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Research Article

Carpolobia lutea Root Extract Improved Steroidogenic Activity in Male Wistar Rats Exposed to Cadmium

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Summary: Cadmium (Cd) is known to affect reproductive functions adversely. *Carpolobia lutea* is a protective herbal derivative due to its antioxidant potential. This study investigates the steroidogenic activities of methanol extract of *Carpolobia lutea* root on cadmium-induced reproductive toxicity in male Wistar rats. *Carpolobia lutea* root was obtained in Ijare via Akure. The plant was authenticated at the herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, with FHI number 109784. The methanol extract *Carpolobia lutea* root (MCL) was obtained by Soxhlet extraction. Thirty male Wistar rats (150-170g) were used in this study (n=5) and treated as follows: Control, Cd (2 mg/kg), Cd+MCL (2 mg/kg+100 mg/kg), Cd+MCL (2 mg/kg+200 mg/kg), MCL (100 mg/kg), and MCL (200 mg/kg). The extract was administered orally for eight weeks, and a single dose of 2 mg/kg Cd was given intraperitoneally. Serum Follicle Stimulating Hormone (FSH), Luteinizing hormone (LH), testosterone levels, testicular hydroxysteroid dehydrogenases (HSDs) activities and Steroidogenic Acute Regulatory protein (StAR) expression were evaluated. Data were subjected to descriptive statistics and analysed using ANOVA at $p<0.05$. Serum FSH, LH, testosterone levels, 3β-HSD, 17β-HSD activities and StAR expression were significantly reduced ($p<0.05$) in Cd group. The co-administration of Cd with MCL (200mg/kg) significantly increased ($p<0.05$) serum FSH, LH, testosterone levels, 3β-HSD, 17β-HSD activities and StAR expression when compared with Cd group. *Carpolobia lutea* root extract improved steroidogenic activity in male Wistar rats exposed to cadmium.

Keywords: *Carpolobia lutea, hydroxysteroid dehydrogenases, Steroidogenic Acute Regulatory protein, Hormone, Wistar rat*

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INTRODUCTION

Steroidogenesis is the enzymatic reactions leading to the synthesis of male steroid hormones. One of the functions of the testes is male sexual hormones (androgens) synthesis. Androgens, especially testosterone is required for normal male fertility, frequency and presence of sexual phantasies, morning erections, the frequency of copulation and sexual activity (Chang *et al*., 2004; Zhang 2006; Xu *et al*., 2007). The testicular functions can be affected by some factors. Heavy metals exposure is one of the environmental factors that can affect testicular steroidogenesis.

Many recent studies have suggested that pathogenesis of male infertility is attributed to oxidative stress (Akinola *et al*., 2020). Spermatozoa produce small amounts of reactive oxygen species (ROS) in physiological conditions and various scavengers help reduce the concentration of these ROS in the seminal plasma. But, increased production and/or decreased clearance causes oxidative stress within the sperm, leading to reduced motility (Kao *et al*., 2008) and defective membrane integrity (Agarwal *et al*., 2003). One of the reactive oxygen species inducers is environmental exposure to toxicants.

The roles of heavy metals in the aetiology of reproductive dysfunctions have been studied for several years (Patrick, 2003). Heavy metals are among one of the most widespread potential chemical contaminants in the environment (Ragan and Mast, 1990). Some heavy metals like lead, cadmium (Cd), mercury and cobalt are toxic to reproductive functions (Anderson *et al*., 1992). Effect of these metals exposure on the testis is of enormous interest as occupational exposure to them impaired reproductive activities (Afonne *et al*., 2002). Cadmium is one of the major heavy metals that increase in ecological systems through mining, smelting and industrial activities. The major sources of human exposure to this metal include food, cigarette smoke, alcoholic beverages and underground water (Jarup *et al.,* 1998). Testes are exquisitely sensitive to cadmium toxicity (Anders, 1990). Stoh *et al.* (2001), El-Demerdash *et al.* (2004) and Massanyi *et al.* (2007) reported that cadmium caused testicular damage, reduction in serum testosterone level, and reproductive dysfunction in Wistar rats. Acute cadmium chloride exposure causes significant reproductive dysfunction via generation of free radical and increased oxidative stress leading to histological alteration, (necrosis, edema etc.) and spermatological damage (decreased sperm motility and sperm concentration, and increased abnormal sperm cells) (Akinola *et al*., 2020). Cadmium toxicity is associated with severe damage to various organs,

particularly the testes, in both humans and animals (Fouad *et al*., 2009). Cadmium disrupt the reproductive capacity by causing serious testicular degeneration, seminiferous tubule damage and necrosis in rats (Burukoğlu and Bayçu, 2008).

