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Lead and Cadmium-Induced Testicular Toxicity: Protective Role of Soursop (*Annona muricata*) Phytowaste Extract via Antioxidant and Anti-Inflammatory Pathways

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ABSTRACT

Lead (Pb) and cadmium (Cd) exposure induces oxidative stress, inflammation, and testicular dysfunction. This study investigated the protective effects of Hydro-Ethanollic Extract of *Annona muricata* phytowaste (HEEAM) in male Wistar rats exposed to Pb (200 mg/kg) and Cd (100 mg/kg) in drinking water for 90 days. Treatment groups received HEEAM (250 or 500 mg/kg) or zinc-selenium (Zn-Se) supplements. Pb-Cd exposure significantly increased malondialdehyde (MDA: 19.58 ± 1.05 nmol/mL), testicular Pb (2.30 ± 0.06 mg/kg), Cd (1.62 ± 0.01 mg/kg), sperm abnormalities (51.20%), interleukin 6 (IL-6 (5.76 ± 1.35 pg/mL), and tumor necrosis factor alpha (TNF- α (23.18 ± 0.69 pg/mL), while reducing super oxide dismutase (SOD (4.72 ± 0.63 U/mL), glutathione (GSH (3.97 ± 0.01 nmol/mL), and testosterone (0.48 ± 0.02 ng/mL) ($P < 0.05$). HEEAM at 500 mg/kg significantly reduced MDA (10.21 ± 0.83 nmol/mL), Pb (0.92 ± 0.04 mg/kg), and Cd (0.65 ± 0.02 mg/kg), improved SOD (9.03 ± 0.52 U/mL), GSH (6.02 ± 0.11 nmol/mL), testosterone (1.85 ± 0.04 ng/mL), and lowered IL-6 (2.10 ± 0.36 pg/mL) and TNF- α (12.12 ± 0.48 pg/mL) ($P < 0.05$). Sperm count, motility, and morphology were also restored. HEEAM outperformed Zn-Se in reducing oxidative stress and metal burden. Phytochemical screening revealed flavonoids, phenolics, and alkaloids as likely active constituents. These findings highlight *Annona muricata* phytowaste as a promising, sustainable intervention against heavy metal-induced reproductive toxicity.

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1. INTRODUCTION

Contamination by heavy metals, particularly lead (Pb) and cadmium (Cd), has become a critical public health concern due to their ability to induce oxidative stress, inflammation, and tissue damage (Peana *et al.*, 2022). These metals are persistent environmental pollutants that bioaccumulate in living

organisms, leading to chronic and prolonged toxic effects. Notably, Pb and Cd are known to disrupt the body's antioxidant defense systems, resulting in excessive generation of reactive oxygen species (ROS) (Goyal et al., 2021). This imbalance promotes oxidative damage to lipids, proteins, and DNA, thereby triggering cellular dysfunction, inflammation, and injury to vital organs such as the liver, kidneys, brain, and reproductive organs (Rajput et al., 2015; Carpena et al., 2022).

Among the affected systems, the male reproductive system is particularly vulnerable. Testicular toxicity induced by environmental toxicants like Pb and Cd is associated with impaired spermatogenesis, hormonal imbalances, and histopathological damage. These adverse effects are largely mediated by oxidative stress mechanisms, including lipid peroxidation, mitochondrial dysfunction, and apoptosis of testicular cells, which compromise male fertility (Rajput et al., 2015).

Parallel to concerns about heavy metal toxicity is the growing issue of agro-industrial waste, especially in the context of a rapidly increasing global population and the need for greater food production. During processing, parts of fruits and vegetables such as peels, seeds, and skins are often rich in bioactive compounds which are typically discarded as phytowaste (Carpena et al., 2022). Improper disposal of this organic waste contributes to environmental pollution, including greenhouse gas emissions, water system contamination, and excessive landfill use (Kumar et al., 2017).

In response, there is increasing interest in sustainable waste management practices within the agro-food sector. Driven by regulatory pressures, health and environmental concerns, and a growing appreciation for the pharmacological potential of bioactive-rich waste materials, industries are exploring strategies for repurposing phytowaste (Carpena et al., 2022). Utilizing phytowaste as a source of natural antioxidants presents a cost-effective and eco-friendly alternative, with potential applications in the development of novel therapeutics and nutraceuticals (Dike et al., 2021). One promising candidate is *Annona muricata*, commonly known as soursop, a tropical fruit belonging to the Annonaceae family. Widely distributed in tropical and subtropical regions, including parts of South America, the Caribbean, Southeast Asia, and Africa, *Annona muricata* has gained attention for its medicinal properties. Various parts of the plant, particularly its fruit, leaves, and seeds, are rich in bioactive compounds such as acetogenins, alkaloids, flavonoids, and polyphenols. These compounds possess potent antioxidant and anti-inflammatory activities, making the plant a viable natural agent against oxidative stress-related pathologies (Moghadamtousi et al., 2015; Santos et al., 2023).

Given the increasing prevalence of testicular toxicity due to environmental exposure to Pb and Cd and the potential health benefits of underutilized plant waste, this study aims to investigate the testicular protective effects of *Annona muricata* phytowaste in Wistar rats exposed to Pb and Cd through drinking water.

2. MATERIALS AND METHODS

2.1. Preparation of the Ethanolic Extract of *Annona Muricata* Phytowaste

Fresh Soursop fruit was bought from New Benin market, Benin City, Edo State. It was identified and authenticated by a plant taxonomist in the Department of Plant Biology and Botany, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. Following a thorough washing of the soursop, the peels, fibers and seeds were taken out and cut into small pieces). The pieces were placed in a dehydrator to dry for 7 days. The fibers, peels, and seeds were separately ground into fine powder using a mechanical blender. In a plastic container, 150 g of the seed, 100 g of the fiber, and 250 g of the peel powder were soaked in a solution of 1000 ml of absolute ethanol and 1000 ml of distilled water, sealed with foil paper to prevent the evaporation of the ethanol and macerated for 72 hours. After 72 hours, the extract was sieved using a sieve cloth and Whatman paper, afterward ensuring a homogenous solution was obtained. The filtrate was poured into stainless containers and was concentrated in a water bath into a paste. The paste form of extract was stored in small containers, properly sealed, labeled, and kept in the refrigerator at 4 °C for preservation prior to usage (Owolabi et al., 2008).

