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Original Article

Protective effects of ethanol seed extract of *Plukenetia conophora* on cadmium-induced reproductive toxicity in male Wistar rats

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Abstract

BACKGROUNDAND AIM: Male infertility is a significant global health concern, with sperm quality deficiencies contributing significantly to its prevalence. Infertility affects approximately 15% of couples globally, with male factors contributing to around 50% of cases. Medicinal plants, including the African walnut (*Plukenetia conophora*), are gaining attention for their potential in addressing reproductive health challenges. The aim of this study was to evaluate protective effects of ethanol seed extract of *Plukenetia conophora* (African walnut) on cadmium-induced reproductive toxicity in male Wistar rats.

METHODS: Thirty-six adult male Wistar Rats were divided into six groups and subjected to different treatments, group A served as the control group, Group B and C received 250 mg/kg body weight and 500 mg/kg body weight of ethanol seed extract of *Plukenetia conophora* only respectively, group D and E received 250 mg/kg body weight and 500 mg/kg body weight of *Plukenetia conophora* in addition to 10mg/kg body weight of cadmium chloride respectively, and group F received 10 mg/kg body weight of cadmium chloride only over a 56-day period, Cadmium and *Plukenetia conophora* were administered daily using orogastric tube.

RESULTS: Cadmium chloride negatively impacts body weight, testicular weight, and sperm quality. *Plukenetia conophora* extract exhibits protective effects, mitigating the detrimental impact of cadmium, particularly at lower doses. Histological analysis confirmed the protective role of the extract in maintaining normal testicular architecture.

CONCLUSION: *Plukenetia conophora* extract shows potential as a protective agent against cadmium-induced reproductive toxicity, highlighting its therapeutic potential for male infertility caused by environmental toxins. Further research is warranted to explore its clinical applications.

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Infertility, *Plukenetia conophora*, Cadmium chloride, Heavy metal toxicity, Reproductive health

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INTRODUCTION

Infertility is a medical condition that affects both men and women, and is defined by the inability to conceive after 12 months or more of regular unprotected sexual intercourse (WHO, 2023). It is a global issue, with an estimated one in six people experiencing infertility in their lifetime (WHO, 2023). The total number of people affected by infertility is estimated to be about 180 million worldwide (WHO, 2023).

Male infertility, in particular, is estimated to affect between 8 and 12% of couples of reproductive ages globally (Vander-Borght and Wyns, 2018). Interestingly, while males are found to be solely responsible for 20–30% of infertility cases, they contribute to 50% of cases overall (Vander-Borght and Wyns, 2018). Interestingly, while males are found to be solely responsible for 20–30% of infertility cases, they contribute to 50% of cases overall (Vander-Borght and Wyns, 2018). This means that male factors play a role in half of the cases where a couple is unable to conceive.

The causes of infertility can be varied and complex. In men, conditions that can affect sperm production or quality, such as undescended testicles, genetic defects, hormone problems, and health issues like diabetes, can lead to infertility (Vander-Borght and Wyns, 2018). Infections such as chlamydia, gonorrhea, mumps, or HIV can also impact sperm (Vander-Borght and Wyns, 2018). In women, infertility can be caused by ovulatory disorders, endometriosis, or abnormalities in the uterus (WHO, 2023).

Fortunately, there are many treatment options available for those struggling with infertility. These can range from medications that help with hormones and ovulation, to minor surgical procedures, to assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and intrauterine insemination (Mascarenhas *et al*., 2012).

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The choice of treatment depends on various factors such as the underlying cause of infertility, the couple's age, and their overall health (Mascarenhas *et al.*, 2012). This study investigates the protective effects of ethanol seed extract of *Plukenetia conophora* (African walnut) on cadmium-induced reproductive toxicity in male Wistar rats.

MATERIALS AND METHODS

Collection and Identification of Plant

Plukenetia conophora (African Walnut) was purchased in New Benin Market in Oredo local government area of Edo State. It was identified by Prof. Akinnibosun Henry Adewale in Department of Plant Biology and Biotechnology (PBB) University of Benin, Benin city (Habarium number: UBH-P402). Cadmium chloride was manufactured by Loba chemie Pvt LTD (Batch no. L231151707) was purchased at Emmytex Biomedical store at 47 new Lagos Road opposite UBTH Benin city, Edo state.

