Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(3): 316-322
Received: 06-03-2018
Accepted: 12-04-2018

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Betulinic acid attenuates hepatic and testicular redox imbalance and DNA damage in male rats exposed to crude oil

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Abstract
This study investigated the effects Betulinic acid (BA) on CO-induced redox imbalance in liver and testes of rats Twenty-four male rats were assigned into four groups and treated with normal saline (control), BA (20 mg/kg), CO (10 mg/kg) and BA + CO (as stated) for 21 days.. Crude oil decreased hepatic and testicular SOD activities (70% and 50%, respectively) and Hepatic GSH (28%). Hepatic catalase activity significantly decreased in CO group (74%). Crude oil elevated MDA levels in liver (194%) and testes (67%) against controls. Supplementation with BA significantly ameliorated the redox changes induced by CO. DNA fragmentation increased in liver (35.70±5.24%) and testes (50.78±7.32%) in CO group, which BA prevented. Remarkable cytomophorlogical distortions occurred in liver and testes of CO group, but ameliorated on BA supplementation. BA induced activation of antioxidant pathways against CO-induced redox changes and DNA damage in rats.

Keywords: crude oil, betulinic acid, oxidative stress, DNA damage, organ degeneration

Introduction
The toxicological implications of crude oil exposure involving humans and animals have been noted with serious challenges on environmental and public health [1, 2]. Crude oil is a complex mixture of organic and inorganic substances including alkanes, cycloalkanes, aromatic hydrocarbons, nitrogen, oxygen, sulphur, iron, nickel, copper, vanadium and organometallic compounds [3-5]. Furthermore, Speight, [6] in the recent time, has reported the scarcity or complete absence of alkenes and alkynes in petroleum. On spillage upon water, crude oil becomes spread over a wide area forming a slick, and later begins to undergo a series of physical, chemical and biological changes [7]. During oil exploration, the vicinity usually becomes contaminated with oil, leading to an exposure of the workers hazardous effects of the pollutant [8]. The toxicity resulting from an oil spill has been associated with the aromatic compounds of the oil [9]. An important class of aromatic compounds in crude oil is polycyclic aromatic hydrocarbons (PAHs), which are metabolized by cytochrome-p450 enzymes to mutagenic diol epoxides [10]. A study by Izawa *et al*. [11] observed that the polycyclic aromatic hydrocarbons (PAHs) present in diesel exhaust particles triggered reproductive toxicity via activation of aryl hydrocarbon receptors (AhRs) and reduction in number of sertoli cells in testes of mice. However, in humans, PAHs have been well implicated in atherosclerosis, mutagenesis and carcinogenesis, involving oxidative and inflammatory mechanisms triggered by their reactive metabolites [12-15].
Natural products have been used for combating human diseases since they exhibit a wide range of biological properties that can be exploited for medical application [16]. One of such products is betulinic acid (BA) (3β-hydroxy-lup-20 (29)-en-28-oic acid), which is a pentacyclic lupane-type triterpene with a myriad of biological properties. BA has been reported to be potent against human immunodeficiency virus (HIV), [17] bacterial infections, [18] malaria, [19] inflammatory processes, [20-22] and cancers [23, 24]. This phytochemical has also been shown to prevent pathologic apoptosis and micronucleus formation [25]. Furthermore, the chemopreventive potential of BA has been demonstrated, showing reduction in the activities of cytochrome p450-dependent enzymes, and elevation in glutathione-S-transferase (GST) and uridyl diphosphoglucurononyl transferase (UDPGT) activities in experimental rats [26]. In the present study, we tested the hypothesis that BA, a triterpene with strong antioxidant potential, could protect against hepatic and testicular oxidative injuries in rats exposed to crude oil.

Materials and Methods

Chemicals

Betulinic acid (BA), hydrogen peroxide, epinephrine, Glutathione, and 5, 5′-dithios-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co., Saint Louis, MO, U.S.A. Trichloroacetic acid and thiobarbituric acid (TBA) were purchased from British Drug House (BDH) Chemical Ltd, Poole, UK. Crude oil (CO) was supplied by the Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria. Other chemicals and reagents were of analytical grade and purest quality available.