2.2. Qualitative and Quantitative Phytochemical Screening of Hydro-Ethanol Extracts of *Annona muricata* Phytowaste

Phytochemical screens were performed on the extracts using standard procedures by Trease and Evans (2002) and Lawal et al. (2014).

2.3. Animal Model

A total of thirty (30) healthy male Wistar rats with weights ranging from 105.4 g to 154.4 g were purchased from the University of Ibadan animal house in Oyo State and housed in adequately ventilated plastic cages in the Department of Science Laboratory Technology (SLT), University of Benin, Benin City, Nigeria. The rats were acclimatized for two weeks prior to the commencement of the experiments. All rats received food and water ad libitum and exposed to 12 hours' light and darkness. The rats were weighed once every week for 90 days. They were handled in accordance with laboratory animal standard protocols (National Institute of health USA public Health service policy on humane care and use of laboratory animals, 2002) and the guidelines of the Institutional Animal Ethics Committee of the Department of Science Laboratory Technology, University of Benin.

2.4. Experimental Design

After 14 days of acclimatizing to laboratory conditions, the Wistar rats were grouped into five (5) groups of six (6) rats in each group. Group I (control) was administered 0.5 ml of distilled water via oral route, Group 2 was exposed to lead (200 mg/kg) + cadmium (100 mg/kg) in 300ml of drinking water, Group 3 was administered zinc (Zn (1 mg/kg) + selenium (Se (1.5 mg/kg) via oral route and exposed to lead (200 mg/kg) + cadmium (100 mg/kg) in 300ml of drinking water, Group 4 and 5 were administered soursop extract (250 and 500 mg/kg) via oral route and exposed to lead (200 mg/kg) + cadmium (100 mg/kg) in 300ml of drinking water respectively. Orogastric tube was used for the administration.

2.5. Blood Sample Collection

After 90 days of administration, the animals were sedated using chloroform-induced anesthesia and humanely sacrificed to expose the abdominal aorta and organs. Blood was collected from the abdominal aorta (4 ml total) and then separated into three parts: 1 ml was placed in a plain bottle for heavy metals analysis, while 3 ml was placed in a plain bottle as whole blood (WB), which was subsequently centrifuged to isolate serum for biochemical and anti-inflammatory analysis. The WB samples were labeled accurately and sent to the laboratory for analysis. The testes were subsequently harvested using dissecting kits, trimmed of all fat and stored in a universal container, it was labeled appropriately. The epididymis was carefully cut for semen analysis.

2.6. Determination of Catalase Activity

Catalase activity was measured by adding 0.05 ml of sample or distilled water (blank) into test tubes, followed by 500 μ L of 30 M hydrogen peroxide. Immediately before reading, 100 μ L of 6M H_2SO_4 and 700 μ L of 0.01 M KMnO_4 were added. Absorbance was recorded within 30–60 seconds using a uv/vis spectrophotometer (UV-1100) zeroed with distilled water. A standard was prepared using phosphate buffer, sulfuric acid, and potassium permanganate.

2.7. Determination of Malondialdehyde (MDA)

Lipid peroxidation was assessed using the TBARS method. A reagent mix of 15% TCA, 0.25 M HCl, and 0.875% TBA was heated to homogeneity. 200 μ L of serum was added to 1.5 ml of this reagent. After cooling, samples were centrifuged at 10,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 534 nm.

2.8. Determination of Superoxide Dismutase (SOD)

SOD activity was evaluated by mixing 1000 μL carbonate buffer with 80 μL of serum and 100 μL of epinephrine. The absorbance was measured every 30 seconds up to 120 seconds at 480 nm. A reference standard was prepared with SOD reagent and adrenaline.

2.9. Determination of Glutathione (GSH)

Trichloroacetic acid (TCA) solution (5%, 0.5 μL) was combined with 1.5 μL of blood sample, forming a precipitate. After centrifugation (10 min), 0.5 μL Ellman's reagent and 1.5 μL phosphate buffer were added to fresh tubes, followed by 2.5 μL of the centrifuged sample. Absorbance was read at 416 nm against a blank (without sample).

2.10. Heavy Metals Analysis

Blood and testes were homogenized and 0.5 ml was digested with a mixed acid solution ($\text{HNO}_3\text{:HClO}_4\text{:H}_2\text{SO}_4$ in 5:2:1 ratio) until clear. After cooling, the mixture was diluted with deionized water, filtered, and made up to 100 ml. Heavy metal concentrations were measured using Atomic Absorption Spectrophotometry (BK-AA320N).

2.11. Anti-Inflammatory, Pro-Inflammatory, and Apoptotic Analysis

Serum was collected after clotting and centrifugation. 100 μL of standards, blanks, and samples were added to ELISA plate wells. After incubation and sequential washing, biotinylated detection antibody, HRP-conjugate, substrate, and stop solutions were added per kit instructions (Elabscience). Absorbance was measured at 450 nm using a microplate reader (BK-EL10A).

2.12. Sperm Cell Morphology and Count

Sperm was extracted from the vas deferens into a petri dish with pre-warmed saline. A drop was placed on a slide and covered for analysis.

2.13. Morphology

Smears were stained with Eosin and Leishman's stain for 15 minutes, rinsed, dried, and viewed under x100 magnification. Sperm morphology was assessed and quantified by percentage.

2.14. Motility

A drop of sperm was placed on a slide and observed under x20 and x40 magnification. Motility was scored based on progressive movement.

2.15. Testosterone Assay

Reagents, calibrators, and samples were equilibrated to room temperature. Samples and controls were pipetted into wells with enzyme reagent and incubated for 60 minutes. After washing, a substrate was added, followed by a stop solution. Absorbance was recorded at 450 nm using a microplate reader (BK-EL10A).

