Comparing the effects of *Cyperus esculentus* hydroethanolic extract and *Euterpe oleracea* on reproductive efficacy against cadmium-induced testicular toxicity in male rats

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**ABSTRACT**

Objective: Cadmium chloride (CdCl$_2$) is an environmentally toxic pollutant that can cause reproductive toxicity. *Cyperus esculentus* and *Euterpe oleracea* are potent antioxidant plants currently used to counteract the action of harmful pollutants. The present experiment was intended to evaluate and compare the role of *C. esculentus* hydroethanolic extract (CHE) and *E. oleracea* in treating the reprotoxity induced by Cd, in rats.

Materials and Methods: Forty adult male rats (160–210 gm) were allocated into five groups equally. Control group: received 5 ml of normal saline (NS); the other treatment groups were injected with CdCl$_2$, as a single dose for two weeks to induce testicular toxicity. After 14 days, the four groups were treated orally daily for two months as follows: The cadmium group (Cd) received NS, the third group (TC) was administered 800 mg/kg BW of CHE, the fourth group (TO) received 500 mg/kg BW of *E. oleracea*, and the fifth group (TCO) received CHE with *E. oleracea*.

Results: The live sperm and motility, serum testosterone, follicle-stimulating hormone (FSH), testicular superoxide dismutase (SOD), catalase (CAT), steriodogenic acute regulatory protein (StAR), 17β-hydroxysteroid dehydrogenase, and 3β-hydroxysteroid dehydrogenase (3β-HSD) were significantly increased in the TCO, TC, and TO groups compared with the Cd group. Testicular nitric oxide and malondialdehyde were elevated significantly in the Cd group compared to the TC, TO, TCO, and control groups. The fold changes of Fshβ, Lhβ, and Gnrh genes were upregulated in the TCO group compared to the Cd and control groups.

Conclusion: The combination of CHE with *E. oleracea* showed improvements in rat testicles affected by cadmium toxicity via upregulated reproductive gene expression and its antioxidant effects.

**Introduction**

Infertility stands as a global concern jeopardizing couples worldwide; several factors contribute to about 40%–50% of male infertility, encompassing sluggish sperm motility, a lowered count of sperm, and heightened sperm morphology defects. Lifestyle (smoking and drinking), environmental toxins, genetic variants, and pathological conditions contribute to sperm deoxyribonucleic acid (DNA) fragmentation due to oxidative stress and apoptosis. Environmental toxins are the main causes of infertility; cadmium “Cd” is a hazardous heavy metal in the environment; it is classified as the seventh of the ten general toxic compounds to human health according to the ATSDR ranking. Cd is used in rubber processing, steel plates, batteries, cosmetics, glass, plastic pigments, pesticides, and many industrial products. It is absorbed in large quantities from air pollution, contaminated water, food, soil, and cigarette smoke, causing tissue toxicity such as respiratory, reproductive, skeletal, and cardiovascular toxicity in animals and humans, as well as being considered a carcinogenic substance for humans by an international agency for cancer research.

Among the deleterious characteristics of cadmium is that it can accumulate in the liver, heart, kidney, brain, testis, and lungs and alter their functions, so it is considered an accumulative toxicant due to its lower excretion...
rate and long biological half-life [5,6]. Cadmium can alter the function and structure of the testis, causing testicular degeneration and toxicity that are well described in [6]. Cd can cause damage to the testicular blood barrier and cause the loss of seminiferous epithelia, interstitial cells, and germinal cells, in turn resulting in reduced testosterone levels, impaired spermatogenesis, and impaired sperm functions as a consequence of Cd toxic effects in Sertoli cells and Leydig cells [7].

Several researchers attempted to counteract the testicular toxicity of cadmium, focusing on the natural products and phytotherapy that have antioxidant properties, such as curcumin [8] and Artocarpus altillis [9]. Cyperus esculentus (tiger nut, nut grass, or chufa sedge) is an herbaceous plant belonging to the Cyperaceae. It is an ancient Egyptian plant; its rhizomes can be grown from tubers and the base of tiger nuts [10]. C. esculentus has several beneficial effects due to its potential biological components, such as alkaloids, sterols, flavonoids, polyterpenes, tannins, polyphenols, salicylic acid, terpenoids, and other phytochemical contents [11]. The tubers are considered a good source of vitamins B (B12, B9, B5, B2, B1, and B6) and minerals like iron, sodium, magnesium, calcium, and zinc [12].