Experimental animals

Male Wistar rats (204.66±7.72 g) were purchased from the Animal house of the Institute for Medical Research and Training (IMRAT), University College Hospital (UCH), Ibadan, Nigeria. They were housed in plastic cages and fed on rats pellets and given drinking water ad libitum. The rats were acclimatized for 7 days before the experiment and subjected to 12-hour light/dark cycle and temperature of 29±2 °C. The study was approved by the Animal Ethics Committee of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

Study design

Twenty-four male Wistar rats were randomly assigned into four groups of six rats each. The first group (Control) was administered with normal saline, BA group was administered with BA alone (20 mg/kg body weight), CO group was administered with CO alone (10 mg/kg body weight), while [BA + CO] group was administered with BA (20 mg/kg body weight) and CO (10 mg/kg body weight). The choice of BA dosage and vehicle was based on previous studies by Adeleke and Adaramoye, [25, 26] while that of CO was guided by our pilot study. BA (for 21 consecutive days) and CO (two times per week) were both administered by oral gavage.

Collection of liver and testes

The rats were fasted overnight on day 21. The rats were then anaesthetized, and liver and testes were excised, washed in ice-cold 1.15% potassium chloride solution to remove blood stains. One portion each of liver and testes was homogenized using a Teflon homogenizer and centrifuged using a high speed refrigerated centrifuge (HITACHI) at 10,000g for 10 minutes to obtain post mitochondrial fraction. Another portion each of the two organs was homogenized in Tris-EDTA (TE) buffer and then centrifuged at 27,000 x g for 10 minutes to separate the intact DNA (pellet) from the fragmented DNA (supernatant) for DNA fragmentation assay. While another portion of liver was fixed in 10% formalin, a portion of testes was fixed in Bouin's solution for histopathology.

Biochemical analyses

Protein determination

Hepatic and testicular protein levels were determined according to the method of Lowry *et al*. [27] using bovine serum albumin as the standard.

Determination of superoxide dismutase activity

Hepatic and testicular superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich. [28] The method was based on the ability of SOD to inhibit the autoxidation of epinephrine (pH 10.2, 30 °C). The increase in absorbance of assay reaction at 480 nm was monitored spectrophotometrically at 30 seconds intervals for 150 seconds. The specific activity of SOD was expressed in units/ mg protein.

Determination of GSH concentration

Hepatic and testicular GSH concentrations were assayed using the method described by Mitchell *et al*. [29] the assay involves oxidation of GSH by the sulfhydryl reagent DTNB to form a yellow product, 5′-thio-2-nitrobenzoic acid, which absorbs maximally at a wavelength of 412nm. GSH level is proportional to absorbance at 412 nm. Values were expressed as µmol/g tissue.

Assay of Catalase activity

The method, as described by Aebi, [30] is based on the ability of catalase to promote the decomposition of hydrogen peroxide in a reaction mixture. The change in absorbance during 3 minutes at 240 nm is a measure of catalase activity. Enzyme activity was expressed as units/mg protein.

Determination of malondialdehyde (MDA) level

Hepatic and testicular lipid peroxidation (LPO) was estimated by determining the concentration of thiobarbituric acid reactive substance (TBARs), as described by Ohkawa *et al*. [31] The method is based on the reactivity of an end product of LPO, malondialdehyde (MDA), with TBA to produce a pink adduct. The absorbance of the clear supernatant was read in a spectrophotometer against a reference blank at 532 nm. LPO was expressed in micromole MDA formed/mg protein using a molar extinction coefficient of 1.56×105/m/ cm.