2.16. Histopathological Examination

The testis specimen was dehydrated in alcohol, processed in xylene and then embedded in paraffin wax before sectioning with a microtome. Sections (5 μm thick) of the tissue were stained with hematoxylin and eosin. All sections were viewed under a Leica light microscope (model DM500) at x400 magnification by a histopathologist who was unaware of the experimental grouping and protocols.

2.17. Statistical Analysis

The data was presented as a mean + standard error of mean. Significant differences in the values between the treatment and control groups were assessed using one-way analysis of variance (ANOVA) after confirming the homogeneity of variance among groups. Significance was determined at $P < 0.05$. The analysis was conducted using Graph Pad Prism 9.0.

3. RESULTS AND DISCUSSION

The qualitative phytochemical screening of *Annona muricata* revealed the following phytochemicals as presented in Table 1. The result obtained from the qualitative phytochemical screening of *Annona muricata* revealed the presence of key bioactive compounds, including glycosides, saponins, alkaloids, phenolics, eugenols, terpenoids, flavonoids, and tannins, with reducing sugars being highly abundant (++) and absent of steroids. These findings align with previous research (Agu *et al.*, 2017; Anaya-Esparza and Montalvo-González, 2020), confirming the plant's rich phytochemical profile and its potential pharmacological benefits, such as antioxidant, anti-inflammatory, and nephroprotective properties. The absence of steroids in this study is consistent with earlier reports (Agu *et al.*, 2017), reinforcing the reliability of the findings.

Table 1: Qualitative phytochemical screening of *Annona muricata* phytowaste extract

Phytochemical compound	<i>Annona muricata</i> phytowaste.
Glycoside	+
Saponins	+
Alkaloids	+
Phenolics	+
Eugenols	+
Steroids	-
Terpenoids	+
Flavonoids	+
Tannins	+
Reducing sugar	++

Key: + = present; ++ = highly present; -= not detected

The quantifiable phytochemical content of alkaloid, saponins, phenolics, flavonoids and Tannins in *Annona muricata* phytowaste extract are presented in Table 2. The quantitative phytochemical estimation of *Annona muricata* phytowaste extract. *Annona muricata* contains significantly higher alkaloids (18.32%). The high alkaloid content in *Annona muricata* confirms their therapeutic potency, particularly in neuroprotection, anticancer activity and anti-inflammatory. This supports the findings of (Agu *et al.*, 2019), where ethanol extracts of *Annona muricata* was effective in protecting against cycad-induced oxidative stress in wistar rats. High alkaloid content aligns with their ability to chelate heavy metals like Pb and Cd, reducing bioavailability and toxicity (Oladele *et al.*, 2019). Tannin content in *Annona muricata* was (25.41 mg/kg). Tannins are polyphenolic chemicals with significant metal-chelating capabilities, meaning they bind to heavy metals and limit their absorption and accumulation in tissues (Zhang *et al.*, 2023). The significant presence of tannins *Annona muricata* waste suggests a protective role in preventing Pb and Cd accumulation. Pb and Cd primarily accumulate in the liver and kidneys, causing chronic nephrotoxicity and hepatotoxicity (Satarug *et al.*, 2020). Tannins *Annona muricata* can reduce metal absorption in the intestines, minimizing their toxic effects on the body. This supports previous findings that tannins prevent heavy metal-induced liver damage and renal dysfunction by forming insoluble metal complexes that are excreted from the body (Matović *et al.*, 2015).

The results in Table 3 indicate levels of Pb and Cd present in the blood of experimental animals before acclimatization, extracts of *Annona muricata*, administered, feed and water given to Wistar rats *ad libitum*. All parameters show minute levels of Pb and Cd exposure.

Table 2: Quantifiable phytochemical content of *Annona muricata* phytowaste extract.

Phytochemicals	<i>Annona muricata</i> phytowaste.
Alkaloid [%]	18.32 ± 0.52
Saponins [mg/kg]	0.93 ± 0.00
Phenolics [mg/kg]	1.45 ± 0.01
Flavonoids [mg/kg]	1.68 ± 0.07
Tannins [mg/kg]	25.41 ± 1.09

Table 3: Baseline study for heavy metals accumulation in Pb and Cd induced Wistar rats

Parameters	Lead (200 mg/kg)	Cadmium (100 mg/kg)
Blood (Control)	0.004	0.002
Extract	0.005	0.003
Water	0.007	0.003
Feed	0.007	0.004

From Table 4, the control group showed a significant increase in the weight at day 30 and day 60 when compared to Pb and Cd exposed group and the combined treatment with Zn and Se ($P < 0.05$). The control group also exhibited very significant increase in the weight at day 90 (243.30 ± 1.89) when compared to Pb and Cd exposed group (189.14 ± 0.07) and the combined treatment with Zn and Se ($P < 0.01$). Heavy metal exposure is known to impair growth and metabolic processes, as evidenced by stunted weight gain in Pb and Cd-exposed subjects (Bersenyi *et al.*, 2003; Sarkar *et al.*, 2013). The partial weight recovery observed with combined Zn and Se treatment suggests their supportive role in mitigating toxicity, though their efficacy was surpassed by *Annona muricata* extract (HEEAM), particularly at the 250 mg dose. This aligns with studies highlighting the role of polyphenols in enhancing nutrient uptake and protecting gastrointestinal integrity (Oladele *et al.*, 2019; Zubaidi *et al.*, 2023). The reduced efficacy of the higher dose (500 mg) may indicate a biphasic response, warranting further investigation into optimal dosing.