Preparation of ethanol seed extract of *Plukenetia conophora* stock solution

Preparation of ethanol seed extract of *Plukenetia conophora* was conducted in the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City. The seeds were pulverized in a British milling machine. 500 g of powder was soaked in ethanol in a conical flask. After twenty-four (24) hours, the solution (mixtures of seed extract powder and ethanol) was filtered with a filter rag and funnel. Before decanting the supernatant, the filtrate was allowed to settle for a time. At 60^{o}C, the supernatant was steamed to dryness in an evaporating dish (Royal Worcester, England) using an H-H Digital Thermometer Water Bath (Mc Donald Scientific International – 22050Hz1.0A). The extracts were kept refrigerated at 4°C in plastic vials until needed.

Dosage: LD50 was carried out to determine the dosage for plant using Lorke's method of dosage determination. The oral LD50 of Cadmium was determined to be 88mg/kg body weight (Siddiqui, 2010).

Experimental *Plukenetia conophora* on cadmium-induced reproductive toxicity in male Wistar rats rats

Thirty-six male adult Wistar Rats weighing between 160 and 180 grams was used for this experiment. The rats were given a two-week acclimatization period before the administration method begun. They were given free access to conventional rat feed and water. The research ethics committee's guidelines for animal handling and treatment at the University of Benin's College of Medicine were espoused and fully implemented.

Experiment Protocol

Thirty-six adult male Wistar Rats were divided into six groups and subjected to different treatments, group A served as the control group, Group B and C received 250 mg/kg body weight and 500 mg/kg body weight of ethanol seed extract of *Plukenetia conophora* only respectively, group D and E received 250 mg/kg body weight and 500 mg/kg body weight of *Plukenetia conophora* in addition to 10mg/kg body weight of cadmium chloride respectively, and group F received 10 mg/kg body weight of cadmium chloride only.

All administrations were done by gavage and lasted for fourteen (56) days.

Administration

The *Plukenetia conophora* and cadmium were given using a gavage with an orogastric tube. The rats were carefully handled to minimize oral or oesophageal injuries.

Sacrifice of animals

During the time of the experiment, the rats were weighed weekly to monitor their weight change as the experiment progressed.

During sacrifice, the final weights of the rats were taken using compact electric weighing scale calibrated in grams. Cotton wools were soaked with chloroform of about 30ml in an enclosed container was used to anaesthetized the animals. After anaesthetizing, the rat was placed on supine position on the dissection trolley and ventral abdomino- thoracic incision was made on the rat to expose the viscerae. Thereafter the vas deferens was harvested and placed in a plain bottle for sperm parameter test and the testes were harvested, weighed and fixed with Bouin's fluid in a universal bottle for histological studies.

Statistical analysis

GraphPad Prism version 9.0 (GraphPad Software Inc.) for Windows was used to analyze the data obtained in the study. Results obtained were expressed as Mean ± SEM (standard error of mean). Differences among the means were compared by one-way analysis of variance (ANOVA). Values were considered statistically significant if P value is less than 0.05 ($p < 0.05$). LSD *Post Hoc* test was used to determine where the significance lay.

RESULTS

Effects of treatment on weight

Table 1 shows results for body weight and testicular weight. From the table, there was no statistically significant difference ($P>0.05$) in initial body weight across all the groups. The final body weight was significantly reduced ($P<0.05$) in Cadmium-only-treated group compared to the control. However, there was a statistically significant increase ($P<0.05$) in final body weight of cadmium chloride group treated with low dose of the extract compared to Cadmium chloride only treated group but there was no statistically significant difference ($P>0.05$) in final body weight of cadmium chloride group treated with high dose of the extract compared to Cadmium chloride only treated group. There was no statistically significant difference ($P>0.05$) in final body weight of the groups treated with low and high doses of the extract compared with the control group.

