Costus Afer Ameliorates Lead-Induced Reproductive Alterations in the Pituitary-Testicular Axis: Histopathological and Biochemical Studies

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Introduction
Lead is one of the multiple reprotoxic agents ever known, and its exposure in places of work has been described as a key public health issue. Empirical evidence has shown that lead has adverse effect on spermatogenesis, chromosomal damage, abnormal prostatic function [1]. Moderate lead build-up in the blood has been implicated in dose-dependent changes in male sex hormones functions, as indicated by the effects of lead on the pituitary-testicular axis (Ng et al., 1991). Treatment for various infertility issues and poisoning due to lead exposure has become a major burden for families in the sub-Saharan Africa, due to the cost of procuring the conventional therapeutics and the numerous adverse effects associated with it. The culminations of the cost and side effects, has necessitated and rekindled massive interest in the search for alternative therapies including herbal medications to mitigate the effects of lead-induced sexual dysfunction in male. Costus afer belongs to the family of Asteraceae. The leaf extract contains flavonoids, steroids, terpenes and antioxidant [2]. Studies has shown that costus afer has infertility enhancing potential (Boison et al., 2019), the leaves and roots are used to treat diabetes, cancer and inflammations (Azhagu et al., 2019; Abd-El-Rahman et al., 2020). The present study aimed to evaluate the potency of costus afer in ameliorating lead-induced reproductive impairment in the pituitary-testicular axis of adult male wistar rats.

Materials and Methods
Collection and Identification of Plant
Sufficient quantity of Costus afer leaves were collected from a plantation in Aro Village, in Uturu community of Isiukwato LGA of Abia State, Nigeria. It was duly identified and authenticated by Prof. M.C. Dike of the Department of Forestry, College of Natural and Environmental Resources, Michael Opara University.
of Agriculture, Umudike (MOUAU). A voucher number-MOUAU/ZEB/20/0011, was assigned to it.

Preparation of Plant Extract
The fresh leaves of the Costus afer were washed with distilled water to remove dirt and Sand. They were air dried at room temperature for 14 days and grounded using an electric blender. One hundred and fiftyfour (154g) of pulverized plant material was macerated in ethanol, and stirred vigorously and left for 3 days, after which the plant material was filtered. The filtrate was placed in hot air oven at a low temperature to allow the ethanol to evaporate. Upon evaporation of ethanol, crude extract which was weighed at 7.5g was obtained and represented a percentage yield of 4.84%. The extract was preserved in a refrigerator until needed and is hereafter referred to as Costus Afer Ethanol Extract (CAEE).

Animals Husbandry
A total of twenty (20) wistar rats (173-180g) were obtained from the animal unit of the College of Natural and Environmental Resources, Michael Opara University of Agriculture, Umudike (MOUAU) and used for this study. The animals were housed in aluminium cages and provided with standard feed (Vital Feed, Nigeria) and water ad libitum. All animal experiments were carried out in compliance with NIH guidelines for Care and Use of Laboratory Animals (Pub.no.85 Revised 1985), (OECD 2001). Prior to the commencement of the study, the animals were allowed to acclimatize to for a period of 14 days.

Experimental Design
Twenty (20) adult male wistar rats were divided into four groups of five rats each, and housed in aluminium cages. Each group was treated for 21 days through the oral route using oral gavage as follows:

Group 1: Normal control received distilled water
Group 2: Treated with 1000mg/kg of Lead acetate
Group 3: Treated with 1000mg/kg of Lead acetate + 400mg/kg of CAEE
Group 4: Treated with 1000mg/kg of Lead acetate + 800mg/kg of CAEE

Animal Sacrifice
At the end of the experimental period, the animals were anaesthetized using chloroform. Blood samples were collected by cardiac puncture into plain tubes, and centrifuged at 4000 rpm for 20 minutes in order to separate the serum. The serum was thereafter kept in a refrigerator at a temperature of -40°C. The testes were harvested, blotted dry, rinsed in saline solution and weighed at 1.62±0.05 (Figure. 1.0) higher when compared with the control in which the percentage weight was 9.43±0.14 and 9.29±0.19ng/ml respectively and were not statistically significant when compared with the control. Serum testosterone value in control was 9.43±0.24 ng/ml while in groups 3 and 4 treated 400mg/kg and 800mg/kg of CAEE the values were 9.00±0.08 ng/ml when compared with control.