Carpolobia lutea G. Don (family: Polygalaceae) is also known as cattle stick (English), Ikpafum (Ibibio, Southern Nigeria), Agba or Angalagala (Igbo, Eastern Nigeria) and Oshunshun (Yoruba, Western Nigeria) (Etekudo, 2003). It is a shrub of about 5cm tall and widely found in tropical Africa (Akpan *et al*., 2012). Ethno-botanically, various parts of the plant have been affirmed to be used in curing several diseases. The leaves have also been used to promote childbirth (Ajibesin *et al*., 2008; Muanya and Odukoya, 2008), while the root bark has been implicated to be used for treating rheumatism, fever, general pain and insanity (Ajibesin *et al*., 2008). The dried stem bark is usually taken as snuff to cure a migraine (Nwidu *et al*., 2011). Furthermore, the root decoction is reputed in Western and Southern Nigeria as ‘‘Ogun aleko’’ meaning sex stimulating tonic or aphrodisiac (Nwafor and Bassey, 2007; Ajibesin *et al*., 2008).

Aphrodisiacs modes of action are majorly classified into three types, which increase libido; potency; or sexual pleasure. *Carpolobia lutea* root extract has been used in folk medicines of different Nigerian cultures to energise, vitalise and improve sexual function, and physical performance in male (Yakubu and Jimoh, 2014; Dare *et al.*, 2015).

Therefore, the present study was designed to examine the possible ameliorative and protective effects of methanol extract *of Carpolobia lutea* (MCL) root against Cd-induced testicular dysfunction in male Wistar rats.

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MATERIALS AND METHODS

Plant Collection and Extraction: *Carpolobia lutea* root was obtained from Ijare a village *via* Akure, Ondo state. The plant was authenticated at the herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, with FHI number 109784. The root was air dried and pulverised. The pulverised root (5.20 kg) was subjected to Soxhlet extraction using pure methanol. The methanol root extract of *C. lutea* was concentrated at 4^0C in a rotary evaporator. The remaining extract was finally dried in a vacuum oven at 30^0C for 2 hours to ensure the removal of any residual solvent. The yield of the powdery mass was 87.88g (1.69% yields).

Animal and Experimental Design: Adult male rats of Wistar strain (150 to 170 gram) housed in well-ventilated cages in the Central Animal House, College of Medicine, University of Ibadan were used for this study. They were maintained under standard laboratory conditions of 12-hour light and 12-hour dark cycle and were fed with standard commercial rat pellets (Ladokun feeds Limited, Ibadan, Nigeria) and allowed access to water *ad libitum*. They were acclimatized for two weeks. The procedures in this study conformed to the guiding principles for research involving experimental animals as recommended by the Declaration of Helsinki as well as the Guiding principles in the Use and Care of Animals (American Physiological Society, 2002).

Thirty male Wistar rats were randomly divided into six groups with five animals per group and treated as follows: Group 1 were given distilled water (Control), group 2 were given Cd (2 mg/kg *b.w.*) single dose intraperitoneally, groups 3 and 4 were pre-treated with Cd (2 mg/kg *b.w.*) single dose intraperitoneally before treating with MCL 100 mg/kg *b.w.* and 200 mg/kg *b.w.*) orally for 8 weeks respectively, groups 5 and 6 were treated with MCL 100 mg/kg *b.w.* and 200 mg/kg *b.w.*) orally for 8 weeks respectively..

Twenty-four hours after the last administration, the animals were anaesthetised with 50 mg/kg *b.w.* of sodium thiopentone. Blood was collected from all the animals via cardiac puncture for serum levels of gonadotropin and testosterone. The testes were harvested and used for determination of androgenic enzymes activities and steroidogenic acute regulatory protein (StAR) expression.