Table 4: The Effect of HEEAM on the weight of Wistar rats exposed to Pb and Cd in drinking water from day one and an interval of 30 days till the final day of treatment

Group	Day 1 (g)	Day 30 (g)	Day 60 (g)	Day 90 (g)
Control	121.82 ± 0.56	204.14 ± 0.39	195.44 ± 0.56 ^{ab}	243.30 ± 1.89 ^{ab}
Pb (200 mg/kg) and Cd (100 mg/kg)	137.26 ± 0.06	181.36 ± 1.10	168.90 ± 0.30 ^a	189.14 ± 0.07 ^a
Zn (1 mg/kg) and Se (1.5 mg/kg)	139.48 ± 0.89	169.35 ± 0.47	164.85 ± 0.85 ^a	191.00 ± 0.47 ^a
HEEAM (250 mg/kg)	119.20 ± 1.38	183.65 ± 0.83	170.70 ± 0.78 ^a	209.03 ± 0.96 ^c
HEEAM (500 mg/kg)	122.43 ± 0.45	165.80 ± 0.07	165.77 ± 0.4 ^a	202.70 ± 0.92 ^c

Values were expressed as Mean ± SD, n=5. Data with same alphabet in a column demonstrate no significant difference ($P > 0.05$) different from each other while data with different alphabet in a column are significantly different from each other.

The accumulation of Pb and Cd in blood across groups is presented in Figure 1. The control group had the lowest levels of Cd and Pb, representing baseline levels. The Pb and Cd exposed group showed significant increase in both Cd and Pb levels compared to control. Zn and Se treatment resulted in significant decrease in Cd and Pb levels compared to Pb and Cd exposed group. HEEAM 250mg showed significant decrease in Cd and Pb levels compared to Pb and Cd exposed group, while HEEAM 500mg demonstrated a more significant decrease in Cd and Pb levels when compared to the 250mg/kg group, proving more effective than Zn and Se treatment. The blood analysis demonstrated significant heavy metal accumulation in Pb and Cd-exposed subjects, consistent with their known toxicity even at low concentrations (Papanikolaou *et al.*, 2005;

Jaishankar *et al.*, 2014). The protective effects of Zn and Se were evident in their ability to reduce metal levels, likely through antioxidant and chelation mechanisms (El-Boshy *et al.*, 2015; Kim *et al.*, 2016). However, comparatively HEEAM, especially at 500 mg, exhibited superior efficacy, suggesting a dose-dependent enhancement of its metal-chelating and antioxidant properties (Oladele *et al.*, 2019; Mutakin *et al.*, 2022).

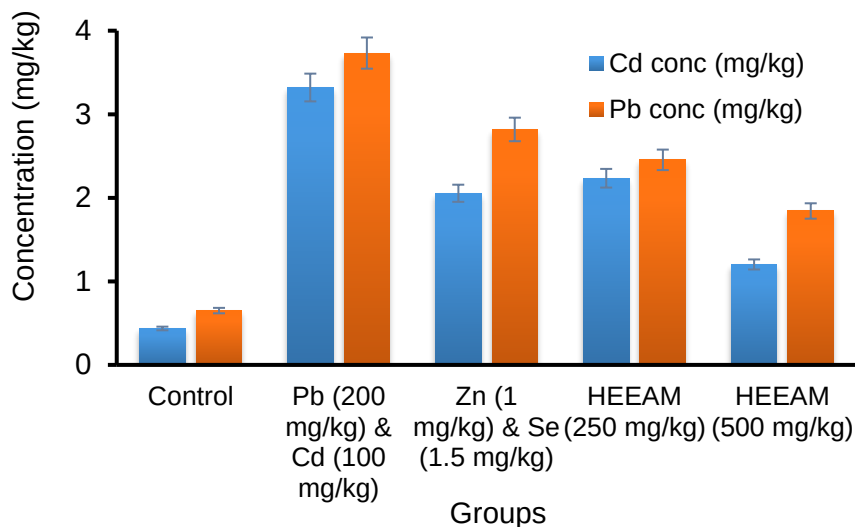


Figure 1: The effect of HEEAM on Pb and Cd accumulation in the blood of Wistar rats exposed Pb and Cd in drinking water

Testicular Pb and Cd levels is presented in Figure 2. The Pb and Cd exposed group exhibited significantly elevated testicular levels of both metals compared to control. The Zn and Se group showed reduced metal levels compared to Pb and Cd group. HEEAM at both 250mg and 500mg demonstrated dose-dependent reductions in metal accumulation, with the 500mg dose achieving Cd and Pb reduction levels comparable to or lower than control levels. Reproductive toxicity, a critical consequence of heavy metal exposure, was evident in elevated testicular metal levels and associated oxidative damage (Järup, 2003; Wirth and Mijal, 2010). The Zn and Se combination mitigated some of these effects, likely through zinc's displacement of Cd and selenium's enhancement of glutathione peroxidase activity (Yamaguchi *et al.*, 2009; Al-Ani *et al.*, 2015). HEEAM, particularly at 500 mg, achieved near-normal metal reduction levels, underscoring the potential of plant-derived antioxidants like flavonoids and acetogenins in restoring testicular function (El-Monem *et al.*, 2020; Mobasher *et al.*, 2024).

Assessed oxidative stress markers including SOD, CAT, and GSH levels are presented in Figure 3. In the Pb and Cd exposed group, SOD and CAT were significantly decreased ($P < 0.05$), and GSH was reduced compared to control. Zn and Se treatment restored SOD and CAT activity, with GSH levels showing similarities to control group. HEEAM 250mg showed SOD lower than control but higher than Pb and Cd group, while CAT and GSH were significantly increased ($P < 0.05$) compared to control. HEEAM 500mg demonstrated CAT and GSH significantly increased ($P < 0.05$) compared to control, and SOD significantly increased ($P < 0.01$). Oxidative stress occurs when harmful molecules (free radicals) injure cells more rapidly than the body can heal them. Exposure to lead and cadmium enhances oxidative stress by increasing harmful compounds like MDA and reducing protective enzymes like SOD and CAT. In the Pb and Cd exposed group, SOD and CAT, were significantly decreased ($P < 0.05$), reflecting impaired enzymatic defense. GSH level was reduced compared to the control group resulting in a disrupted cellular redox balance as Cd directly conjugates with GSH to generate inert complexes (Sarkar *et al.*, 2013).