Tiger nuts can be used to treat dysentery, indigestion, and flatulence, which extends into interference with colon cancer [12]. Several studies have reported the activity of the aqueous extract of tiger nut on kidney, adrenal gland, liver, and testis functions; this action can be attributed to the large quantities of antioxidants in C. esculentus, which exhibit effects in combating hepatic, renal, and testicular problems caused by toxic substances like carbon tetrachloride [13–15]. Furthermore, another study by Udoh et al. [16] illustrated the ability of the aqueous extract of C. esculentus to reverse the testicular damage triggered by radiation and enhance spermatogenesis and steroidogenesis [16].

Euterpe oleracea fruit, commonly known as an acai, is a potent antioxidant plant due to anthocyanins, phenolic acid, and flavonoids. The pulp and seeds of acai contain various quantities of phytochemicals; acai seeds contain 28.3% polyphenols (oligomeric, polymeric, proanthocyanidins, epicatechin, and catechin), 2% lipids, 5% protein, and 65% fiber [17]. The acai pulp contains 9% anthocyanin, 11% lignoids, 31% flavonoids, and 25.5% polyphenol [17]. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside are the major anthocyanins responsible for fruit color [18].

Evidence leans toward supplementing acai, which could suppress reactive oxygen species (ROS, generation in morphonuclear cells and reduce cerebral and pulmonary oxidative stress [19,20]. E. oleracea has antioxidant activity, which reduces sperm DNA fragmentation and enhances fertility [21].

Studies have investigated the deleterious effects of cadmium chloride (CdCl₂) on testicular tissues, and the ameliorating effects of E. oleracea or CHE when administered individually, as illustrated by Udefa et al. [22] reported the effect of C. esculentus on sperm functions and the antioxidant activity after exposure to lead acetate; whereas Muro et al. [23] investigated the antioxidant role of E. oleracea on testicular tissues after exposure to cadmium, but their reports do not show their additive effects when given together, as well as those reports do not investigate the possible mechanisms by which both C. esculentus and E. oleracea can be improved testicular functions at the gene expression level. Based on these limitations, the current study was designed to explore the possible mechanism(s) at the hypothalamic-pituitary-testicular axis by which E. oleracea with CHE can improve and enhance testicular functions in CdCl₂-treated rats.

Material and Methods

Ethical approval

The ethical approval for the current experiment was obtained from the ethics committee of Veterinary Medicine at Al-Qasim Green University and approved to guide the care and use of laboratory animals (ESCVM, No. 1192022).

Preparation of tiger nut hydroethanolic extract

The tubers of C. esculentus were obtained from the Babylon market for herbal medicine in Babylon province. The hydroethanolic extract was prepared according to the established methodology [32]. 500 gm of tubers were ground and then dissolved in 5,000 ml of hydro-ethanolic solution (7:3 ethanol: H₂O) for 48 h at room temperature. The solution was filtered and concentrated by a rotary evaporator at 45°C and then kept at 4°C in the refrigerator. The percentage yield of the extract was 163.5 gm.

Acute toxicity “LD₅₀” of CHE

The acute toxicity of the hydroethanolic extract of C. esculentus, CHE, was detected according to the Lorke method [24] with two phases. In the initial trial, initial trial (INT) phase, nine male rats were allocated equally into three groups and administered C. esculentus extract at doses of 10, 100, and 1,000 mg/kg BW. Rats were manifested for 48 h for mortality and any toxicity signs. Depending on the results of the INT stage, the second phase comprises 16th male rats, which are divided into four equal groups and receive orally a graded dose of CHE (1,250, 2,500, 3,750, and 5,000 mg/kg) and monitor the clinical signs and mortality every 4 h for 24 h. This method uses geometric means to determine the lowest lethal dose and the highest nonlethal dose.

**Analysis of sperm function**

To determine the count of sperm, the epididymal tail was harvested and cut into tiny incisions with 2 ml of saline at 37°C, and one drop of semen suspension was put in a Neubauer chamber to calculate the count according to a previously outlined protocol [26]. The sperm motility was assessed by putting semen suspension on the slide, covered with a glass slip, and manifested by a light microscope, as described in [28].

The abnormality of sperm was determined using the approach detailed by [29] via placing a drop of epididymal suspension and smearing after that, stained with eosin-nigrosine, then manifested under a microscope. The live and dead sperm were calculated by placing a drop of suspension in the slide, mixed with eosin-nigrosine, and calculated under a microscope; the live sperm heads did not retain stain, whereas those dead were stained [30].