Hormonal Analysis
Serum testosterone was analysed using commercial enzyme-linked immunosorbent assay (ELISA) kit, and following the instruction of the manufacturer (LSBio).

Semen Analysis
The caudal end of the epididymis was incised to obtain spermatozoa, employing the method previously used by El-Desoky et al. (2013). Briefly, the epididymis was immersed in 3ml phosphate buffered saline at PH of 7.4, centrifuged and incubated at 37°C. The supernatants were thereafter assayed for sperm quality and characteristics using the method described by [3]. The assessment of progressive sperm motility was done using the method described by Zemjanis (1970). Spermatozoa were counted using improved Neubaur-hemocytometer Chamber (Deep 1/10mm, LABART, Germany).

Biochemical Analysis
The testicular catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) assays were determined using the methods described by [4,5].

Statistical Analysis
The results were analysed using one-way analysis of variance (ANOVA)-statistical package for social sciences (SPSS) version 13. Data were expressed as mean±standard error of mean (S.E.M) and multiple comparism were done using Bonferoni post-hoc tests. The significance levels were set at p<0.05

Results
Effect of CAEE on relative organ (Testis) weight (ROW) in percentages
The result showed a significant increase in the testes to body weight ratios in the Lead + high dose of CAEE group when compared with the control (p=0.05). The Lead only group and Lead + low dose group had a percentage weight of 1.85±0.02 and 1.72±0.04 respectively. The values so obtained were slightly higher when compared with the control in which the percentage weight was 1.62±0.05 (Figure. 1.0)

Effect of CAEE on Serum Testosterone
Serum testosterone was slightly lowered in the Lead only group with the value, 9.00±0.08 ng/ml when compared with control. Testosterone value in control was 9.43±0.24 ng/ml while in groups 3 and 4 treated 400mg/kg and 800mg/kg of CAEE the values were 9.43±0.14 and 9.29±0.19ng/ml respectively and were not statistically significant when compared with the control (Figure. 1.1)
Effect of CAEE on Serum Antioxidant Enzymes

Results of the effects of CAEE on antioxidant enzymes of Lead-induced toxicity showed that the extract significantly increased the concentration on antioxidant enzymes following treatment when compared with Lead-only group (p<0.05). Superoxide dismutase (SOD) concentrations in the control and Lead-only groups were 31.29±0.51 and 25.31±0.91 U/L. Treatment with low and high doses of CAEE elevated the serum levels of SOD in groups 3 and 4 as follows, 31.55±0.85 and 33.40±1.04 U/L. Changes in the concentration of catalase (CAT) showed similar increase as that of the SOD. There was slight significant decrease in the serum levels in the Lead-only group when compared with the control (p<0.05). The slightly elevated values of CAT in the groups treated with low and high doses of CAEE were 13.38±0.43 and 14.23±0.30 U/L respectively. However, there was a statistically significant increase in the serum levels of malondialdehyde (MDA) in the Lead-only group (1.21±0.04mmol/L) when compared with the control (0.36±0.06mmol/L) (p<0.05). Lowered levels of MDA were observed in groups 3 (0.88±0.06mmol/L) and 4 (0.73±0.02mmol/L) (Figure. 1.2, 1.3 & 1.4).