Serum Hormone Analysis: Serum was obtained from a blood sample collected and used for testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) analysis. The analysis was carried out using an Enzyme-Linked Immunosorbent Assay (ELISA) based on the manufacturer’s manual. (Ulloa-Aguirre and Timossi, 1998; Oyeyemi *et al*., 2019).

Assay of Testicular Androgenic Enzyme Activities: The testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was measured according to the method of Talalay (1962). The testicular tissue of each animal was homogenized in 15% spectroscopic grade glycerol (BDH, Mumbai, India) containing 5 mmol potassium phosphate (Loba, Mumbai, India) and 1 mmol EDTA (Organon, Calcutta, India.) at a tissue concentration of 100 mg/ml. The homogenized mixture was centrifuged at 10,000 × g for 30 min at 4° C. The supernatant (1 ml) was mixed with 1 ml of 100 µmol sodium pyrophosphate buffer (pH 8.9) and 30 µg of dehydroepiandrosterone (Sigma) in 40 µl of ethanol and 960 µl of 25% BSA (Sigma), making the incubation mixture a total of 3 ml. The enzyme activity was measured after addition of 0.5 µmol of NAD (Sigma) to the tissue supernatant mixture in a U 2000 spectrophotometer (Hitachi, Japan) cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

The activity of testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) was done according to Jarabak *et al*. (1962). The same supernatant prepared for the assay of 3β-HSD (above) was used. The supernatant (1 ml) was mixed with 1 ml of 440 µmol sodium pyrophosphate buffer (pH 10.2), 40 µl of ethanol containing 0.3 µmol of testosterone (Sigma) and 960 µl of 25% BSA (Sigma), making the incubation mixture a total of 3 ml. The enzyme activity was measured after addition of 1.1 µmol NAD (Sigma) to the tissue supernatant mixture in a U 2000 spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was equivalent to a change in absorbance of 0.001/min at 340 nm.

Extraction and Amplification of Steroidogenic Acute Regulatory Protein Gene Expression by Polymerase Chain Reaction: Testicular DNA was purified using Zymo research DNA extraction kit. *Taq* Polymerase chain reaction (PCR) was carried out on purified testicular DNA samples. The DNA samples were amplified using

Steroidogenic Acute Regulatory protein (StAR) primer (Forward -CGT GGC TGC TCA GTA TTG AC and backward- AGT CCT TAA CAC TGG GCC TC) which was designed with Primer 3 software, and its specificity was ascertained with the help of National Centre of Biotechnology Information (NCBI)-Blast.

Digital Image Analysis: The PCR plate was analysed and quantified using ImageJ software (Version 1.49, National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis: Results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to assess the statistical significance of the data, and Fisher´s Least Significant Difference (LSD) test was used for post hoc analysis (Multiple comparisons). P<0.05 was considered significant.

RESULTS

Effect of methanol extract of *Carpolobia lutea* root on serum testosterone level in male Wistar rats exposed to cadmium: Table 1 shows that serum testosterone level was significantly decreased (p<0.05) in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) treated groups when compared with control. There was a significant increase (p<0.05) in serum testosterone level of MCL (200 mg/kg) group when compared with the control group. Serum testosterone level was significantly increased (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group.

Effect of methanol extract of *Carpolobia lutea* root on serum follicle stimulating hormone level in male Wistar rats exposed to cadmium

Table 2 shows that serum follicle stimulating hormone was significantly decreased (p<0.05) in Cd (2 mg/kg) treated group, while there was a significant increase (p<0.05) in MCL (200 mg/kg) group when compared with control group. Alternatively, there was a significant increase (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group.

Effect of methanol extract of *Carpolobia lutea* root on serum luteinizing hormone level in male Wistar rats exposed to cadmium: Serum luteinizing hormone was significantly decreased (p<0.05) in Cd (2 mg/kg), Cd+MCL (100 mg/kg) and Cd+MCL (200 mg/kg) treated groups when compared with control. Alternatively, there was significant increase (p<0.05) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group (Table 1). .