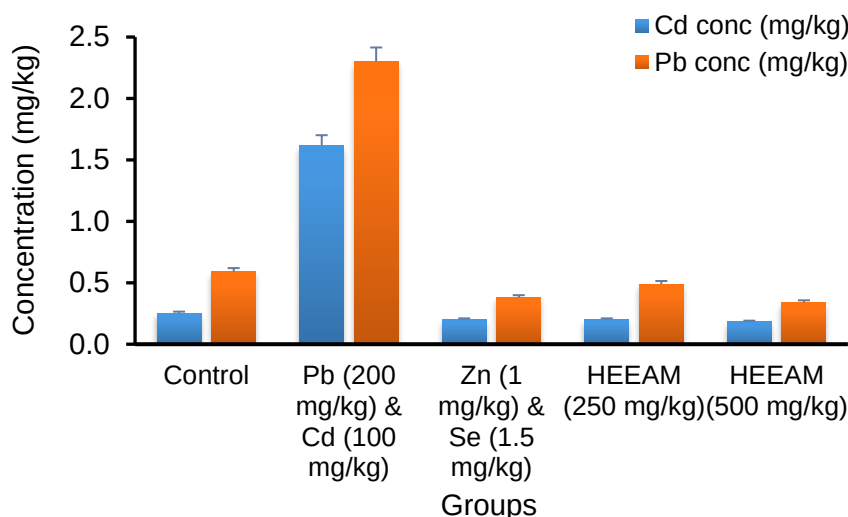


Figure 2: The effect of HEEAM on Pb and Cd accumulation in the Testes of Wistar rats exposed Pb and Cd in drinking water

In the combined treatment with Zn and Se, the SOD and CAT activity were restored, indicating enhanced antioxidant defenses, which is in line with their roles in mitigating heavy metal toxicity by activating antioxidant enzymes (El-Boshy *et al.*, 2015; Rehman *et al.*, 2018; Das and Al-Naemi, 2019). GSH levels were similar when compared to the control group. The SOD level in the HEEAM 250 mg was lower compared to the control group but higher than the Pb and Cd exposed group which indicates partial restoration of SOD activity. This may be due to the presence of flavonoids, and this aligns with previous research that suggests that flavonoids present in *Annona muricata* may stabilize catalase structure or directly scavenge hydrogen peroxide (Oladele *et al.*, 2019).

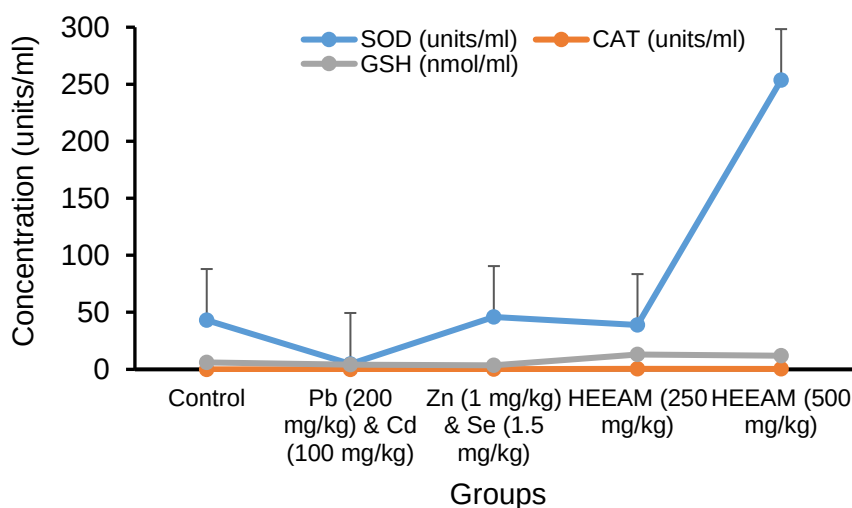


Figure 3: The Effect of HEEAM on anti-oxidant markers in the serum of Wistar rats exposed to Pb and Cd in their drinking water

CAT and GSH showed a significant increase ($P < 0.05$) compared to the control group. This suggests that *Annona muricata* effectively restores GSH (Agu *et al.*, 2019). The HEEAM 500mg group levels of CAT and GSH showed significant increases ($P < 0.05$) when compared to the control group, indicating a potent antioxidant effect probably as a result of the induction of Nrf2-mediated antioxidant genes.

This aligns with previous research that linked *Annona muricata*'s protective effects to its potent antioxidant contents (Agu *et al.*, 2019). The significant SOD increase ($P < 0.01$) in HEEAM 500 mg may be due to dose-dependent antioxidant enzymes stimulation, though the high variability points to individual variations in metabolic response to phytochemicals like annonacin and quercetin.

The result of MDA levels as a lipid peroxidation marker is presented in Figure 4. Malondialdehyde (MDA) is a lipid peroxidation marker that confirms oxidative membrane damage. In the Pb and Cd exposed group, MDA levels were elevated indicative of severe oxidative stress, lipid peroxidation, and cell membrane damage (Ara and Usmani, 2015; Matović *et al.*, 2015; Dobrakowski *et al.*, 2017; Genchi *et al.*, 2020). In the combination treatment with Zn and Se, MDA reduced compared to the lead and cadmium group but was still significantly increased ($P < 0.05$) compared to control suggesting that less damage occurred. The MDA level in the HEEAM 250 mg was significantly increased ($P < 0.05$) compared to the control, combined treatment of Zn and Se, and HEEAM 500mg group but is lesser than the Pb and Cd exposed group. The MDA levels in the HEEAM 500 mg were lesser compared to the Pb and Cd exposed group but were slightly higher than the control group indicating less oxidative damage. These results support earlier studies that indicated that *Annona muricata* can assist in preventing organ damage and reducing oxidative stress in cases of heavy metal poisoning (Oladele *et al.*, 2019; Agu *et al.*, 2019). The natural antioxidants found in abundance in the fruit protect the body from harmful toxins (Sears, 2013).