Effect of CAEE on Testicular Antioxidant Enzymes

Treatment with CAEE slightly lowered the testicular level of SOD when compared with the control. The values of SOD in the control and Lead-only groups were 7.16±0.16 and 6.50±0.19U/L. When compared with the Lead-only group, the groups 3 and 4 had slight elevated values of SOD as follows, 7.08±0.21 and 7.13±0.16U/L. A significant decrease in CAT level was observed in Lead-only group when compared with the control. Moderate to highly significant increase was observed in groups treated low and high doses of CAEE when compared with Lead-only group. However, there was a highly statistically significant increase in the testicular levels of malondialdehyde (MDA) in the Lead-only group when compared with the control group. Also, significant decrease was observed in the Lead + low and high doses groups when compared with Lead-only group (Figure. 1.5, 1.6 & 1.7).
Figure 1.5: CAEE on Testicular Superoxide Dismutase (SOD)

Figure 1.6: CAEE on Testicular Catalase (CAT)

Figure 1.7: CAEE on Testicular Malondialdehyde (MDA)

Effect of CAEE on Seminal Indices
Table 1.0 showed the multiple comparison of levels of seminal indices in study groups. It was observed that there was reduced level of sperm counts, normal morphology, viable sperm cells and progressive motility in group 2 (1000mg/kg lead acetate) compared with control (p<0.05) but non-significant difference between group 3 (100mg/kg lead acetate +400mg/kg CAEE) and group 4 (100mg/kg lead acetate +800mg/kg CAEE). It was also observed that CAEE showed significant difference (p<0.05) in changing the abnormal morphology of the sperm cells to normal form induced by lead acetate toxicity.

Table 1.0: Multiple Comparison of Levels of Seminal Indices in Study Groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 N=5</th>
<th>Group 2 N=5</th>
<th>Group 3 N=5</th>
<th>Group 4 N=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (x10^6)</td>
<td>114.58±4.34</td>
<td>82.90±5.49</td>
<td>93.24±2.75</td>
<td>95.24±1.82</td>
</tr>
<tr>
<td>Sperm PH</td>
<td>7.12±0.03</td>
<td>6.49±0.32</td>
<td>6.76±0.18</td>
<td>6.91±0.09</td>
</tr>
<tr>
<td>Abnormal form(%)</td>
<td>1.25±0.08</td>
<td>5.38±0.46</td>
<td>3.61±0.31</td>
<td>2.85±0.25</td>
</tr>
<tr>
<td>Normal form(%)</td>
<td>98.74±0.09</td>
<td>94.64±0.44</td>
<td>96.39±0.31</td>
<td>97.14±0.25</td>
</tr>
<tr>
<td>Viable sperm cell(%)</td>
<td>90.00±1.92</td>
<td>54.20±1.92</td>
<td>64.80±4.44</td>
<td>70.40±4.51</td>
</tr>
<tr>
<td>Non-viable sperm cells(%)</td>
<td>10.00±2.07</td>
<td>45.80±1.92</td>
<td>35.20±4.44</td>
<td>29.60±4.50</td>
</tr>
<tr>
<td>Progressive motility(%)</td>
<td>81.00±2.12</td>
<td>45.60±1.12</td>
<td>50.40±23.15</td>
<td>49.60±23.15</td>
</tr>
<tr>
<td>Sluggish motility(%)</td>
<td>19.00±2.12</td>
<td>54.40±1.12</td>
<td>49.60±23.15</td>
<td>44.40±24.50</td>
</tr>
</tbody>
</table>

Plate 4.1: Photomicrograph of Group 1 (Control) Showing Normal Testicular Cytoarchitecture with Seminiferous Tubule (ST) Having an Abundant Population of Spermatogonia, Spermatocytes and Spermatids. H&E X400

Plate 4.2: Histological Photomicrograph of Group 2 Rats That Received 1000mg/kg of Lead Acetate Showing Abnormal Testicular Cytomorphology Characterized by Interstitial Space Degeneration, Wider Interstitial Space, Reduced Spermatogenic Cells. H&E X400
Plate 4.3: Histological Section of Group 3 Rats That Received 1000mg/kg Lead + 400mg/kg of CAEE Showing Similar Histological Organization as That of the Control. H&E X400

