted as described by Handa *et al.* (2008). Briefly, 450g of the sample was weighed, mixed with 1200ml of absolute ethanol (BDH) and allowed to stand for 4 days with intermittent shaking. The mixture was filtered and the soluble extract was concentrated *in vacuo,* using a rotary evaporator (NYC), to obtain the ethanol leaf extract of *C. zenkeri* (CZEE) concentrate. The concentrated ethanol extract so obtained was stored in a refrigerator at 4°C until used.

Acute toxicity study (LD_{50})

A procedure that required few animals was followed for this determination using the method of Lorke, (1983) as described in Chinedu *et al.* (2013). The first phase employed nine male albino rats. The rats were divided into three groups of three animals each. Each group of animals were administered different doses (10, 100 and 1000 mg/kg) of ethanol leaf extract of *Combretum zenkeri*. The rats were placed under observation for 24 hours to monitor their behavior and any mortality. There was no observable behaviour or mortality. In the second phase three rats were used, which were distributed into three groups of one animal each. The rats were administered higher doses (1600, 2900 and 5000 mg/kg) of ethanol leaf extract of *Combretum zenkeri* and then observed for 24 hours for behavioural changes and mortality. As well, no observable behaviour or mortality was noticed. Based on the above observations, we decided to use a dose of 400 mg/kg for the study.

Animals

Thirty adult male albino rats weighing 80-100g were purchased from a laboratory animal farm (Animal Friend Co., Owerri). They were housed in standard cages and left to acclimatize for 7 days to standard laboratory conditions - light/dark regimen (12hr light: 12 hr darkness) at a temperature of 25°C, before the commencement of the experiment. The animals were maintained on standard laboratory feed and portable water *ad libitum*. The rats were divided into five groups of 6 rats each.

Experimental design

i) Group I (Control): They were provided with standard laboratory feed and portable water *ad libitum.*

ii) Group II, Benzo(a)pyrene group (BaP group). Apart from feed and water, they were administered intra-peritoneally with benzo(a)pyrene dissolved in olive oil (200 µl) at 200 mg/kg bw without being treated.

iii) Group III, Post Treatment group (PST group). They were administered benzo(a)pyrene dissolved in olive oil (200 µl) at 200 mg/kg bw by intra-peritoneal route at the beginning of the experiment, and later treated with *C. zenkeri ethanol* extract dissolved in 200µl olive oil at 400mg/kg bw administered every two days by gavage using an intubator after two weeks of intoxication. This treatment was on alternate days and ran through the last two weeks of the experiment.

iv) Group IV, Pre Treatment group (PRT group). They were initially treated with the *C. zenkeri* ethanol extract dissolved in 200µl olive oil at 400mg/kg bw administered on alternate days by gavage using an intubator for two weeks before being administered with benzo(a)pyrene dissolved in olive oil (200µl) at 200mg/kg bw by intra-peritoneal route. The treatment with *C. zenkeri* extract was then continued for the remaining two weeks.

v) Group V (Plant Extract Only Group). The animals in this group were exposed to the plant extract dissolved in 200µl olive oil at 400mg/kg bw administered on alternate days by gavage using an intubator throughout the duration of the experiment.

Animal Sample Preparation

At the end of the treatment period (28 days), the animals were made to fast overnight. After which they were sacrificed following mild ether anaesthetics. Blood was drawn by cardiac puncture into both EDTA tubes for plasma, and dry plain tubes for serum samples. Blood haemolysate was obtained after washing the whole blood as described in Bowmanand Gillespie(1982). The kidney and liver tissues were excised and stored in freshly prepared formalin for histological processing and microscopy.

Biochemical Analyses

Antioxidant Parameters

Glutathione (reduced) was measured according to Ellman's method as described by Raja *et al.* (2007). Blood malondialdehyde (MDA) concentration was determined in terms of thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa *et al.* (1979).

Liver Function Tests

The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of the test animals were assayed using commercial test kits (Randox Laboratories, UK) based on the Reitman and Frankel method (1957).The serum albumin concentration was determined with Biosystems test kit using the bromocresol green method as described by Doumas *et al.,* 1971. Serum total protein test was carried out using Biuret method as described in Tietz, 1995. Total serum bilirubin was determined using the Pearlman and Lee method as described in Tietz, (1995).