Colorimetric assay of DNA Fragmentation by Diphenylamine (DPA) method

The Diphenylamine (DPA) method described by Wu *et al*. [32] (with some modifications) was used to determine DNA fragmentation level in both hepatic and testicular organs The tissues were homogenized in Tris-EDTA (TE) buffer and then centrifuged using cold (refrigerated) centrifuge at 27000 x g for 10 minutes to separate the unfragmented DNA (pellet) from the fragmented DNA (supernatant). Each of the pellet and supernatant was treated with freshly prepared DPA reagent and the reaction mixture was incubated at 37°C for 16-24 hours for colour development. The absorbance was read spectrophotometrically at 620nm. The percentage fragmented DNA was calculated according to the formula:

$$\% \text{ Fragmented DNA} = \frac{\text{Absorbance of Supernatant x 100}}{\text{Absorbance of Pellet + Absorbance of Supernatant}}$$

Histological examination

A section of the liver was fixed in 10% formalin, while that of the testicular tissue was fixed in Bouin's solution for histology. Microsections (3 µm) of the tissues were prepared and stained with hematoxylin and eosin (H&E) dye, and then examined under a light microscope.

Statistical analysis

All values were expressed as the mean ± standard deviation of six animals per group. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test for the analysis of biochemical data using SPSS (10.0). Statistically significant values were taken at $P<0.05$.

Results

Effects of Betulinic acid (BA) on body weight gain in the CO-treated rats

Table 1 shows the changes in body weights of the experimental rats. Treated with CO alone significantly (p<0.05) elevated the body weight gain relative to controls (26.90±8.93 versus 13.03±6.43 g, respectively). A supplementation with BA significantly attenuated the increase in weight gain compared with the CO-treated rats (15.10±5.00 versus 26.90±8.93 g, respectively).

Table 1: Changes in body weights of rats treated with crude oil and BA (alone and in combination

Treatments	Initial weight (g)	Final weight (g)	Body weight gain (g)
Control	210.12±14.10	223.15±25.88	13.03±6.43
BA	212.20±21.02	230.10±30.10	17.90±3.42
CO	196.22±17.32	223.12±20.82	26.90±8.93*
BA + CO	200.10±05.01	215.20±17.32	15.10±5.01**

Data expressed in Mean± SD, n = 6, CO= Crude oil, BA= Betulinic acid

* Statistically different compared with control

** Statistically different compared with crude oil.

Effects of BA on antioxidant status of CO-treated rats

Treatment with CO alone was observed to cause a significant imbalance in the redox status of both hepatic and testicular organs of the experimental rats. The activities of SOD in the respective organs of CO-treated rats significantly (p<0.05) decreased by 70% and 50% compared with controls, as show in table 2. On supplementation with BA, there were significantly elevated activities of SOD in liver (329%) and testes (135%) compared with CO-intoxicated group. The treatment with CO alone lowered the hepatic GSH level relative to controls (56.50±7.87 versus 72.50±9.00 µg /mg protein, respectively), while a supplementation with BA increased the level comparable to controls (Table 2). However, the testicular GSH level remained unchanged during the treatment period. Further investigation on the redox status revealed the effects of the treatment on the hepatic and testicular catalase activities and MDA levels in the rats. The treatments caused the hepatic catalase activity to significantly decrease in the CO-treated rats by 74% compared with the controls, while the testicular catalase activity showed no remarkable changes (Figure 1). From the result presented in figure 2, CO treatment significantly elevated the MDA levels in liver and testes by 194% and 67%, respectively compared with control rats. A supplementation with BA was noticed to significantly (p<0.05) attenuate the effects relative to the toxicant group.