**Determination of hormones, antioxidants, and oxidant parameters**

Blood samples were collected at the end of the experiment and centrifuged at 5,000 rpm for 10 min to obtain sera to determine biochemical concentrations [31]. Serum concentrations of LH, FSH, and testosterone were evaluated utilizing the ELISA technique described by manufacturer instruction (AFG Biosceince LLC, Co., US.). In addition, testicular malondialdehyde (MDA), nitric oxide (NO), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) were measured following established protocols for available kits (AFG Biosceince LLC, Company, US.).

**Total RNA extraction and determination of its purity**

Total Ribonucleic acids (RNAs) from rat anterior pituitary and hypothalamus tissues were extracted using a TRizol® kit (“Bioneer, Korea”). To measure the RNA purity and quantity, a nanodrop spectrophotometer, “Thermos, USA,” was utilized for this purpose.

**cDNA synthesis, preparation of master mix, and analysis of RT-qPCR**

To remove the trace quantities of DNA from eluted RNA, the total extracted RNA was treated with the DNase-I enzyme, which was consistent with the instructions explained by Promega Company, USA.

After that, total samples were converted into cDNA; the master mix was prepared depending on the instructions described by “Bioneer Company, Korea.” The primers used in the current study are illustrated in Table 1. SYBER Green dye was used to evaluate the amplification of genes by the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gnhr</td>
<td>Forward</td>
<td>“CCA GCA CGT GTC CTA TGG GT”</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>“AGA GCT CCT CGC AGA TCC CT”</td>
<td></td>
</tr>
<tr>
<td>Fshβ</td>
<td>Forward</td>
<td>“TTG CAT CCT ACT CTG GTG CT”</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>“AGC TGG GTC CTT ATA CAC CA”</td>
<td></td>
</tr>
<tr>
<td>Lhβ</td>
<td>Forward</td>
<td>“ATC ACC TTC ACC ACC AGC AT”</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>“GAC CCC CAC AGT CAG AGC TA”</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>“AAG GTC ATC CCA GAG CTG AA”</td>
<td>[32]</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>“ATG TAG GCC ATG AGG TCC AC”</td>
<td></td>
</tr>
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</table>
real-time PCR machine “Bioneer Company, Korea.” The relative changes (fold changes) of mRNA levels were estimated by ∆∆Ct method as described previously [32].

Assessment of StARs and steroidogenic enzyme activities

To determine the testicular steroidogenic enzyme activities and StAR levels, approximately 100 mg of testicular tissues were washed and homogenized separately in a solution of phosphate-buffered saline (PBS). Samples were centrifuged at 5,000 g for 5 min in a cold centrifuge at 4°C. After that, the supernatant was utilized to assess the StAR level, 3β-Hydroxysteroid dehydrogenase (3β-HSD), and 17β-Hydroxysteroid dehydrogenase activities (17β-HSD) according to the manufacturer’s instructions for rats-specific ELISA kits (Elabscience, USA).

Histological examination

Testis specimens were taken and prepared for histological study in accordance with a previously outlined method [33].

Statistical analysis

The current data were statistically performed by ANOVA-I and using statistical package for the social sciences (SPSS-16). The current results were presented as mean (M) ± standard deviation (SD). The statistical differences are significant at a level of p-value <0.05 [34].

Results

Acute toxicity of CHE

The acute toxicity results of the CHE are illustrated in Table 2. It showed that the CHE can be given up to 5,000 mg/kg BW with no vital behavioral signs or mortality in both phases.

Effects of CHE and E. oleracea on body weight and relative genital weights in male rats exposed to CdCl₂

Table 3 displays body and relative genital weights in rats exposed to CdCl₂ in various experimental groups. The IWs appeared to have non-significant (p > 0.05) variances among experimental groups that indicate the rats’ weights were matched. The FW, body weight changes (BWG), relative testis weight (RTW), and relative epididymis weight (REW) significantly declined (p < 0.05) in rats exposed to CdCl₂ compared with all experimental groups (Table 3). Interestingly, the FWs and body weight changes were significantly (p < 0.05) elevated and enhanced in TC, TO, and in rats receiving CHE with E. oleracea in comparison with the Cd group (Table 3).