Comparing initial and final weights within each group, there was a statistically significant increase ($P<0.05$) in body weight in the

control group, low dose only treated group and high dose only treated group; there was a statistically significant decrease (P<0.05) in body weight in the high dose plus CdCl group and CdCl only treated group. However, there was no statistically significant difference (P>0.05) between initial and final body weight in low dose plus CdCl treated group. This is also reflected in the body weight change (final body weight minus initial body weight) where there was no statistically significant difference (P>0.05) in body weight change of the groups treated with low and high doses of the extract compared with the control group but there was weight loss in the CdCl only group, low dose plus CdCl group and high dose plus CdCl group which was statistically significant (P<0.05) compared to the control group.

There was no statistically significant difference (P>0.05) in testicular weight of the groups treated with low and high doses of the extract compared with the control group but there was statistically significant (P<0.05) decrease in testicular weight in the CdCl only group, low dose plus CdCl group and high dose plus CdCl group compared to the control group. There was no statistically significant difference (P>0.05) in testiculosomatic of the groups treated with low dose of the extract, low dose plus CdCl and high dose plus CdCl compared with the control group. Testiculosomatic index was significantly increased (P<0.05) in high dose only group and significantly decreased (P<0.05) in CdCl only group compared to the control group.

Effect of treatment on sperm analysis

Table 2 shows result of sperm analysis. From the table, there was no statistically significant difference (P>0.05) in sperm count of the groups treated with low and high doses of the extract compared with the control group. Sperm count was significantly decreased (P<0.05) in the CdCl only group compared with the control but on co-administration with the extract, there was a statistically significant increase (P<0.05) in sperm count compared with the CdCl only treated group with the low dose being more potent.

There was no statistically significant difference (P>0.05) in progressive motility, non-progressive motility and immotile sperm cells in the groups treated with low and high doses of the extract with the control group. Progressive motility was significantly decreased (P<0.05) while non-progressive motility and immotile sperm was significantly increased (P<0.05) in CdCl only treated group compared with the control group. However, on co-administration with the extract, there was a statistically significant increase (P<0.05) in progressive motility and statistically significant decrease (P<0.05) in non-progressive motility and immotile sperm compared with the CdCl only treated group with the low dose being more potent.

Effect of Treatment on the Histology of the Testes

Fig 1 portrays the histology of rat's normal testes, showing normal architecture including seminiferous tubules lined with spermatogenic series (ST), Sertoli cells (SC) and interstitial cells of Leydig (LC).

Fig 2 portrays the histology of rat's testes administer with 250mg/kg of Walnut only, with "a" showing tubules lined by spermatogenic series (ST), Sertoli cells (SC), and interstitial cells of Leydig (LC) while "b" shows testicular lysis, Sertoli cells (SC), Leydig cells (LC) and immature spermatocytes (SP).

Fig 3 represents the histology of rat's testes treated with 500mg/kg of walnut only, with "a" showing normal spermatogenic series (TS), Sertoli cells (SC) and Leydig cells (LC) while "b" shows patchy spermatogenic arrest (SA) and Sertoli cells degeneration (SD).

Fig 4 represents histology of rat's testes treated with 250mg/kg of walnut + cadmium chloride, showing normal spermatogenic series in normal sequential maturation (SS), Sertoli cells (SC) and Leydig cells (LC).

Fig 5 represents the histology of rat's testes treated with 500mg/kg of walnut + cadmium chloride, showing severe testicular hyalinization (TH) and calcification.

Fig 6 portrays the histology of rat's testes administered with cadmium chloride, showing severe testicular hyalinization (TH) and calcification (CA).