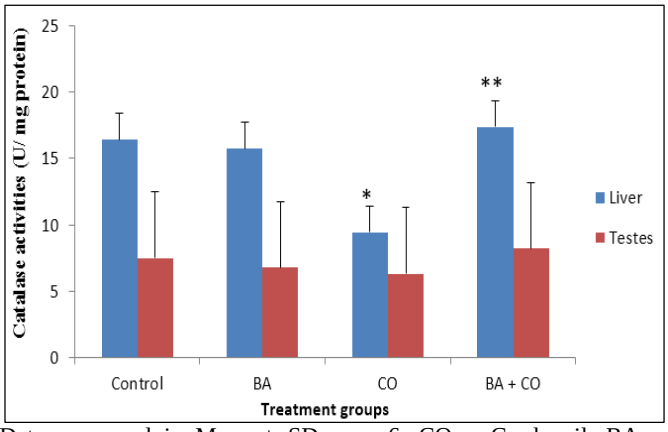
Table 2: Hepatic and testicular superoxide dismutase (SOD) activities and reduced glutathione (GSH) levels in rats treated with crude oil and BA (alone and in combination)

Treatments	Superoxide dismutase (U/mg protein)		Reduced glutathione (µg/mg protein)	
	Liver	Testes	Liver	Testes
Control	06.14±2.13	30.45±1.46	72.50±9.00	32.88±7.75
BA	05.53±2.60	38.84±0.00	73.00±6.87	34.50±4.00
CO	03.61±0.00*	20.29±2.91*	56.50±7.87*	29.00±8.87
BA + CO	15.48±6.59**	47.62±1.87**	74.17±3.76**	31.00±5.23

Data expressed in Mean± SD, n= 6, CO= Crude oil, BA= Betulinic acid

* Statistically different compared with control

** Statistically different compared with crude oil.

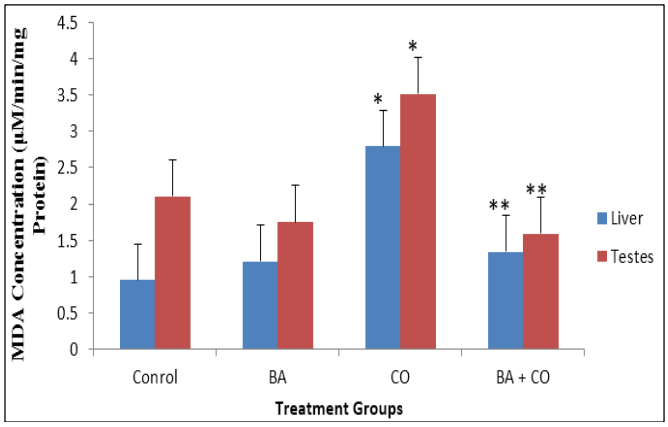


Data expressed in Mean ± SD, n = 6, CO = Crude oil, BA = Betulinic acid

* Statistically different compared with control

** Statistically different compared with crude oil.

Fig 1: Hepatic and testicular Catalase (CAT) activities in rats treated with crude oil and BA (alone and in combination)



Data expressed in Mean ± SD, n = 6, CO = Crude oil, BA = Betulinic acid

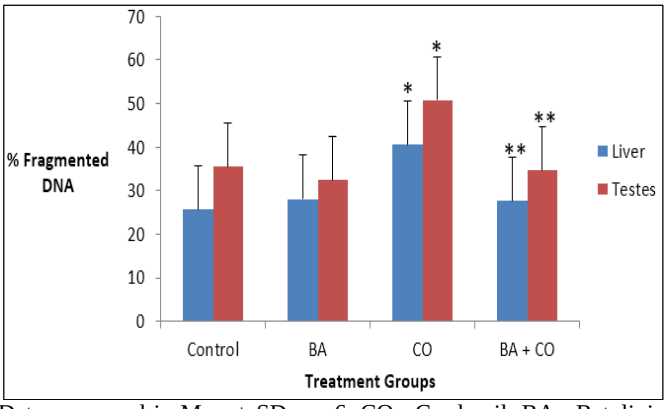
* Statistically different compared with control

** Statistically different compared with crude oil.

Fig 2: Hepatic and testicular malondialdehyde (MDA) levels in rats treated with crude oil and BA (alone and in combination)

Effects of BA on DNA damage and histopathology of liver and testes of CO-treated rats