The relative weights of genitalia were significantly increased (p < 0.05) in rats that received an extract of C. esculentus with E. oleracea in comparison with all experimental groups. Additionally, TC and TO showed a significant (p < 0.05) elevation in RTW and REW parameters compared with rats exposed to Cd only (Table 3).

Effects of CHE and E. oleracea on sperm parameters in adult male rats exposed to CdCl₂

The current results showed significant (p < 0.05) elevations in testicular MDA (tMDA) and tNitric Oxide levels in the Cd rats compared with the TC, TO, TCO, and control groups. When compared with all experimental groups, the TCO rats recorded a decline (p < 0.05) in testicular MDA and nitric oxide levels (Fig. 1A and B, respectively). Interestingly, the statistical analysis of current data revealed there was a significant (p < 0.05) elevation in testicular SOD (tSOD) and testicular CAT (tCAT) activities in TCO compared with other treatment groups (Fig. 1C and D). tSOD and tCAT recorded a significant (p < 0.05) raise in TC and TO in comparison with the Cd and control groups; the Cd group recorded a significant (p < 0.05) drop in these parameters compared with all experimental rats.

Table 2. Acute toxicity of Cyperus esculentus hydroethanolic extract in rats.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Number of rats used</th>
<th>Rats dead/Rats used</th>
<th>Percentage of mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>Second phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1250</td>
<td>4</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>2500</td>
<td>4</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>3750</td>
<td>4</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>5000</td>
<td>4</td>
<td>0/4</td>
<td>0</td>
</tr>
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</table>
The fold change of hypothalamic Gnrh and pituitary Fshβ and Lhβ gene expression in adult male rats exposed to CdCl₂.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>Cd</th>
<th>TC</th>
<th>TO</th>
<th>TCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>IW (g)</td>
<td>162.86 ± 4.02a</td>
<td>164.96 ± 3.89a</td>
<td>164.54 ± 4.75a</td>
<td>162.7 ± 3.99a</td>
<td>163.8 ± 5.357a</td>
</tr>
<tr>
<td>FW (g)</td>
<td>226.08 ± 3.26a</td>
<td>117.2 ± 5.019b</td>
<td>217.6 ± 1.673a</td>
<td>216.6 ± 3.782c</td>
<td>247.54 ± 2.933a</td>
</tr>
<tr>
<td>BWG (g)</td>
<td>63.22 ± 4.486a</td>
<td>-47.76 ± 4.415a</td>
<td>50.06 ± 5.591a</td>
<td>53.9 ± 5.249a</td>
<td>83.74 ± 3.959a</td>
</tr>
<tr>
<td>RTW (%)</td>
<td>1.751 ± 0.046a</td>
<td>0.682 ± 0.147e</td>
<td>1.566 ± 0.022b</td>
<td>1.507 ± 0.038b</td>
<td>2.51 ± 0.129a</td>
</tr>
<tr>
<td>REW (%)</td>
<td>0.5142 ± 0.056a</td>
<td>0.076 ± 0.011a</td>
<td>0.341 ± 0.019b</td>
<td>0.335 ± 0.033c</td>
<td>0.967 ± 0.18b</td>
</tr>
</tbody>
</table>

Values represented mean ± standard deviation. Dissimilar letters refer to significant differences (p < 0.05) among groups. C: control group, Cd: 1mg/kg b.w. of cadmium chloride, TC: Cd+ C. esculentus hydroethanolic extract (800mg/kg b.w.), TO: Cd+ E. oleracea (500mg/kg b.w.), TCO: Cd+ C. esculentus hydroethanolic extract with E. oleracea 800 and 500 mg/kg b.w., respectively.

Effects of C. esculentus hydroethanolic extract and E. oleracea on sperm parameters in adult male rats exposed to CdCl₂.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>Cd</th>
<th>TC</th>
<th>TO</th>
<th>TCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (million/ml.)</td>
<td>47.994 ±0.707b</td>
<td>22.98 ± 1.184a</td>
<td>41.80 ± 1.303a</td>
<td>41.54 ± 0.953a</td>
<td>64.94 ± 0.639a</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>73.40 ±2.408b</td>
<td>34.60 ± 3.435c</td>
<td>71.60 ± 1.14a</td>
<td>71.0 ± 1.224a</td>
<td>89.00 ± 1.870a</td>
</tr>
<tr>
<td>Live sperm %</td>
<td>71.50 ±3.162b</td>
<td>32.70 ± 4.35a</td>
<td>68.82 ± 3.15a</td>
<td>65.80 ± 4.816c</td>
<td>88.10 ± 1.511a</td>
</tr>
<tr>
<td>Dead sperm %</td>
<td>28.5 ±3.162b</td>
<td>67.3 ± 4.35a</td>
<td>31.18 ± 3.156bc</td>
<td>34.2 ± 4.816c</td>
<td>11.9 ± 1.517a</td>
</tr>
<tr>
<td>Abnormal morphology (%)</td>
<td>9.36±0.321b</td>
<td>38.62 ± 1.837a</td>
<td>11.48 ± 0.37a</td>
<td>11.98 ± 0.609b</td>
<td>7.24 ± 0.929a</td>
</tr>
</tbody>
</table>