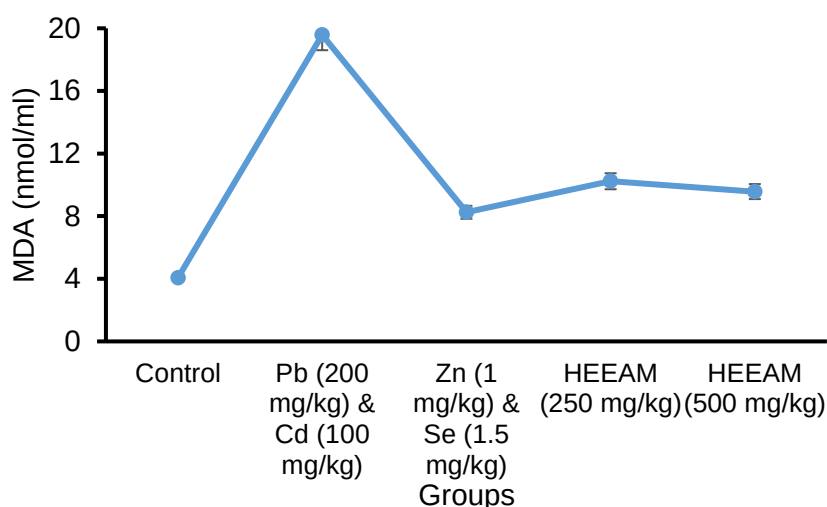


Figure 4: The effect of HEEAM on the oxidative stress marker MDA, in the serum of Wistar rats exposed to Pb and Cd in their drinking water

Examined inflammatory and apoptotic markers results are presented in Table 5. The control group exhibited low IL-6 levels (1.01 ± 0.58 pg/ml), high IL-10 levels (7.21 ± 0.61 pg/ml), low Caspase-3 levels (0.63 ± 0.11 ng/ml), and low TNF- α levels (6.72 ± 1.50 pg/ml). The Pb and Cd exposed group showed highly significant increase ($P < 0.001$) in Caspase-3 compared to control, significant increase ($P < 0.01$) in TNF- α compared to control, increased IL-6 levels, and reduced IL-10 levels. Zn and Se treatment resulted in increased IL-10 levels compared to control, TNF- α elevated but lower than Pb and Cd group, and Caspase-3 marginally higher than control but reduced compared to Pb and Cd group. HEEAM groups showed IL-6 reduced compared to control in both 250mg and 500mg doses. For TNF- α , the 250mg dose showed significant decrease ($P < 0.01$) compared to Pb and Cd group, while 500mg showed reduced levels but slightly higher than 250mg. IL-10 levels in 250mg were slightly increased compared to Pb and Cd but reduced compared to control, while 500mg showed higher levels than 250mg dose.

Table 5: The effects of HEEAM on the anti-inflammatory, pro-inflammatory, and apoptotic factors in Pb and Cd exposed Wistar rats

Group	IL-6 (pg/ml)	IL-10 (pg/ml)	Caspase-3 (ng/ml)	TNF- α (pg/ml)
Control	1.01 $\pm$ 0.58 ^a	7.21 $\pm$ 0.61 ^a	0.63 $\pm$ 0.11 ^a	6.72 $\pm$ 1.50 ^a
Pb (200 mg/kg) and Cd (100 mg/kg)	5.76 $\pm$ 1.35 ^{ab}	3.07 $\pm$ 1.18 ^{ab}	0.89 $\pm$ 0.13 ^{ab}	23.18 $\pm$ 0.69 ^{ab}
Zn (1 mg/kg) and Se (1.5 mg/kg)	1.84 $\pm$ 1.06 ^a	16.21 $\pm$ 1.74 ^c	0.75 $\pm$ 0.06 ^a	17.00 $\pm$ 1.25 ^{ac}
HEEAM (250 mg/kg)	0.79 $\pm$ 0.80 ^a	4.50 $\pm$ 0.54 ^{ab}	0.71 $\pm$ 0.10 ^a	9.16 $\pm$ 0.77 ^a
HEEAM (500 mg/kg)	0.74 $\pm$ 1.38 ^a	8.22 $\pm$ 1.31 ^a	0.73 $\pm$ 0.07 ^a	11.56 $\pm$ 1.0 ^a

Values were expressed as Mean $\pm$ SD, n=5. Data sets with the same alphabet in a column demonstrate no significant difference ($P>0.05$) between each other, while data sets with different alphabets in the same column demonstrate significant difference between each other.

Both doses reduced Caspase-3 compared to Pb and Cd group. Inflammatory and apoptotic markers (IL-6, TNF- α , Caspase-3) were elevated in Pb and Cd-exposed subjects, indicating systemic inflammation and cell death (Sarkar *et al.*, 2013; Fenga *et al.*, 2017). Zn and Se partially mitigated these effects, but HEEAM, particularly at 500 mg, significantly reduced pro-inflammatory cytokines and Caspase-3 levels, aligning with its known anti-inflammatory and anti-apoptotic properties (Coria-Tellez *et al.*, 2018; Zubaidi *et al.*, 2023). The result of the measured testosterone serum levels across groups are presented in Figure 5. The control group maintained healthy testosterone levels, while the Pb and Cd group showed sharp decline in testosterone. The Zn and Se group indicated elevated testosterone levels, and HEEAM groups exhibited partial recovery in testosterone levels.

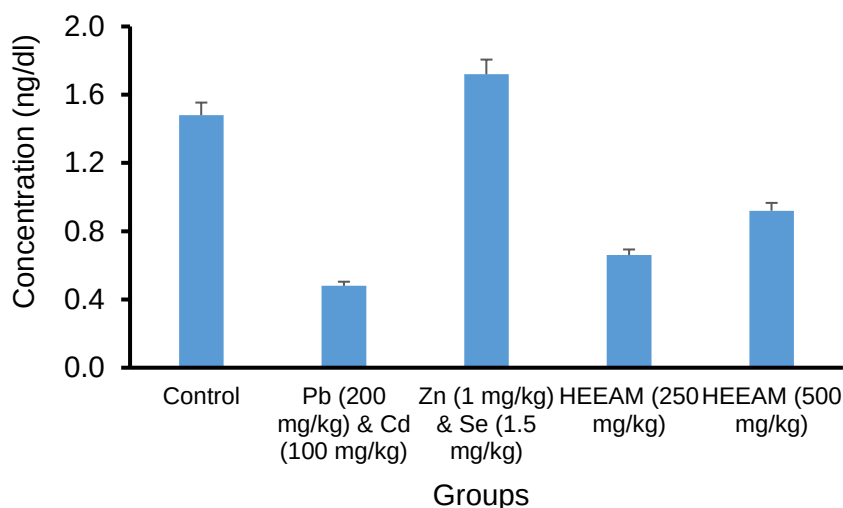


Figure 5: The effects of HEEAM on testosterone level in Pb and Cd exposed Wistar rats