The treatment with CO significantly elevated the level of DNA fragmentation in both liver (35.70±5.24) and testes (50.78±7.32) compared with controls (25.60±3.55 and 35.50±6.21, respectively), as shown in figure 3. However, on supplementation with BA, the levels of fragmented DNA were significantly (p<0.05) ameliorated in both hepatic (27.60±6.31) and testicular (34.61±7.90) organs compared with CO-treated rats (35.70±5.24 and 50.78±7.32, respectively). The present study also demonstrated the histological examination of the liver and testes of the experimental rats. Figure 4 reveals that CO induced sinusoidal congestion and vacuolar degeneration in the liver, while a significant amelioration was observed in [BA + CO] group, comparable to control and BA alone groups. Treatment with CO caused severe germinal cell necrosis in the testes of rats (Figure 5), while the combination of BA and CO showed no observable lesions, similar to the control and BA alone groups.



Data expressed in Mean± SD, n= 6, CO= Crude oil, BA= Betulinic acid

* Statistically different compared with control

** Statistically different compared with crude oil.

Fig 3: Hepatic and testicular % fragmented DNA in rats treated with crude oil and BA (alone and in combination)

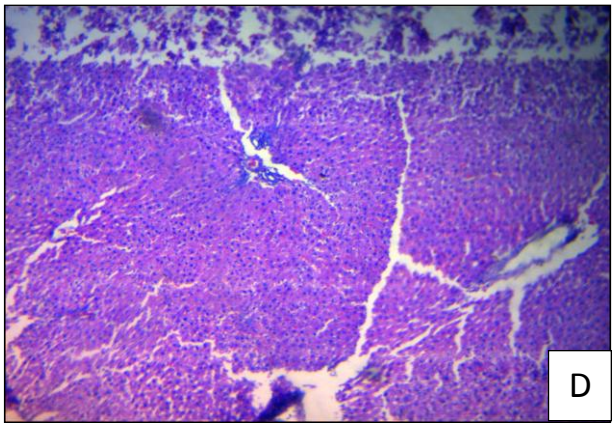
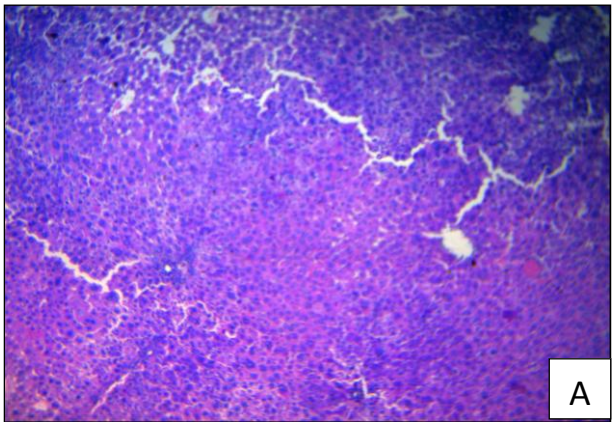
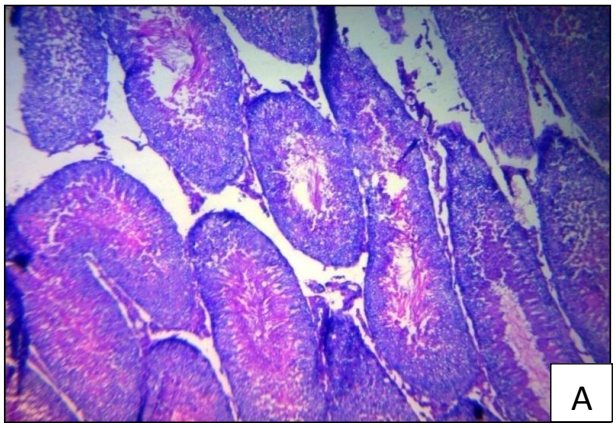
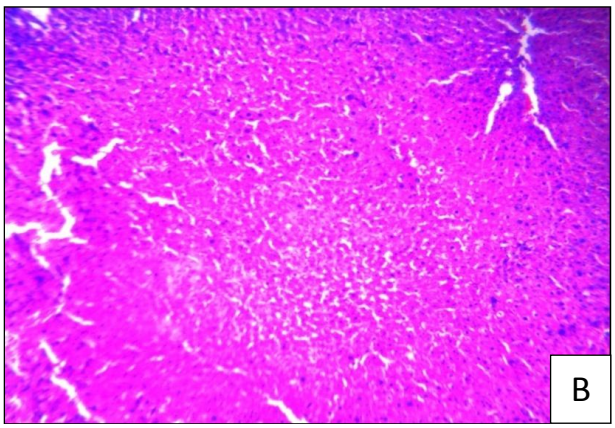
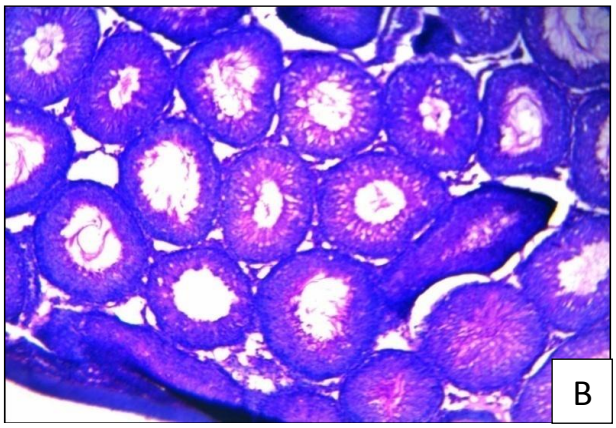