TABLE 1: Weights across experimental groups

	initial body weight	final body weight	body weight change	Testicular weight	Testiculosomatic index
Control	167.00±3.62^{a}	206.00±3.69^{a}*	39.00±1.05^{a}	2.38±0.11^{a}	1.16±0.04^{a}
LD only	177.80±6.83^{a}	212.80±2.87^{a}*	35.00±4.67^{a}	2.56±0.07^{a}	1.20±0.03^{a,b}
HD only	171.75±3.79^{a}	207.25±4.92^{a}*	35.50±5.36^{a}	2.60±0.07^{a}	1.28±0.03^{b}
LD±CdCl	179.33±4.06^{a}	167.67±2.96^{b}	-11.67±2.73^{b}	1.90±0.06^{b}	1.17±0.03^{a,b}
HD±CdCl	180.33±7.26^{a}	153.67±2.33^{b,c}*	-26.67±5.21^{b,c}	1.73±0.07^{b}	1.17±0.03^{a,b}
CdCl	176.00±10.00^{a}	143.00±15.31^{c}*	-33.00±10.21^{c}	1.20±0.20^{c}	0.80±0.06^{c}

Unlike superscripts down a row means statistically significant (P<0.05) across the group while like superscripts down a row means not statistically significant (P>0.05) across the group. *means statistically significant comparing initial and final body weight within a group.

TABLE 2: Sperm analysis across experimental groups

	Sperm count ($\times 10^6$)	Progressive motility (%)	Non-Progressive motility (%)	Immotile (%)
Control	939.00±5.57[a]	92.00±0.58[a]	5.67±0.88[a]	2.33±0.33[a]
LD only	961.33±6.67[a]	90.67±0.88[a]	6.00±0.58[a]	3.33±0.67[a]
HD only	999.33±6.36[a]	95.33±1.20[a]	3.00±1.53[a]	1.67±0.33[a]
LD±CdCl	1022.33±44.11[b]	95.00±0.58[a]	3.00±0.00[a]	2.00±0.58[a]
HD±CdCl	186.67±34.80[c]	35.00±2.89[b]	37.33±1.76[b]	27.67±2.19[b]
CdCl	29.00±8.08[d]	22.33±3.71[c]	41.67±2.33[c]	36.33±1.33[c]

Unlike superscripts down a row means statistically significant (P<0.05) across the group while like superscripts down a row means not statistically significant (P>0.05) across the group.

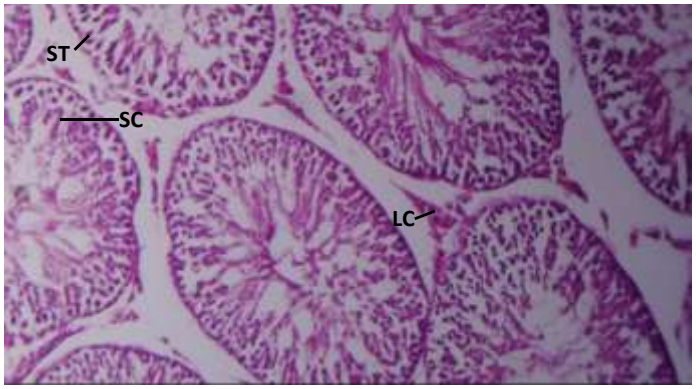

Fig 1: Rat testis. Control. Composed of normal architecture showing: seminiferous tubules lined by spermatogenic series (ST), Sertoli cells (SC), interstitial cells of Leydig (LC): HandE x 100

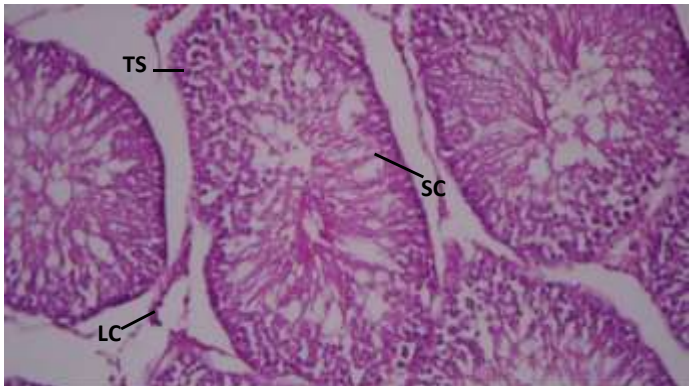

Fig 3: Rat testis given 500mg Walnut only showing normal spermatogenic series (TS), sertoli cells (SC), Leydig cells (LC): HandE x 100