Serum testosterone (ng/dL) reflects Leydig cell function and endocrine health. Testosterone levels in the control group reflects healthy endocrine and Leydig cell function. Pb and Cd group showed a sharp decline, consistent with heavy metal disruption of the hypothalamic-pituitary-gonadal (HPG) axis and Leydig cell apoptosis (Eum *et al.*, 2008). The Zn and Se group indicated elevated testosterone, likely due to the role of Zn in supporting steroidogenesis and Se antioxidant effects on Leydig cells (Yamaguchi *et al.*, 2009; Al-Ani *et al.*, 2015). HEEAM groups exhibited partial recovery. Indicating its antioxidants reduce oxidative stress in Leydig cells but may require longer treatment to fully restore hormonal balance (El-Monem *et al.*, 2020).

This aligns with studies showing phytochemicals like flavonoids improve endocrine function gradually (Mobasher *et al.*, 2024).

Table 6 shows the sperm count and the sperm motility of rats across the experimental groups. Sperm count indicates the number of sperm cells per milliliter of semen, reflecting overall sperm production. Sperm motility indicates the percentage of sperm with progressive movement, crucial for fertility.

Table 6: The effects of HEEAM on sperm count and motility in Pb and Cd exposed Wistar rats

Group	Sperm count (million/mg)	Progressive motility (%)	Non-progressive motility (%)	Immotile (%)
Control	624.00 ± 0.82 ^a	69.00 ± 0.10 ^a	21.75 ± 0.84 ^a	11.75 ± 1.65 ^a
Pb (200 mg/kg) and Cd (100 mg/kg)	398.80 ± 1.66 ^{ab}	48.20 ± 0.29 ^{ab}	30.40 ± 0.04 ^{ab}	21.40 ± 1.36 ^{ab}
Zn (1 mg/kg) and Se (1.5 mg/kg)	500.00 ± 0.63 ^c	55.40 ± 0.76 ^c	25.60 ± 1.69 ^c	19.00 ± 0.45 ^c
HEEAM (250 mg/kg)	657.20 ± 1.66 ^a	71.60 ± 1.29 ^a	18.00 ± 1.27 ^a	10.40 ± 1.63 ^a
HEEAM (500 mg/kg)	707.80 ± 0.23 ^a	72.00 ± 0.95 ^a	18.80 ± 0.58 ^a	9.20 ± 1.28 ^a

Values were expressed as Mean ± SD, n=5. Data sets with the same alphabet in a column demonstrate no significant difference ($P>0.05$) between each other, while data sets with different alphabets in the same column demonstrate significant difference between each other

The control group maintained high sperm count and progressive motility, indicative of a healthy reproductive function. Pb and Cd group showed a significant reduced count and motility compared to control, aligning with studies showing that heavy metals impair mitochondrial energy production and disrupt the blood-testis barrier (Wong *et al.*, 2010; Wagner *et al.*, 2018). The Zn and Se group had a count and motility across than the Pb and Cd group but lower than the HEEAM groups possibly due to antagonistic interaction or suboptimal dosing, as Zn deficiency exacerbates Cd toxicity (Yamaguchi *et al.*, 2009). HEEAM treatment groups restored sperm count and motile surpassing control group. This suggests soursop's flavonoids and acetogenins enhance antioxidant defences, protecting spermatozoa from ROS-induced damage (Rady *et al.*, 2018; Mobasher *et al.*, 2024) and beyond baseline levels as seen in a study by Agarwal *et al.* (2014). The measured sperm morphology effects are presented in Figure 6. The control group exhibited high percentage of normal morphology with minimal abnormalities. The Pb and Cd group showed decreased normal morphology and increased abnormal morphology compared to control.

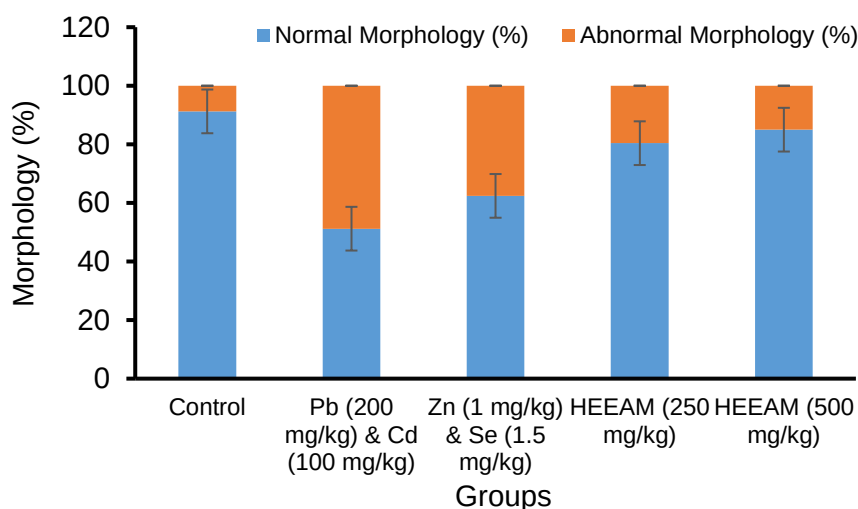


Figure 6: The effect of HEEAM on sperm morphology in Pb and Cd exposed Wistar rats.