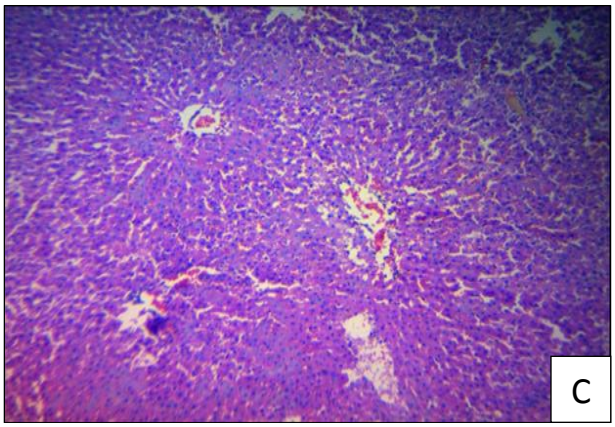
Fig 4: Representative photomicrographs of liver from control and CO-treated rats. Control liver (A), BA-treated rats (B) and [BA + CO] (D) showed no visible lesions, while CO-treated rats showed sinusoidal congestion vacuolar degeneration (C) (x 100). BA-Betulinic acid; CO-Crude oil

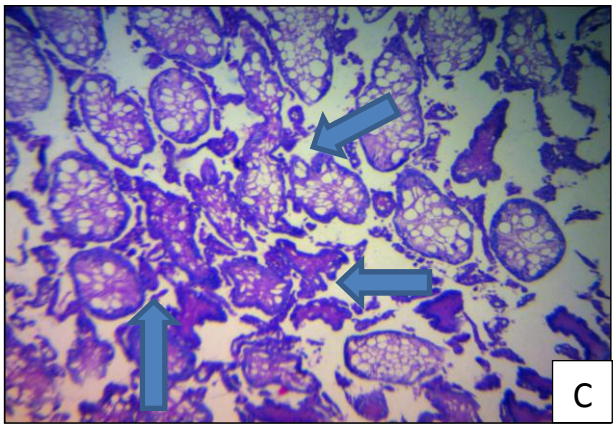












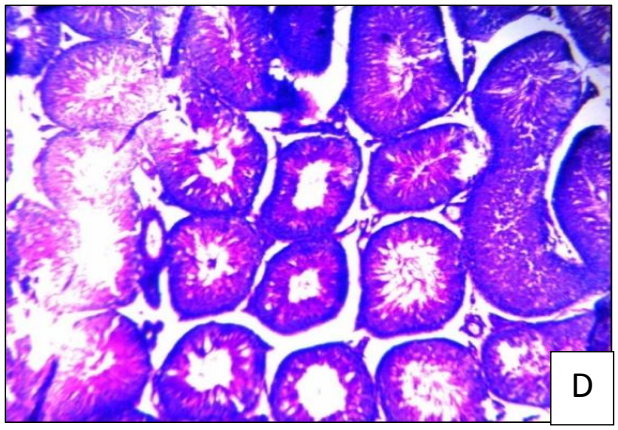


Fig 5: Representative photomicrographs of testes from control and CO-treated rats. Control testes (A), BA-treated rats (B) and [BA + CO] (D) showed no visible lesions while, CO-treated rats showed severe germinal cell necrosis (C) (x 200). BA- Betulinic acid, CO-Crude oil

Discussion

Exposures to crude oil through several means have been noted with serious environmental and public health challenges as reported by Cooney *et al.* [1] and Akfopure *et al.* [2] The present study has shown that crude oil caused a significant increase in body weight gain of the experimental rats relative to control, suggesting the tendency of this toxicant to induce overweight or even obesity in mammals. On supplementation with BA, the body weight gain was significantly lowered in the rats. Petroleum, a fraction of crude oil, has been observed by Val and Almeida-Val [33] and Achuba and Osakwe [34] to induce oxidative stress via generation of deleterious free radicals like reactive oxygen species (ROS). Superoxide Dismutase (SOD) catalyzes the dismutation of superoxide anion radicals ($O^{\cdot}_2$) to hydrogen peroxide (H_2O_2) in cellular systems. SOD is a ubiquitous chain breaking antioxidant enzyme, playing an important role in protection against deleterious effects of oxidative damage. The hydrogen peroxide, in turn, is either metabolized by catalase or by glutathione peroxidase [35]. Superoxide ion ($O_2^{\cdot}$) and hydroxyl radicals are known to cause marked oxidative injuries to surrounding tissues and organs. In the present study, SOD activity was observed to decrease significantly in liver and testes compared with control. A reduced activity of SOD allows accumulation of highly reactive free radicals capable of deleterious actions that compromise the integrity and functions of cell membrane [36, 37]. In a related study, Oyebisi *et al.* [38] reported a significant reduction in SOD activity on exposure to petroleum hydrocarbons. Surprisingly, our study could not show significant effects on the activity of catalase in both hepatic and testicular organs of the rats. A supplementation with BA was observed to elevate SOD activity in liver and testes compared with the CO-treated rats. This finding therefore suggests the potential of this triterpenoid to induce SOD activity to attenuate the effects of reactive oxidants. This present study also examined reduced glutathione (GSH), a low molecular weight thione antioxidant that serves as a substrate for glutathione peroxidase (GPx) that detoxifies lipid peroxides and hydrogen peroxides during oxidative stress. On detoxification of