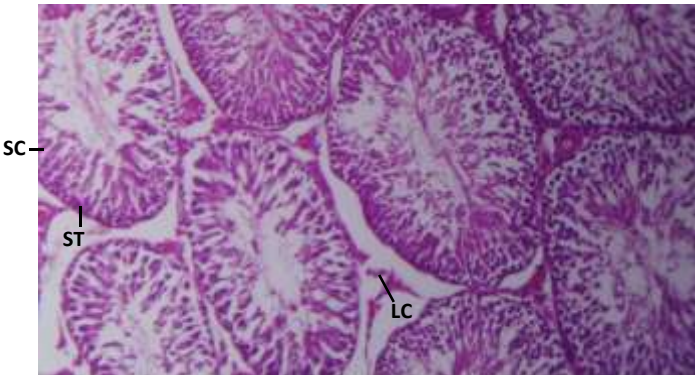

Fig 2: Rat testis given 250mg Walnut only showing: tubules lined by spermatogenic series (ST), sertoli cells (SC), interstitial cells of Leydig (IL), all normal: HandE x 100

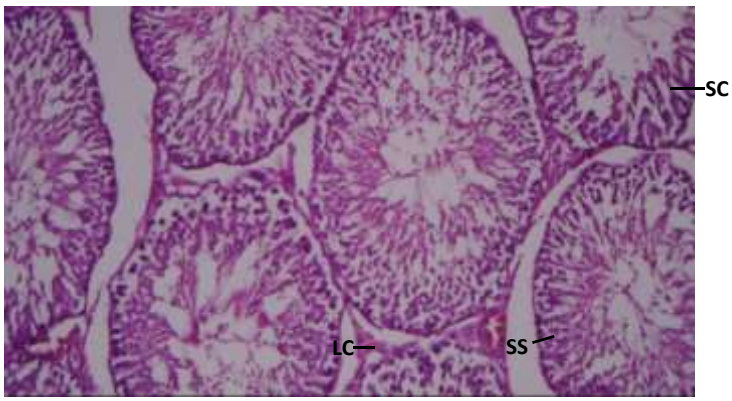

Fig 4: Rat testis given 250mg Walnut + Cadmium showing normal series in normal sequential maturation (SS), sertoli cells (SC), cells (LC): HandE x 100

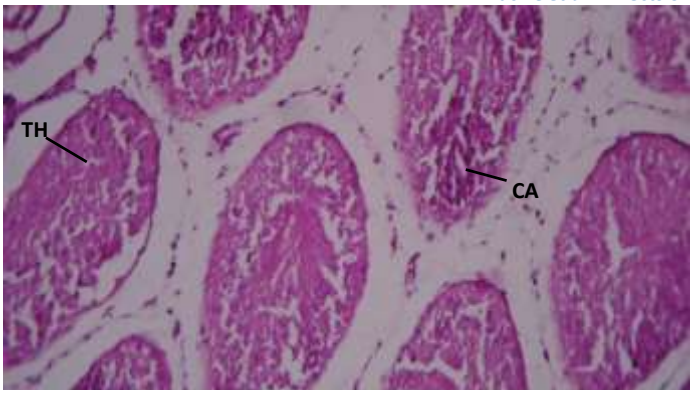

Fig 5: Rat testis given 500mg Walnut + Cadmium showing: severe testicular hyalinization (TH), calcification (CA): HandE x 100

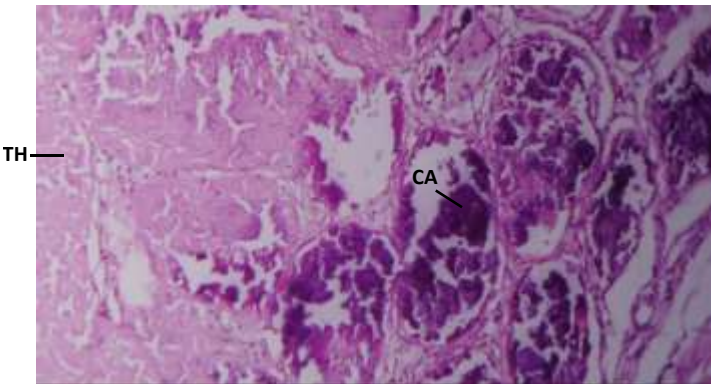

Fig 6: Rat testis given Cadmium only showing: severe testicular hyalinization (TH), calcification (CA): HandE x 100

DISCUSION

Cadmium is a heavy metal element with long biological half-life, slow metabolism and strong toxicity which easily accumulates in the body and thus causing a variety of damages to tissues and organs of the body (Renu *et al*., 2021). Cadmium is present in most foodstuffs because of its high rates of soil-to-plant transfer hence human exposure is inevitable (Franz *et al*., 2008).