Table 1:
Effects of Methanol Extract of *Carpolobia lutea* root on Serum Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone in Male Wistar Rats Exposed to Cadmium

S/N	Groups	Control	Cd(2 mg/kg)	Cd+MCL (100 mg/Kg)	Cd+MCL (200 mg/Kg)	MCL (100 mg/Kg)	MCL (200 mg/Kg)
1	Testosterone	8.6±0.58	3.8 ± 0.52*	5.5±0.58*	8.5±0.86^{+}	8.3±0.46	11.8±1.01*
2	FSH	6.4±0.42	3.1 ± 0.47*	4.0±0.24*	7.4 ± 0.48^{+}	7.8± 0.76	12.5±1.26*
3	LH	12.1±0.44	7.9 ± 0.58*	9.3±1.11*	10.3±0.20*$^{+}$	11.8±0.37	12.3± 0.20

*Values expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

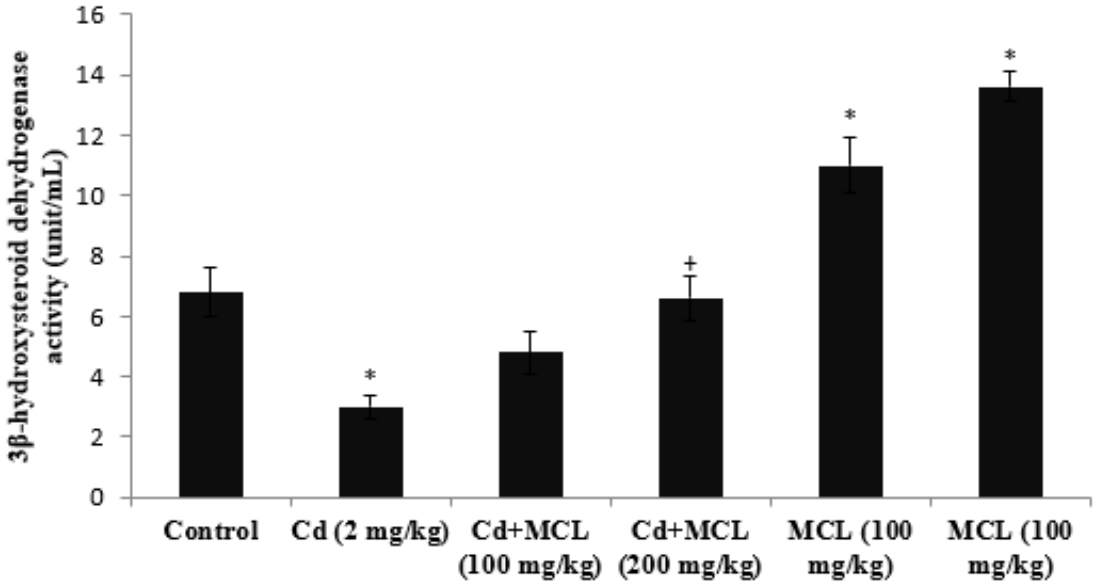

Figure 1:
Effect of Methanol extract of Carpolobia lutea root on 3β-hydroxysteroid dehydrogenase activity in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

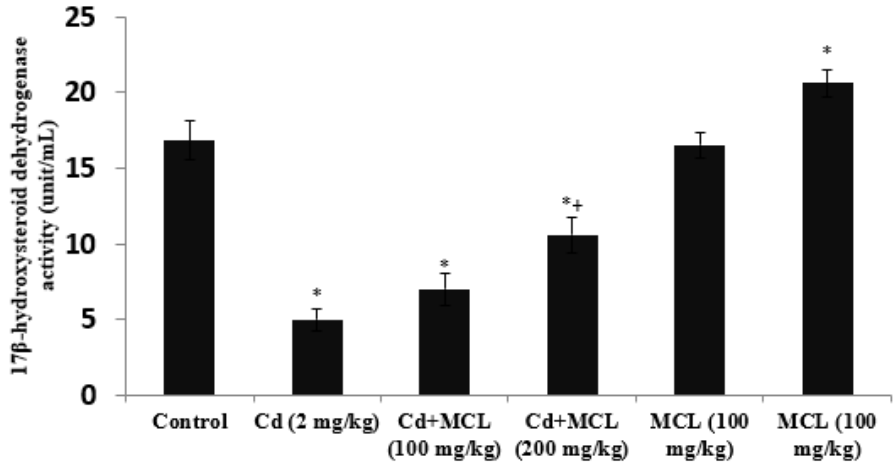

Figure 2:
Effect of methanol extract of *Carpolobia lutea* root on 17β-hydroxysteroid dehydrogenase in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