toxicants, GSH becomes oxidized to GSH disulfide (GSSG), which is converted back to GSH by glutathione reductase (GPr) [39]. Thus GSH prevents the damaging effects of intracellular reactive oxygen species (ROS) [40]. Our findings from the present study show that GSH was significantly depleted in the liver of the CO-treated rats compared with control, an indication of a tendency of this toxicant to induce oxidative damage. Depletion of GSH may be associated with impaired GSH synthesis due to reduced availability of cysteine molecules, which in turn is a result of defective transulfuration characteristic of hepatic damage [41]. There were no significant changes noticed in testicular GSH levels of the rats. A recent study has shown that GSH plays an important role in the maturation of sperm cells in male experimental animals [42]. Reactive oxygen species (ROS) have been implicated in lipid peroxidation, a process forming several products, such as malondialdehyde (MDA) and 4-hydroxymethylnonenal (4-HNE), which are of toxicological importance [43]. The peroxidation of lipids and proteins could result in inactivation of antioxidant enzymes as noticed by Pigeolet *et al*, [44] Romero *et al.* [45] and Souza *et al.* [46] An elevated level of lipid peroxides indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function and cell death. [47] Lipid peroxidation has therefore been regarded as a basic mechanism of oxidative cellular damage resulting in several diseases caused by free radicals, such as ROS. MDA (a major product of LPO) was much later suggested to be a tumour promoter [48]. In the present study, levels of Thiobarbituric acid reactive substances (TBARS) were measured as a marker of lipid peroxidation. There were significant increases in MDA levels in both hepatic and testicular organs of the rats compared with controls. A similar observation made by Oyebisi *et al.* [38] noticed that petroleum hydrocarbons elevated MDA level in experimental rats. In addition, Arafa *et al.* [49] found out that benzo [a] pyrene, a member of the PAHs, reduced the testicular activities of SOD and GST, and GSH level, while MDA level was elevated. Overall, the decreases in SOD activity and GSH level, coupled with an increase in MDA level, observed in the CO-treated rats may suggest a causal link between this toxicant and oxidative stress in the rats. This suggested CO-induced oxidative stress may have resulted from the reactive metabolites, such as superoxide radical, hydrogen peroxide and impaired GSH synthesis generated during the treatment. However, a supplementation with BA was observed to remarkably elevate SOD activity and GSH level, as well as, reduce MDA level in the hepatic and testicular organs, indicating the ameliorative role of this triterpenoid against oxidative stress. Several studies by Senthikumar *et al.* [50] Qian *et al.* [51] and Adeleke and Adaramoye [26] have corroborated the finding from the present study, showing the prevention of oxidative imbalance in experimental rats by BA.