Histological Studies of Liver Tissues

Histological study was carried out on liver tissue samples from the different groups of animals using the modified method described by Okoro (2002).

Statistical Analysis

The data generated were expressed in mean and standard deviation, and analysed using One–way analysis of variance (ANOVA) with the aid of SPSS 17.0 software. The PostHoc test was done with Least Significant Difference (LSD). Means with $p < 0.05$ was considered statistically significant and were indicated by different alphabets in superscript.

3. Results

The effect of *C. zenkeri* (*CZEE*) treatment on benzo(a)pyrene-induced oxidative toxicity was studied by analysing the concentrations of reduced glutathione (Table 1) in the blood haemolysate. The positive control group (BaP-grp) and the group pre-treated with CZEE before administration of benzo(a)pyrene (PRT grp) showed non-significant ($p>0.05$) reductions in GSH concentrations in comparison with the untreated control group. The post-treatment group (PST grp) and the group administered only the plant extract had significantly ($p<0.05$) higher GSH concentrations when compared to the BaP group.

Malondialdehyde concentration was analysed as a marker of lipid peroxidation and the result is also shown in Table 1. The positive control (BaP-grp) showed a significant ($p< 0.05$) increase in the concentration of MDA compared to the normal control, while the other groups showed no significant difference ($p> 0.05$) compared to the control.

The effect of the different treatment plans on serum AST and ALT activities are shown on Table 1. The BaP- and PRT-groups showed a significant ($p<0.05$) increase in AST activity in comparison with the normal control. Administration of the plant extract only, significantly ($p<0.05$) decreased both serum AST and ALT activities when compared with the control and the other treatment groups. Similarly, ALT activities of the PST- and PRT-groups were significantly ($p<0.05$) lower than those of the normal control and the BaP group.

Table 1 also shows the results of the serum total protein, albumin and bilirubin concentrations of the animal groups. It shows that, while serum albumin and total protein concentrations increased significantly ($p<0.05$) in the BaP-, PST- and PRT-treatment groups in comparison with the control group respectively. On the other hand, the serum bilirubin concentrations of the BaP-, PST- and PRT-groups increased significantly ($p<0.05$) when compared with the normal control and the plant extract only group.

Plates 1-5 show the micrographs of liver tissue sections of the normal control, BaP-group, PST-group, PRT-group and Plant extract only group respectively. While Plates 1 and 5 show normal hepatic venules, Plates 2, 3 and 4 show enlarged hepatic venules, which were prominently more in the BaP- and PRT-groups than the PST-group. The micrograph of the BaP-group also showed blood-congested vessels.

Table 1. Concentrations of reduced glutathione andmalondialdehyde in the treatment groups.

Treatment Groups	GSH (mg/dL)	MDA (nmole)
Normal Control	135.8±5.99^{abc}	0.112±0.01^{a}
BaP Group	129.6±2.29^{a}	0.171±0.02^{b}
PST Group	137.2±1.23^{bc}	0.127±0.02^{a}
PRT Group	131.0±3.65^{ab}	0.121±0.01^{a}
Plant extract only	141.5±2.30^{c}	0.129±0.01^{a}