Values represented mean ± standard deviation. Dissimilar letters refer to significant differences (p < 0.05) among groups. C: control group, Cd: 1mg/kg b.w. of cadmium chloride, TC: Cd+ C. esculentus hydroethanolic extract (800mg/kg b.w.), TO: Cd+ E. oleracea (500mg/kg b.w.), TCO: Cd+ C. esculentus hydroethanolic extract with E. oleracea 800 and 500 mg/kg b.w., respectively.

Testicular GPx (tGPx) activity exhibited a significant (p < 0.05) increment in TCO rats compared with all experimental groups. The results of tGPx activity reported a significant (p < 0.05) decline in Cd rats compared with all treatment groups (Fig. 1E).

Effects of CHE and E. oleracea on reproductive hormones in adult male rats exposed to CdCl₂.

The levels of serum FSH, LH, and testosterone were significantly (p < 0.05) elevated in the TCO group in comparison with the Cd, TC, TO, and control groups, whereas their concentrations were significantly (p < 0.05) lowered in the Cd group compared with other treatment groups. FSH, LH, and testosterone levels were enhanced significantly (p < 0.05) in TC and TO rats compared with the Cd rat (Fig. 2A–C).

Effects of C. esculentus hydroethanolic extract and E. oleracea on StAR steroidogenic enzyme activities in adult male rats exposed to CdCl₂.

In the Cd-treated group, the testicular level of StAR protein and the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-Hydroxysteroid dehydrogenase activities (17β-HSD) were significantly (p < 0.05) declined relative to TCO, TO, TC, and control groups (Fig. 4A–C). Intriguingly, the StAR protein level and steroidogenic enzyme activities were significantly (p < 0.05) increased in the TCO group compared to the TO, TC, Cd, and control groups (Fig. 4A–C), respectively.

Histopathological changes

The testicular sections of the control group appeared to have a typical architecture without histological alteration (Fig. 5A), with the normal appearance of seminiferous epithelia, Sertoli cells, and interstitial cells with series spermatogenesis recorded. On the contrary, the testicular sections of rats exposed to cadmium showed congestion in interstitial capillaries with damage to the seminiferous tubules and Sertoli cells, with degenerative changes...
accompanied by the complete absence of spermatozoa and reduced spermatogenesis in degenerated tubules (Fig. 5B). The testicular sections of rats receiving cadmium and *C. esculentus* or *E. oleracea*, TC, and TO groups revealed that the seminiferous tubules return to normal appearance with moderate spermatogenesis (Fig. 5C and D). The testicular sections obtained from the TCO group appear to have a typical architecture similar to that shown by control rats (Fig. 5E), with a typical structure of seminiferous tubules and interstitial cells. Primary and secondary spermatocytes with series spermatogenesis were evident.

**Discussion**

The current experiment has been carried out to compare and evaluate the additive effects of the hydroalcoholic extracts of *C. esculentus* and *E. oleracea* on CdCl$_2$-induced testicular degeneration in adult male rats. The present study showed that exposure to CdCl$_2$ caused a lowering in FW, body weight changes, as well as the relative weight of the epididymis and testis, due to the ability of CdCl$_2$ to suppress the digestion process and absorption via inhibition of the digestive enzymes [35], which in turn reduced feed efficacy and nutrient absorption. Additionally, CdCl$_2$...
can induce oxidative stress and cellular DNA damage that negatively reflects on body weight and testicular and epididymal relative weight [35].