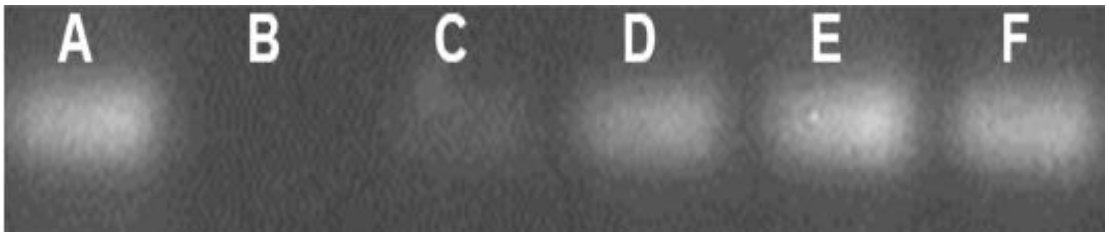

Plate 1:
Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium
Lanes: A: Control; B: Cd (2 mg/kg); C: Cd+MCL (100 mg/kg); D: Cd+MCL (200 mg/kg); E: MCL (100 mg/kg) F: MCL (200 mg/kg)

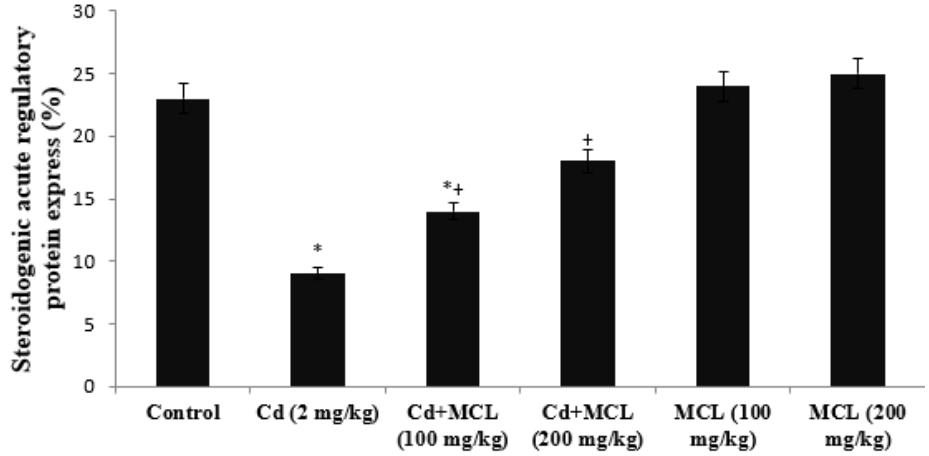

Figure 3:
Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium
*Data expressed in mean ± SEM, *,+ p<0.05 show a significant difference when compared with control and Cd respectively.*

Effect of methanol extract of *Carpolobia lutea* root on 17β-hydroxysteroid dehydrogenase in male Wistar rats exposed to cadmium: Figure 2 shows that 17β-hydroxysteroid dehydrogenase activity was significantly decreased ($p<0.05$) in Cd (2 mg/kg), Cd+MCL (100 mg/kg) and Cd+MCL (200 mg/kg) treated groups when compared with control. Also, there was a significant increase ($p<0.05$) in MCL (200 mg/kg) when compared with control. Alternatively, there was a significant increase ($p<0.05$) in Cd+MCL (200 mg/kg) group when compared with Cd (2 mg/kg) treated group

Effect of methanol extract of *Carpolobia lutea* root on testicular steroidogenic acute regulatory protein expression in male Wistar rats exposed to cadmium: Plate 1 and Figure 3 show the qualitative RT-PCR products and relative expression of testicular steroidogenic acute regulatory protein (StAR) expression. Steroidogenic acute regulatory protein expression was moderately expressed in the control, Cd+MCL (200 mg/kg), MCL (100 mg/kg) and MCL (200 mg/kg) groups while it was mildly expressed in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) groups respectively (Plate 1). The figure 3 shows that the StAR expression was significantly reduced ($p<0.05$) in Cd (2 mg/kg) and Cd+MCL (100 mg/kg) groups when compared with control group, while significant increase ($p<0.05$) was observed in Cd+MCL (200 mg/kg) group when compared with control.