Poisoning caused by long-term chronic accumulation of Cadmium has attracted more and more social attention, but the pathogenesis of short-term toxicity of high-dose Cadmium is not completely clear (Li and Shen, 2023).

There was no significant difference in the initial body weight across all groups, indicating a similar starting point for all. However, the Cadmium-only-treated group showed a significant reduction in final body weight compared to the control, suggesting a detrimental effect of Cadmium on body weight. Cadmium is known to be a toxic heavy metal that can cause damage to various organs and systems in the body, which could contribute to weight loss. In a study where Wistar rats were fed with combined levels of Cadmium chloride and zinc chloride, it was observed that the rats had the lowest growth rate after 12 weeks (Rhman *et al*., 2011). This suggests that the effects Cadmium chloride can lead to a decrease in body weight. Another study investigated the effect of an herbal preparation on Cadmium-induced antioxidant system in female Wistar rats (Dailiah and Padmalatha, 2011). Two different doses of Cadmium (50 ppm and 200 ppm) were given to the rats. The study found that Cadmium is one of the elements that damage the antioxidant systems in mammals, which could potentially lead to weight loss (Dailiah and Padmalatha, 2011). Cadmium chloride can have a significant impact on body weight in Wistar rats. The exact mechanisms through which Cadmium chloride induces weight loss are not fully understood and may involve multiple pathways (Poli *et al*., 2022). Interestingly, the group treated with Cadmium and a low dose of Walnut (*T. conophora*) extract showed a significant increase in final body weight compared to the Cadmium-only group, indicating a potential protective effect of the Walnut extract against Cadmium-induced weight loss. Research by (Lesmana *et al*., 2021) reported a significant increase in the adipose tissue of rats treated with Walnut extract which could explain the increase in the body weight. This protective effect was not observed in the group of animals treated with high dose of the Walnut extract.

The groups treated with low and high doses of the Walnut extract showed no significant difference in testicular weight compared to the control group, suggesting that the Walnut extract itself did not affect testicular weight. However, the Cadmium-treated groups showed a significant decrease in testicular weight compared to the control, indicating that Cadmium negatively affects testicular weight. Similar finding was reported by (Skolarczyk *et al*., 2017), attesting that exposure to cadmium chloride even at a dose as low as 2mg/kg can cause significant decrease in the weight of the testes. Cadmium chloride is a toxic substance that exhibits cytotoxic effects on rapidly proliferating cells, leading to cell death and tissue damage (Genchi *et al*., 2020). When cadmium chloride is present in the body, it can disrupt normal cellular function and cause harm to cells that are rapidly dividing and growing (Rani *et al*., 2013). This is particularly concerning for tissues with high cell turnover rates, such as testicular tissue (Xiong *et al*., 2021).

The testiculosomatic index was significantly increased in the high dose-only group of extract and significantly decreased in the Cadmium-only group compared to the control group. This suggests that Cadmium chloride negatively impacts the testiculosomatic index and this result is in agreement with the finding of (Wang *et al*., 2021).

These results suggest that Cadmium has a negative impact on both body weight and testicular weight. The extract appears to have a protective effect against Cadmium-induced weight loss at low doses, but this effect is not observed at high doses.