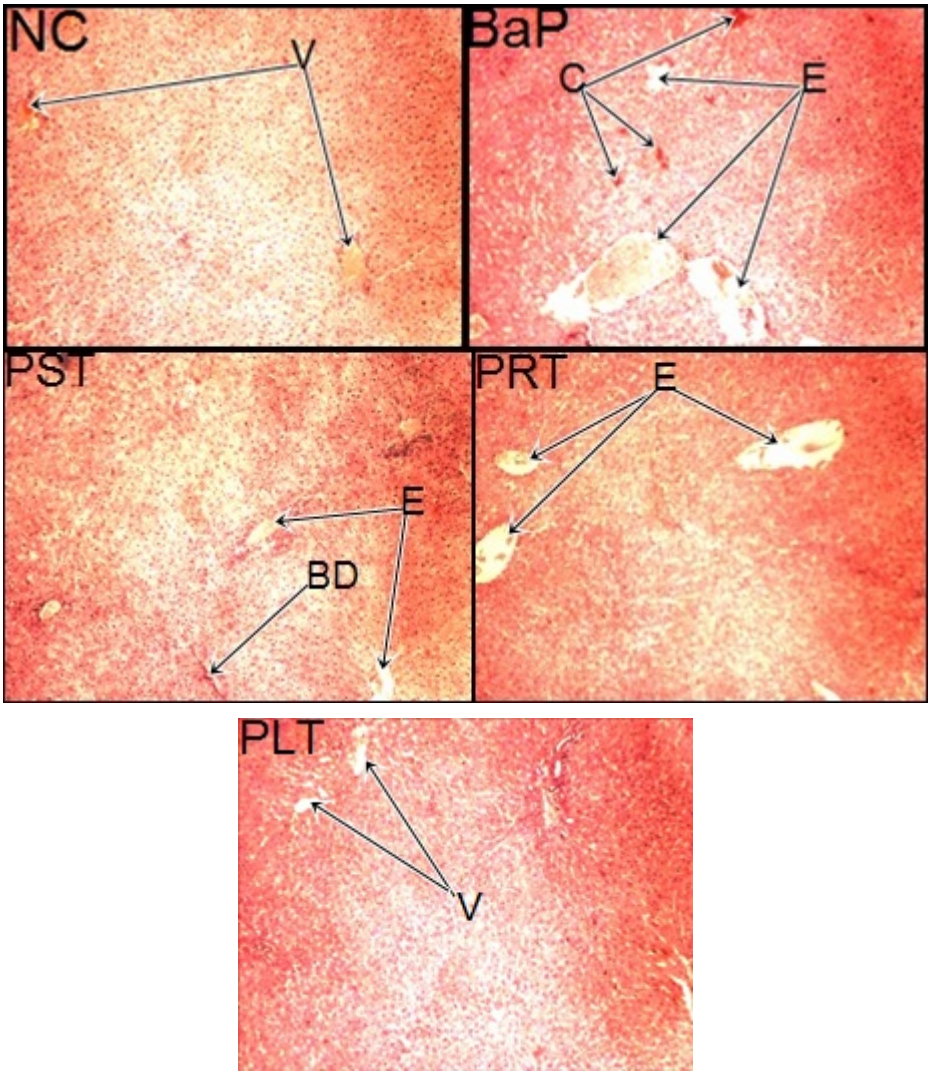
Values are mean ± standard deviation. Values with different superscripts per column are significantly different ($p<0.05$). GSH, glutathione; MDA, malondialdehyde; BaP, Benzo(a) pyrene; PST, Post-treatment; PRT, Pre-trreatment.

Table 2. Serum Protein, Bilirubin and liver enzymes activities of the treatment groups.

Treatment Groups	AST (U/L)	ALT (U/L)	Albumin (g/L)	Total Protein (g/L)	Bilirubin (mg/dL)
Normal Control	147.3±2.03^{b}	41.33±1.90^{c}	33.83±1.07^{c}	71.72±0.18^{d}	0.94±0.09^{a}
BaP Group	174.0±3.31^{d}	40.25±1.38^{c}	29.38±0.63^{a}	65.54±0.18^{a}	1.67±0.03^{c}
PST Group	150.6±2.17^{b}	30.40±0.65^{a}	31.80±1.03^{b}	70.66±0.19^{c}	1.12±0.67^{b}
PRT Group	160.7±3.18^{c}	38.98±0.38^{b}	31.56±1.75^{b}	66.51±0.12^{b}	1.22±0.05^{b}
Plant extract only	124.5±5.03^{a}	29.82±0.34^{a}	36.35±1.10^{d}	74.22±0.11^{e}	0.86±0.03^{a}

Values are mean ± standard deviation. Values with different superscripts per column are significantly different ($p<0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; BaP, Benzo(a) pyrene; PST, Post-treatment; PRT, Pre-treatment.

4. Histological Results



Low-power Micrograph of liver tissue section; **NC** = normal control group showing normal liver tissue architecture.**V** = Hepatic venules; **BaP** = Bap-treated group showing some congested blood vessels. **E** = enlarged hepatic portal veins and hepatic venule, **C** = blood-congested vessels; **PST** = Post-treated group.Some areas appear with enlarged hepatic portal veins. **E** = enlargements seem to have reduced. **BD** = bile duct; **PRT** = Pre-Treated group showing normal tissue architecture. **E** = enlarged central veins; **PLT**CZEE-only group.

Discussion

Studies have shown that increased production of reactive oxygen species (ROS) may be one of the underlying causes of most degenerative diseases (Adesegun *et al.,*2008; Wasson *et al.,* 2008; Martin and Appel, 2010) including cancer. Inhibition of the activities of reactive oxygen species by the ethanol leaf extracts of *C. Zenkeri* is another leap in the path to relief.