However, the current results found a significant enhancement in FWs, body weight changes, testis, and epididymal relative weights in TC, TO, and TCO rats that can be attributed to the active biological components of each *E. oleracea* and/or *C. esculentus* can ameliorate the adverse effects of CdCl₂ via protecting cells membrane and enhancing functional and structural proteins biosynthesis causing to improve body organs functions [21,36], previous studies reported elevation in body weight changes resulting from enhanced the appetite, food conversion rate, and some digestive enzymes like amylase and lipase in rats give *C. esculentus* extract/or oil and *E. oleracea* [21,36]. Additionally, these effects in TCO rats might be attributed to an elevation of androgen synthesis that significantly affects testicular and epididymal relative weights in rats, which agrees with previously reported results [19,37].

Sperm is sensitive to ROS production due to its membrane components of polyunsaturated fatty acids, leading to sperm dysfunctions, decreased sperm motility, and infertility; this is in line with another study that has concluded that CdCl₂ caused defects in sperm production, morphology, viability, and decline in steroidogenic and antioxidant enzymes [38] via induced oxidative stress and lipid peroxidation leading to damage in protein and DNA of sperm, degeneration and damaged of testicular cells [39], the current results were consistent with prior research [40] who found a decline in sperm functions after exposure to CdCl₂ for 15–30 days which occurred due to generate large quantities of ROS that reduced antioxidants synthesis thereby resulting in lipid peroxidation in testicular tissues causing harmful effects on membrane integrity of spermatogonia and spermatozoa and spermatogenesis disruption [40].

At the same time, accordance to current results, *C. esculentus* hydroethanolic extract with *E. oleracea* “CHE-EO” can demonstrate an additive effect at the specified dosages; however, their effectiveness is evident when administered
individually in comparison to the control, due to their abilities to improve the sperm functions and reduce the adverse effects of exposure to CdCl₂ as reduced the dead and abnormal sperm with increasing motility, count, live sperm and decrease abnormal sperm in CdCl₂-CHE-E0-treated rats that contributed to the ability of anthocyanin, major natural antioxidant of *E. oleracea*, to lower CdCl₂ accumulation in blood and testis, suppress the oxidative stress and reduce the sperm deformities, that consistent with finding of [37], as well as, administrated *C. esculentus* extract with *E. oleracea* can enhance and improve the testicular antioxidant defense system which aid in protection of cell membranes from oxidative damage due to their contents from quercetin, zinc, vitamins E and C that protect DNA of sperm from ROS and oxidative stress [41], besides, *E. oleracea* with *C. esculentus* extract could induce the signal alteration between spermatozoa and calcium that improve sperm movements [22,23] that positively affect sperm motility in TCO treated rats.

The statistical analysis of current results recorded a significant elevation in tMDA and nitric oxide “tNO” with a reduction in testicular SOD, GPx, and CAT in rats administered CdCl₂ which is regarded as a toxic damage marker in testis. These results align with the previous study [7], which attributed the ability of CdCl₂ to damage cell membranes, causing pathological alteration and oxidative stress resulting from excessive production of ROS and nitrogen radicles, which in turn impaired the DNA, proteins, and lipids of testicular cells [40].

Cadmium can cause testicular toxicity via GSH exhaustion, and chelating proteins contain amino groups or hydroxyl and sulfhydryl groups; moreover, CdCl₂ can alter protein biosynthesis by competing and displacing the essential minerals and metals, resulting in lipid
peroxidation leading to an increasing MDA level [8]. The elevation of testicular NO in rats receiving CdCl₂ results from an upregulated eNOS level that affects the biosynthesis of antioxidants, suppresses synthesis, and releases testosterone from Leydig cells [39]. In current results, the activities of tSOD, tGPx, and CAT declined in rats exposed to CdCl₂ due to the ability of Cd to interact with iron, which led to iron deficiency because iron is considered a structural building block of the CAT active site. Besides, CdCl₂ can interact with divalent ions such as zinc, copper, and manganese, which led to a decline in testicular SOD [40].

Interestingly, the current results revealed a significant elevation in the activities of the antioxidant enzyme in rats that received both C. esculentus hydroethanolic extract and E. oleracea, which may be attributed to their ability to induce the synthesis of the antioxidants with diminished NO and MDA levels, as well as the additive effects of phytochemicals in C. esculentus hydroethanolic extract and E. oleracea like flavonoids, anthocyanins, quercetin, phenolic acid, vitamin C, and vitamin E that potentially enhance the antioxidant system in rats [21,42].