Walnut (*T. conophora*) extract, at both low and high doses, did not significantly affect the sperm count compared to the control group. This suggests that Walnut (*T. conophora*) extract itself does not have a detrimental effect on sperm count. However, Cadmium chloride alone significantly decreased the sperm count. Decrease in sperm count induced by cadmium chloride could be as a result

of various conditions, according to (Xiong *et al*., 2021), oxidative stress, inflammation of the testes, and epigenetic changes are some of the mechanisms at which cadmium chloride can reduce sperm count. Impairment of zinc transporters like ZIP8 and ZIP14 in the male genital tract can lead to decrease in sperm count (Pappalardo *et al*., 2023). Similarly, impairment of Ion channels can also lead to decreased viability and motility of sperm (Ali *et al*., 2022). These mechanisms could have collectively contributed to the decrease in sperm count observed with cadmium chloride exposure (Xiong *et al*., 2021). Interestingly, co-administration of Walnut (*T. conophora*) extract with Cadmium chloride significantly increased the sperm count compared to the Cadmium chloride only group, with the low dose of the extract being more potent. This implies that Walnut (*T. conophora*) extract may have a protective effect against Cadmium chloride-induced decrease in sperm count. Cadmium chloride.

Similar to the sperm count, Walnut (*T. conophora*) extract did not significantly affect the progressive motility, non-progressive motility, and immotile sperm cells compared to the control group. However, Cadmium chloride alone significantly decreased progressive motility and increased non-progressive motility and immotile sperm. On co-administration with Walnut *(T. conophora*) extract, there was a significant increase in progressive motility and decrease in non-progressive motility and immotile sperm compared to the Cadmium chloride only group, again with the low dose being more potent. This suggests that Walnut (T. conophora) extract may also have a protective effect against Cadmium chloride-induced changes in sperm motility.

These findings suggest that Walnut (*T. conophora*) extract could potentially be used to mitigate the harmful effects of Cadmium chloride on sperm count and motility.

The histological evidence from rat testes treated with cadmium chloride and walnut underscores the toxic impact of environmental pollutants on reproductive health and the potential for dietary interventions to offer protective benefits. This research has significant implications for both individual health choices and broader environmental policies.

The group, which received cadmium chloride only showed severe testicular hyalinization (TH) and calcification (CA). These conditions suggest that cadmium chloride caused significant damage to the testicular structure, leading to the hardening of the seminiferous tubules and deposition of calcium salts (Zhu *et al*., 2020). The mechanism behind this damage is likely due to cadmium's ability to induce oxidative stress and disrupt the blood-testis barrier, leading to cell death and tissue degeneration (Zhu *et al*., 2020).

Low dose extract of walnut exhibited a healthy and damage testicular structures. 'a' plate showed normal spermatogenic series (ST), Sertoli cells (SC), and Leydig cells (LC), indicating that at this dosage, walnuts may support normal testicular function and spermatogenesis (Ghorbani *et al*., 2014). However, 'b' plates revealed testicular lysis, which could suggest that while walnuts have protective effects, they may not completely prevent testicular damage at this concentration.

At a higher dose of walnut, 'a' plate showed normal spermatogenic series (TS), Sertoli cells (SC), and Leydig cells (LC), which implies a protective or even restorative effect of walnuts on testicular health (Ghorbani *et al*., 2014). 'b' plates with patchy spermatogenic arrest (SA) and Sertoli cells degeneration (SD) indicate that despite the higher dose, some areas of the testes were still affected, possibly due to inherent sensitivity or other factors not mitigated by walnut intake.

Interestingly, the co-administration of a low dose walnut with cadmium chloride at this dosage showed normal spermatogenic series in normal sequential maturation (SS), Sertoli cells (SC), and Leydig cells (LC). This suggests that walnuts at this concentration can counteract the harmful effects of cadmium, maintaining normal testicular structure and function (Ghorbani *et al*., 2014).

In contrast to the previous group, the higher dose of walnuts combined with cadmium chloride resulted in severe testicular hyalinization (TH) and calcification. This outcome might indicate that the protective effect of walnuts has a threshold and that beyond a certain level of damage induced by cadmium, walnuts are unable to provide adequate protection (Ghorbani *et al*., 2014).

Conclusion: These results demonstrate the complex interactions between toxic substances like cadmium chloride and protective agents like walnuts. They highlight the importance of understanding dosages and combinations when considering the therapeutic potential of natural products against environmental toxins.

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