Plate 4.4: Photomicrograph of Group 4 Rats Treated with 1000mg/kg Lead + 800mg/kg of CAEE Showing Tight Interstitial Space, Increased Number of Spermatogonia, Spermatocytes and Spermatids in the Seminiferous Tubules. H&E X400

Plate 4.5: Photomicrograph of Anterior Pituitary Gland (adenohypophysis) Show Cords of Cells with Barely Discernible Connective Tissue Stroma but with Prominent Artefactual Tissue Separation. The Cellular Composition is Variable Including (A) Acidophils, (B) Basophils and (C) Chromophobes. H&E X400

Plate 4.6: Histological Section of Group 2 Wistar Rats Anterior Pituitary Gland That Received 1000mg/kg of Lead Acetate Showing Severe Reduction in Cellularity with Prominent Depression of the Acidophils and the Basophils. H&E X400

Plate 4.7: Photomicrograph of Group 3 Rats Pituitary Gland Showing no Significant Change in Pathology When Compared to Group1 H&E X400

Plate 4.8: Photomicrograph of Group 4 Rats Pituitary Gland That Received 1000mg/kg Lead + 800mg/kg of CAEE Showing Mild Increase in Cellularity. H&E X400

Discussion
It has been well established that infertility affects up to 15% of the world population and about 20% of cases are associated with male infertility [6]. Heavy metals (lead, cadmium and mercury) at workplaces have been implicated as one of the major causes of male infertility through the generation of reactive oxygen species (ROS). The severe testicular toxicity associated with lead has necessitated investigation into alternative medications, including natural compounds to ameliorate its effects. Pituitary-testicular
disruption is one of the effects resulting from lead toxicity. It has been shown that lead-induced pituitary-testicular toxicity is mediated by oxidative damage and generation of reactive oxygen species (ROS) [7, 8]. ROS has been shown to counteract and alter normal sperm function through lipid peroxidation in the sperm plasma membrane [9]. The present study, showed that lead slightly increased the weight of the testis in the group administered with lead when compared to the control (Figure 1.0). Finding is in contrast with the work done by and Anthonet and Orish (2019) [10]. Both researchers postulated that lead significantly reduced testicular weight when compared with normal control. The increase in weight may be due to degeneration of cells and subsequent infiltration of fluid into the interstitial spaces. Also, our study showed that the administration of low dose of costus afer slightly reduced the weight of the testes.

The organs of the human system depend on good and potent antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA), for normal metabolic activities. reported that SOD scavenges superoxide anion and catalyses its conversion to hydrogen peroxide (H2O2) and oxygen (O2) [11]. It has been reported that SOD protects sperm against lipid peroxidation, prevents DNA damage and promotes sperm motility [12]. In our study, lead significantly reduced the levels of SOD in both serum and testicular homogenate assays. CAT was also significantly decreased in both assays, while there was significant increase in MDA, also in both assays when compared with the normal control (Figure 1.2-1.7). The results were consistent with results of Anthonet and Orish (2019), [10, 13]. However, the treatment with costus afer significantly reduced the level of MDA, and elevated the levels of SOD and CAT. The ability of the extract to attenuate the deviations in the antioxidant biomarkers, as compared with the lead-only group, suggest that its antioxidant properties may be linked to active phytochemicals, like flavonoid, phenolic substances, terpenes [2].