DISCUSSION

The present study demonstrated that Cd toxicity induced serious alterations in the testes, which were protected against by co-administration of methanol extract of *Carpolobia lutea* root. Cd administration induced significant decreases in the serum levels of testosterone, FSH, LH, androgenic enzymes activities and expression of steroidogenic acute regulatory protein (StAR) in the rat. Furthermore, methanol extract of *Carpolobia lutea* root ameliorated all alterations induced by Cd toxicity. Cadmium is a toxic metal that is widely distributed in the environment (Singh *et al*., 2012). Humans are exposed to Cd toxicity by either inhalation or ingestion; skin absorption of Cd is relatively insignificant (Zalups and Ahmad, 2003; Mead, 2010). Cd is reported to be contained in industrial emissions, cigarette smoke and agricultural fertilizers (Zalups and Ahmad, 2003). It facilitates early oxidative stress, disrupts hypothalamic pituitary testicular axis and testicular steroidogenesis (Massanyi *et al*., 2007). Cd accumulation may also occur in the testes, where it causes testicular oxidative stress by two mechanisms i.e. by reacting with the sulfhydryl groups of various proteins or by glutathione depletion (Valko *et al*., 2005). It has also been previously reported that Cd acts through overproduction of ROS and enhanced lipid peroxidation (Sen Gupta *et al*., 2004). In addition, chronic Cd toxicity leads to impairment of the H_2O_2 removal system, which leads to inhibition of steroidogenesis in the Leydig cells due to an accumulation of H_2O_2 (Diemer *et al*., 2003). The result of this study showed a significant reduction in serum testosterone level in cadmium-treated rats. This result is consistent with the previous reports of Habeebu *et al*. (1998), Stoh *et al*. (2001), Massanyi *et al*. (2007) who reported a reduction in testosterone levels and reproductive dysfunction in Wistar rats exposed to cadmium. Also, Yang *et al*. (2003) and El-Demerdash *et al*. (2004) also reported that in the male gonad, cadmium could cause testosterone suppression, failure of spermiation, reduced sperm motility, increased incidence of Leydig cell tumours, and at high doses testicular damage. The marked decrease in the serum levels of testosterone detected in the present study may also possibly be due to Cd-induced decreased synthesis and availability of cholesterol for steroidogenesis. Consequently, decreases in cholesterol biosynthesis result in downregulation of steroid biosynthesis (Barlow *et al*., 2003). Testosterone level was significantly increased in cadmium group treated with high dose of C. lutea extract. Cadmium has been proposed to reduce testosterone production by testes through feedback inhibition of hypothalamic-pituitary-testicular axis (Waalkes *et al*., 1997). Therefore, treatment of C. lutea root extract with cadmium may prevent the feedback inhibition of the hypothalamic-pituitary-testicular axis, and hence facilitate testosterone synthesis in the testes. C. lutea root extract may avert the inhibitory effect of cadmium in testosterone biosynthesis in Leydig cells. It has been reported that cadmium decreases serum testosterone level in male Wistar rats and this may be a reflection of the accumulation and direct toxic effect of the cadmium in the testes (Salama and El-Bahr, 2007).