The Zn and Se group had better outcome than Pb and Cd group but lower than control. HEEAM at 250mg and 500mg improved morphology with dose-dependent effect. Sperm morphology indicates percentage of sperm with normal structure; abnormalities impair fertilization. Control group exhibited high percentage of normal morphology and minimal abnormalities, reflecting healthy sperm development. The Pb and Cd group showed a decreased normal morphology and increased abnormal morphology when compared to control group consistent with oxidative stress and DNA fragmentation caused by heavy metals (Wirth and Mijal, 2010; Tchounwou *et al.*, 2012; Jiang *et al.*, 2016). The Zn and Se group had a better outcome than the Pb and Cd group but lower than the control group suggesting that low doses of Zn and Se were insufficient to counteract Cd-induced damage, as higher Zn levels are typically required to restore spermatogenesis (Al-Ani *et al.*, 2015). HEEAM at 250 mg and 500 mg improved morphology, likely due to its phenolic compounds scavenging reactive oxygen species (ROS) and reducing lipid peroxidation in testicular cells (Ugwoke *et al.*, 2017; El-Monem *et al.*, 2020). The dose dependent effect highlights soursop's capacity to mitigate oxidative damage. (Ogunlade *et al.*, 2020) demonstrated that antioxidant therapy reduces sperm abnormalities in Cd-exposed rats, consistent with the findings in this study.

Histopathological analysis, as presented in Figure 7, revealed that exposure to lead (Pb) and cadmium (Cd) induced marked testicular damage, characterized by shrunken seminiferous tubules, thickened basement membranes, and reduced germ cell populations, features consistent with testicular atrophy.

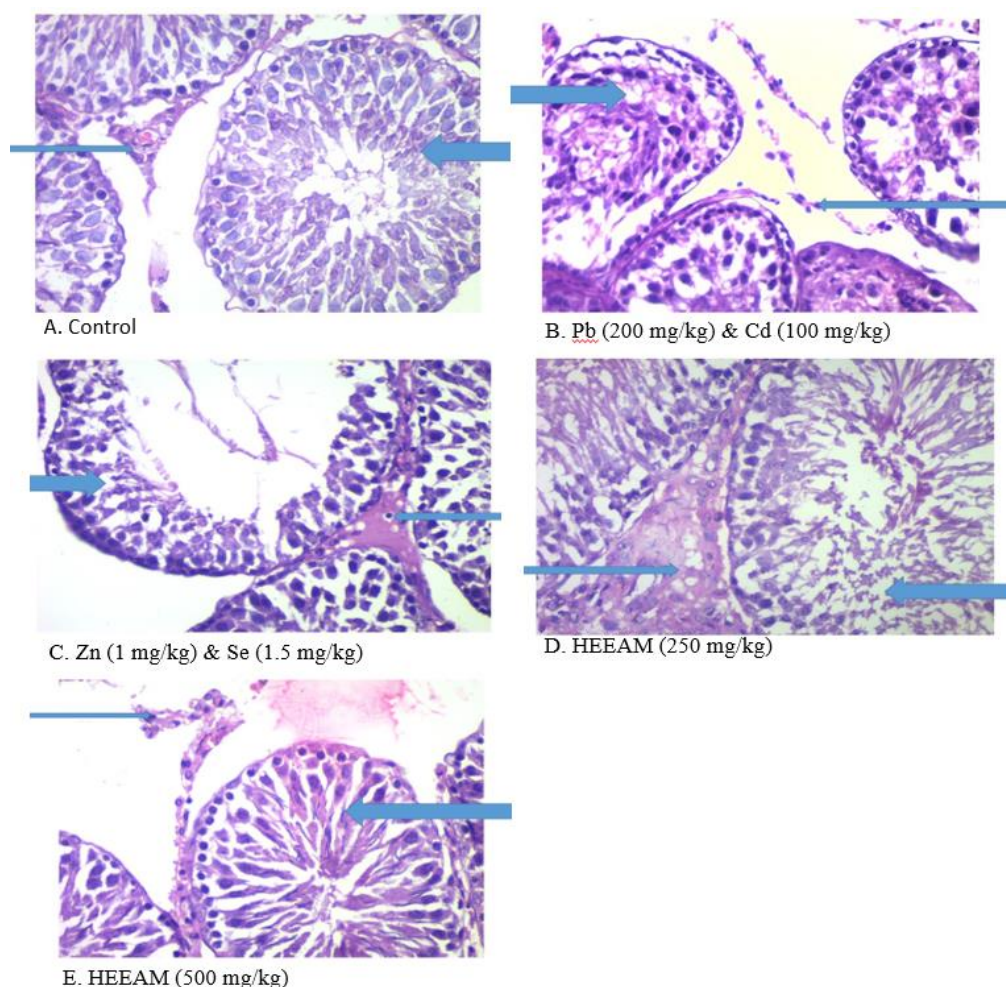


Figure 7: Histopathological observations of Wistar rat testes exposed to Pb and Cd

These findings align with established evidence that heavy metals disrupt testicular structure through oxidative stress and hormonal interference. Although zinc (Zn) and selenium (Se) are known antioxidants, their co-administration did not substantially improve testicular morphology, suggesting that the protective effect at the given dosage was insufficient against the severity of Pb and Cd toxicity. Conversely, treatment with HEEAM at both 250 mg/kg and 500 mg/kg preserved normal testicular architecture, including intact seminiferous tubules and Leydig cells. This protective effect is likely attributed to the extract's rich phytochemical content particularly flavonoids, alkaloids, and acetogenins which have documented antioxidant and anti-inflammatory properties (Mutakin *et al.*, 2022). The findings support HEEAM's potential as a natural therapeutic agent against heavy metal induced reproductive toxicity.

4. CONCLUSION

This study underscores the therapeutic potential of *Annona muricata* phyto-waste in alleviating Pb and Cd induced toxicity. The hydro-ethanolic extract (HHEAM) demonstrated dose-dependent efficacy in reducing oxidative stress, enhancing antioxidant defenses, and lowering heavy metal accumulation in blood and reproductive tissues. Notably, HEEAM at 500 mg/kg restored sperm quality and testosterone levels while modulating inflammatory and apoptotic markers, outperforming traditional Zn-Se supplementation. These results advocate for the valorization of agricultural waste like soursop peels and seeds as a sustainable strategy to combat environmental heavy metal exposure.

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6. CONFLICT OF INTEREST

The authors disclose no conflict of interest.

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