Studies have shown that the endonuclease G activation taking place in apoptosis (programmed cell death) causes the cleavage of nuclear chromatin to form oligonucloeosomal DNA fragments (strand breaks) with intense chromatin condensation [52-54] This study also investigated the effect of crude oil on apoptosis in liver and testes of the rats, by measuring the level of DNA fragmentation using the diphenylamine (DPA) assay described by Wu *et al.* [32] The present study observed that DNA fragmentation was significantly increased in the respective organs in CO group of the rats compared with controls. Although DNA fragmentation is a typical event in apoptotic changes, the higher level of fragmentation observed in the CO-treated rats relative to controls, may indicate excessive apoptosis, normally described as pathologic apoptosis. Moreover, the excessive DNA fragmentation noticed in the CO group of the rats may indicate a significant damage to DNA molecules in

the organs, suggesting a genotoxic potential of the toxicant. However, the ability of BA supplementation to suppress the level of DNA fragmentation against CO, as observed in the present study, suggests that this triterpenoid may exert a role in preventing both excessive (pathologic) apoptosis and DNA strands breakage in liver and testes of rats. BA was demonstrated to downregulate DNA fragmentation in rats [25]. Histopathological examinations of both hepatic and testicular organs of the rats showed sinusoidal congestion and vacuolar degeneration in the former and severe necrotic germinal cells in the latter, as against the controls. A study by Arafa *et al*. [49] observed that benzo [a] pyrene, a member of PAHs, caused pyknosis and necrotic changes in testes of rats. In an earlier investigation carried out by Sunmonu and Oloyede [55], it was observed that rats fed with crude oil-contaminated cat fish had hepatocytes degeneration. A supplementation with BA showed only mild sinusoidal congestion in liver and no observable degeneration in testicular tissue of the rats. In conclusion, we have shown from the present study that Crude oil induced oxidative imbalance and DNA strands breakage in liver and testes of rats, which supplementation with Betulinic acid was able to attenuate.

Acknowledgement
The authors hereby appreciate the effort of Dr. O. Aina (Department of Veterinary Anatomy, University of Ibadan, Nigeria) for the Histological examination of the tissues.

Conflicts of interests
No conflict of interests exist among the authors.

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