Furthermore, the phenolic compounds in these extracts can act as a donor of one atom of hydrogen or an electron to neutralize the reactive species, reducing oxidative stress, MDA, and NO levels [43]. A recent study [44] reported the role of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside of açaí hydroethanolic extract to upregulate CAT, GPx, and SOD, in turn reducing ROS and reactive nitrogen species (RNS).

Cadmium exposure in rats caused a significant decline in LH, FSH, and testosterone concentrations, suppressed the levels of the pituitary Fshβ, Lhβ gene, and hypothalamic Gnrh gene, and caused histopathological alteration of testicular tissues. A decrease in these hormone concentrations is an indication of spermatogenesis disruption. CdCl₂ is considered a toxic substance for the brain; it accumulates in pituitary and hypothalamic tissues, causing deformity and damage in hypothalamic and pituitary cells, leading to disruption of gene expression of pituitary Gnrhr, Fshβ, and Lhβ, and hypothalamic Gnrh [41], thus affecting FSH and LH secretion as observed in Cd exposure rats (Fig. 2A and B). The drop-in serum FSH concentration in rats exposed to CdCl₂ could have been causing a nutrient deficiency that is essential for sperm development and, in turn, suppresses spermatogenesis and live sperm, as observed in Cd rats [9]. Testosterone released from Leydig cells is necessary for the proliferation and growth of testicular germ cells; a decline in testosterone secretion is evidence of male reproductive toxicity [40].

Testicular toxicity, as found in histopathological changes and a reduction in testosterone level after exposure to CdCl₂ could be a reason for the decrease in spermatogenesis and sperm count [38]. In addition, Iqbal and colleagues [35] reported that cadmium caused disorganization of mitochondrial Leydig cells, a decrease in the number of Leydig cells, and damage to DNA Leydig cells, causing testicular degeneration and a decrease in testosterone secretion, as seen in Figure 5B. As well, exposure to CdCl₂ can suppress the expression of Cyp11a1, Scarb1, StAR, Hsd17b, Hsd11b1, Hsd17a1, Hsd3b, and Lhcg, which lowers testosterone levels [38].

Interestingly, upon the co-administration of CdCl₂ and C. esculentus hydroethanolic extract with E. oleracea, the levels of reproductive hormones increased significantly compared with Cd rats due to the ability of C. esculentus hydroethanolic extract to induce gene expression of pituitary Lhβ, Fshβ, Gnrhr, and hypothalamic Gnrh resulting in elevating the levels of LH and FSH which effected on Leydig cells and Sertoli cells to stimulate testosterone synthesis and spermatogenesis, respectively [37], as well as, C. esculentus hydroethanolic extract can upregulate 17β-HSD, 3β-HSD and steroidogenic acute regulatory protein (StAR) in testicular tissues, in turn, elevated testosterone levels [16,45], this effects might be attributed to the extract phytochemical components from trace elements such as zinc and vitamin C and E and antioxidant such as quercetin [46], that ameliorated the Leydig and Sertoli cells function as proved by current study (Fig. 4).

Besides, E. oleracea can recover seminiferous epithelium and normalize the Leydig cells, which is attributed to the ability of E. oleracea constituents to upregulate the StAR proteins 3β-HSD, 17β-HSD and CYP11A1 in Leydig cells that facilitate steroidogenesis and, in turn, increase testosterone levels (Fig. 4) [23,46]. Interestingly, the biological constituents of both C. esculentus hydroethanolic extract and E. oleracea act as potent factors for upregulating StAR protein and steroidogenic enzymes that positively affect spermatogenesis and androgen biosynthesis, which ameliorates histological changes in the TOC group.

**Conclusion**

The results reported in this study revealed a potential role of E. oleracea with a hydroethanolic extract of C. esculentus to treat the damage to testicular tissues induced by CdCl₂ via ameliorating the antioxidant activity that improved the hypothalamic-hypophysal-testis axis, resulting in upregulated gene expression of reproductive hormones and improved testicular steroidogenesis and spermatogenesis. Therefore, several studies are required to investigate the roles of E. oleracea and C. esculentus on gene expression of thyroid hormones in hypothyroidism and hyperthyroidism in male and female rats. This study didn’t report the anti-inflammatory effects of both E. oleracea and the hydroethanolic extract of C. esculentus, as well as their effects on gene expression of inhibin and activin hormones.


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Author contribution
The author has designed, conceptualized, analyzed, and interpreted the data and finalized the research for publication.