Lim *et al.* (2013), reported that *in utero* exposure to BaP induced increased sensitivity to premature ovarian failure and ovarian tumourigenesis as a result of deficiency of GSH due to deletion of the modifier subunit of glutamate cysteine ligase (Gclm), the rate-limiting enzyme in GSH synthesis. The groups treated with the plant extract after

benzo(a)pyrene exposure showed significantly raised GSH showing how potent the ethanol extract of *C. Zenkeri* can be in the restoration of depleted endogenous antioxidants, so as to reduce possible free radicals-mediated damage. Raised levels of GSH protect cellular proteins against oxidation through the glutathione redox cycle and also detoxify ROS directly, neutralizing reactive intermediate species generated from exposure to xenobiotics, including chemical carcinogens (Ketterer, 1998).

The hydroxyl radical is highly reactive and can damage biological molecules. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids that eventually yield carbonyl products like malondialdehyde (MDA) (Chaudhuri *et al.,* 2012). This agrees with Gao *et al.* (2011) who reported MDA levels increased significantly and dose dependently in the BaP-treated groups compared with the control groups. The significant ($p<0.05$) reduction in MDA concentration in blood hemolysate of the *C. zenkeri* ethanol extract treated groups shows how antagonistic the extract can be to lipid peroxidation. If there is a positive correlation between lipid peroxidation (LPO) status and genotoxicity (Mayer *et al.,* 2000), reduction in MDA concentration by ethanol leaf extracts of *C. zenkeri* which indicates a reduction in lipid peroxidation suggests that it has protection agaist genotoxicity.

It is the liver's role to metabolize nutrients, detoxify harmful substances and make blood clotting proteins and other metabolism related functions. When liver tissues are damaged, enzymes domiciled in the liver cells leak into the blood. AST and ALT are two major enzymes assayed in such conditions. Their elevation in blood signal liver damage, even due to peroxidation of the liver cell membranes. The AST activity in the positive control (BaP grp) was significantly higher than the other groups. This suggests high serum enzyme activity and cellular leakage possibly due to oxidative damage of liver tissues (Mukherjee, 2003). The observed increase in AST activity correlates other works that reported increase in AST activity due to damage to liver tissue (Cosan *et al.,* 2008; Osman *et al.,* 2009; Gao *et al.,* 2011). However, groups treated with only the ethanol extract of *C. zenkeri* show significant reduction in AST and ALT activities.

The -SH moiety of cysteine is highly prone to oxidative attack by several mechanisms, leading to the formation of disulfide bonds and thiyl radicals (Shacter, 2000). Significant reduction in serum total protein and albumin concentrations in the group exposed to benzo(a)pyrene without treatment suggests damage of protein molecules by BaP-derivative oxidants. On the other hand, groups treated with the plant extract before and after exposure to BaP showed significant rise in serum total protein concentration. This result indicates the potential of the plant extract to ameliorate protein damage due to free radicals. Also, glutathione contains free -SH moiety of cysteine, glutathione reduction in the group exposed to benzo(a)pyrene without treatment can be attributed to free radical attack on the free -SH moiety of cysteine.

The study by Cantin *et al.,* (2000), shows that albumin modulates NF-κB activation and cellular GSH levels. According to Ramos *et al.* (2013), albumin and albumin-like proteins are critical regulators of vascular redox signalling. In other words, a significant reduction ($p<0.05$) of albumin proteins could expose cells to oxidative stress. Since protein metabolism is a major project of the liver, low protein concentration in the group exposed to the toxicant without treatment may indicate liver damage.

A raised total bilirubin was reported among diesel engine workers who were exposed to high-pressure resistant lubricants containing lead naphthanate (Seema *et al.,* 2013) and workers exposed to petroleum station pollutants (Al-Helaly and Ahmed, 2014). This could possibly indicate damage to liver tissues, excessive heme destruction and biliary tract blockage. So, there is a mass inhibition of the conjugation reaction channelling unconjugated bilirubin from damaged hepatocytes (Reddy *et al.,* 2012).

We have been able to show that ethanol leaf extract of *Combretum zenkeri* can protect the liver from benzo(a)pyrene-induced oxidative damage.

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