Cadmium is known to directly destroy the hypothalamus-pituitary-gonadal axis, thus destroying the secretory organs of hormones (Massanyi *et al*., 2007) and compromising hormonal release. As regards the serum levels of the gonadotrophins, both follicle stimulating hormone (FSH) and luteinizing hormone (LH) were significantly reduced in cadmium-exposed rats. The reduction in serum FSH and LH in cadmium-exposed rats may indicate destruction in the pituitary testicular axis (Sadik, 2008). The observed reduction in serum FSH and LH hormonal level of cadmium-exposed rats may be responsible for observed decreased in serum testosterone since gonadotrophins induce the signals for testosterone synthesis (Habeebu *et al*., 1998; Massanyi *et al*., 2007). The administration of *Carpolobia lutea* root extract reversed observed reduction in serum FSH and LH in cadmium-exposed rats. The antioxidant activity of *Carpolobia lutea* leaf extract (Nwidu *et al*., 2012) may be responsible for protecting pituitary gland from cadmium toxic and enhance gonadotrophins secretion.

In testicular steroidogenesis, 3β - and 17β - hydroxysteroid dehydrogenase are the prime enzymes that play a critical regulatory function in testicular androgenesis (Ghosh *et al*., 1990; Jana and Samanta, 2006). The diminution in these enzymes by cadmium treatment in our study is in agreement with the findings of others (Sen-Gupta *et al*., 2004; Sadik, 2008). A decrease in serum concentration of testosterone in cadmium-treated rats may occur due to the inhibition of these testicular androgenic enzymes activities because these enzymes are involved in the regulation of testosterone biosynthesis (Ghosh *et al*., 1990; Jana *et al*., 2005). The activity of *C. lutea* root extract in improving these steroidogenic enzymes activities may be associated with cytochrome P450 side-chain cleavage complex (P450scc) because cadmium has been reported to decrease (P450scc) (Sen-Gupta *et al*., 2004). Also, C. lutea root extract may prevent cadmium to bind with the thiol group of proteins and enzymes (Hassoum and Stohs, 1996) hence increase the activity of 3β - and 17β - hydroxysteroid dehydrogenase. The observed increase in serum testosterone level in *C. lutea* root extract co-administered with cadmium treated rats may be due to the stimulatory action of these testicular androgenic enzyme activities since these enzymes are responsible for the regulation of testosterone biosynthesis (Sen-Gupta *et al*., 2004, Sadik, 2008). Moreover, the increase of testicular androgenic enzymes in animals co-treated with C. lutea root extract and cadmium may be as a result of raised in serum level of LH, the prime regulator of testicular androgenic enzymes activities (Kerr and Sharpe, 1986).

The steroidogenic acute regulatory (StAR) protein has been shown to perform a critical function at this step via the transfer of cholesterol (Lin *et al*., 1995). It has been reported that a decrease in StAR activity is associated with a decrease in steroidogenesis (Adel *et al*., 2016). In this study, the steroidogenic acute regulatory protein (StAR) was mildly expressed in the cadmium treated rats. Zhang and Jia, (2007) opined that mechanistic studies have revealed that cadmium inhibits progesterone synthesis in granulosa cells by down-regulation of StAR and p450scc. The same article reported that co-treatment with 8-bromo-cAMP blocked the decline in progesterone secretion, indicating that cadmium exerts its action by interfering with cAMP synthesis and signalling, which in turn leads to a reduced expression of StAR and p450scc. The delivery of substrate cholesterol to the inner

mitochondria membrane in which the P450SCC enzyme is located is considered to be the rate-limiting step in steroidogenic cascade (Stocco and Clarke, 1996). The upregulation of StAR expression in the C. lutea root extract plus cadmium may be responsible for the observed increase in the serum testosterone level. Steroidogenic acute regulatory protein (StAR) up-regulation by C. lutea extract might be attributed to its ability to prevent interference of cadmium with testicular luteinizing hormone receptor, messenger ribonucleic acid, and cyclic adenosine monophosphate synthesis and signalling (Lin *et al*., 1995; Gunnarsson *et al*., 2003; 2007).

From the present results, it can be concluded that administration of methanol extract of C. lutea root can improve testicular steroidogenesis in cadmium-exposed male Wistar rats via steroidogenic activity and gonadotrophin.

In conclusion, methanol extract of C. lutea root ameliorated Cd-induced alterations in reproductive hormones, androgenic enzymes activity and StAR protein expression. Future studies are required to outline the direct beneficial effect of methanol extract of C. lutea root and its availability for treatment of testicular dysfunction during Cd toxicity.

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