Alterations in seminal parameters has been linked to increases in reactive oxygen species [14]. Spermatozoa membrane fluidity, which is relevance to for sperm-oocyte fusion has been attributed to the high level of polyunsaturated fatty acid (PUFA). Consequently, and with the presence of carbon-carbon double bonds, makes such lipids highly prone to oxidative insult. It is well established that ROS induces the formation of lipid peroxide radicals which ultimately reacts with other radicals, triggering a self-mediating chain reaction and cascading to lipid peroxidation [15]. The damage of lipid membrane caused by peroxidation significantly impairs membrane structure and fluidity, dysregulation of membrane associated processes [16, 17]. In our study, seminal parameters were significantly altered in lead-only group when compared with the normal control (Table 1.0), in consonant with previous studies conducted by Anthonet and Orish (2019). However, administration of the CAEE significantly restored seminal pH, morphology and viability of the sperm cells when compared with the lead-only group. Sperm motility is the first to be impaired after mitochondrial membrane damage elicited by lipid peroxidation. This event causes the reduction of mitochondrial membrane potential and defects in the sperm middle-piece and axonemal region [17, 18]. showed that protein peroxidation of α-central carbon generates radical amino acid and induces the cleavage of peptide skeletons. This further enhances the vulnerability of SH-rich chains of cysteine and methionine to oxidation, with the generation of disulphides and methionine sulphide respectively. In the process, proline, arginine, lysine and threonine are oxidized, resulting in the formation of aldehyde and ketones, which result in oxidation status. These alterations in the normal cellular processes after the cellular protein structure with effects on fertility. In the present, sperm motility was significantly restored after the administration of CAEE in a dose-dependent fashion after been altered by lead (Table 1.0), and in parallel with the study of Anthonet and Orish, 2019.

Testosterone is produced through steroidogenesis, a multi-step process catalysed by different enzymes using cholesterol as substrate. The binding of luteinizing hormone (LH) to G-protein-coupled receptors expressed on Leydig cells initiates the transport of cholesterol by steroidogenic acute regulatory protein (StAR) into the mitochondria, where cytochrome P450 enzymes and several hydroxysteroid dehydrogenase facilitate the conversion of this molecule into androgenic hormones (testosterone, androsterone etc) [19]. Testosterone is essentially needed for normal spermatogenesis and fertility. It has been reported that a decline in the serum level of testosterone adversely affect primary and secondary sexual maturity and fertility in male (Anthonet and Orish, 2019). In our study, there was a slight reduction in the level of testosterone in the lead-only group which differs from the normal group. This result is consistent with report of Anthonet and Orish, 2019,[10]. Reported that the decline in serum testosterone level is an indicator of chemical toxicity on the reproductive system. However, the administration of CAEE elevated the level of testosterone a dose dependent fashion to the level seen in the normal control (Figure 1.1) [20]. This finding implies that the antioxidant potential of costus afer may have attenuated the impact of ROS on the steroidogenic pathway.

Light microscopic examination of the adenohypophysis reveals degenerative changes, reduction in cellularity, with depression of acidophils and basophils caused by lead. These changes were in contrast with the normal control (Plate 4.6 & 4.6). The alterations in the cytoarchitecture of the pituitary gland correlate with the reduced level of testosterone and degenerative changes observed in the histomorphology of the testes, since the pituitary gland controls cellular integrity and functions of the testes. Administration of CAEE moderately reversed these alterations. This shows that costus afer has neuroprotective potentials. Histomorphological alteration is one of the effects resulting from lead-induced oxidative stress insult. It has led to seminiferous tubule epithelial apoptosis, necrosis vacuolations. In this study, lead caused significant interstitial space degeneration, and this resulted in wider than normal interstitial spaces, reduced spermatogenic cells and cellular vacuoles. These alterations were also reported in earlier studies done by and Anthonet and Orish, (2019) [10,13]. Costus afer was able to restore these mitigate these changes observed in the lead-only group.

Conclusion
The present study has demonstrated that lead is a reprotoxican, as evidenced in its ability to disrupt the cytoarchitecture of the pituitary and testicular tissues, biochemical and seminal parameters in adult male wistar rats. However, the administration of CAEE (costus afer ethanol extract) showed remarkable tendencies in attenuating the reproductive alterations caused by lead. This result shows that costus afer has therapeutic effect, and can serve as a potential candidate for the treatment of pituitary and testicular dysfunction [21-23].

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Conflict of Interest
The authors declare no conflict of interest.
References


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