Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 30 (1-2)
Sofia ● 2023

Immunochemical Evaluation of Biomarkers of Carcinogenesis, Angiogenesis, Neuro-Cancer Interactions and Demyelination in Cadmium Chloride-Induced Testicular Toxicity in Rats.

*Adelaja Akinlolu[*1], Mubarak Ameen[2], Gabriel Ebito[3], Nnaemeka Asogwa[4], Raheem Akindele[5], Bamidele Fagbohunka[6]*

[1] Department of Anatomy, Faculty of Basic Medical Sciences, Federal University of Health Sciences Otukpo, Benue State, Nigeria
[2] Department of Chemistry, Faculty of Physical Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria
[3] Department of Anatomy, Faculty of Basic Medical Sciences, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria
[4] Central Research Laboratory, Ilorin, Kwara State, Nigeria
[5] Department of Physiology, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria
[6] Department of Biochemistry, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria

*Corresponding author e-mail: adelaja.akinlolu@fuhso.edu.ng

Cadmium is a carcinogen. Neurotransmitter-cancer interaction and tissue-innervation impact cancer survival. This study examined repro-protective and neuro-protective potentials of MO11 (isolated from *Moringa oleifera* leaves) and MS06 (isolated from *Musa sapientum* suckers) in cadmium chloride ($CdCl_2$)-induced testicular toxicity. Twenty-four adult male rats were randomly divided into 6 groups. Group 1 was control. Groups 2-4 and 6 received intraperitoneal single-dose of $CdCl_2$ (Day 1). Groups 3, 4 and 6 were post-treated with MO11-dose, MO11+MS06-doses and Doxorubicin-dose respectively, while Group 5 received Olive Oil-dose (vehicle) from Days 1-17. Quantitative tissue enzyme-linked-immunosorbent-assays of biomarkers of carcinogenesis and neuro-cancer interaction in testicular homogenates were evaluated. Data were analysed using Mann-Whitney-U test ($p \leq 0.05$). Results showed downregulations of MBP, Caspase-3 and sVEGFR, but upregulations of Dopamine, Glutamate and Cytochrome-p450 in Groups 3, 4 and 6, compared with Group 2. Overall, $CdCl_2$-induced testicular toxicity, angiogenesis and neuro-cancer interaction were ameliorated by post-treatments with MO11 and MS06.

Key words: Cadmium, testicular toxicity, drug candidates, neuro-protection, anticancer effects

Introduction

Cadmium (Cd) is one of the 10 chemicals of concern for human health and a human carcinogen as categorized by the World Health Organization, National Toxicology program and the International Agency for Research on Cancer [7, 16, 37]. Commercially, Cd is used in home appliances such as television screens, lasers, batteries, paint pigments and cosmetics [8], hence Cd is an environmental toxin of concerns. Cd is equally an established carcinogen in animals [7, 16, 37]. Cd-exposure resulted in decreased testicular weight [7, 30], necrosis of spermatogenic epithelium and degenerations of testicular seminiferous tubules [7], depletion of germ cells, testicular necrosis, carcinogenesis and sterility in *in vivo* models, and adverse effects on testicular cells in *in vitro* models [1, 33]. Specifically, Cd-induced testicular toxicity resulted in significant decreases of sperm count, sperm motility, testosterone synthesis, spermatogonia, Sertoli cells and Leydig cells in rat testis [23, 40]. In addition, human Cd-exposure was linked with nervous system dysfunctions resulting in impaired learning capacity, headache and vertigo, decreased cognitive functions, olfactory dysfunction, poor vasomotor functioning, peripheral neuropathy, poor equilibrium and balance coordination, and developments of neuro-degenerative diseases (such as Parkinson's disease and Alzheimer's disease) [16].

Neurotransmitters (such as Dopamine and Glutamate) released by nerve fibres within tumour micro-environment influence cancer metastasis via complex neurotransmitter-cancer interactions; and have become targets for therapeutic interventions [19]. Both Dopamine [29] and Glutamate [35] are expressed in the testis. Myelination is required for proper functioning of nerve fibres supplying tissues, and Myelin Basic Protein (MBP) is a biomarker of de-myelination [3]. The mechanism underlying metastasis remains poorly understood. Hence, the biology of innervation of tissues is relevant in the search for anticancer drugs from plants or other sources.

Moringa oleifera (MO) and *Musa sapientum* (MS) are ethno-medicinal plants which are well grown across the world [5]. 'MOF6' is fractionated from ethanolic extract of MO leaves. *In vivo* neurobiological analyses showed that MOF6 possessed significant antioxidant and neuro-protective potentials against Cuprizone-induced cerebellar damage in rats [28], and ameliorated Sodium arsenite-induced neurotoxicity and dysregulations of Acetylcholinesterase concentrations in rats [4]. Furthermore, MOF6 and 'MSF1' (fractionated from ethanolic extract of MS suckers) exhibited hepato-protective and anticancer potentials in 7,12-Dimethylbenz[a]anthracene-induced hepato-toxicity in rats [5].

Will Cd-induced toxicity and decreased testicular weight of the testes change testicular levels of biomarkers of Dopamine and Glutamate? Is de-myelination associated with Cd-induced toxicity and decreased testicular weight? Is angiogenesis associated with Cd-induced toxicity and decreased testicular weight? What are the effects of post-treatments with MO and MS on possible mechanisms underlying Cd-induced toxicity and decreased testicular weight?

Single intraperitoneal administration of 1.5 mg/kg body weight of $CdCl_2$ resulted in significantly decreased diameters of seminiferous tubes and thickness of the germinal layer, and decreased numbers of spermatogonia, Sertoli and Leydig cells in rat testis compared with control group which received sterile distilled water on Days 13, 25 and 49 [23]. In addition, single intraperitoneal administration of $CdCl_2$ resulted

in significantly decreased sperm motility, sperm count and testosterone levels in the $CdCl_2$-only treated group compared to control group on Days 13, 25 and 49 in rats [23]. Furthermore, there was significantly increased level of Malondialdehyde in the $CdCl_2$-only treated group compared to control group on Days 13, 25 and 49 in rats [23]. Sustained increased Malondialdehyde level implied increased oxidative stress which is implicated in carcinogenesis [3, 22].

Toxin-induced cyto-toxicity resulting in decreased tissue or organ weight is associated with necrosis which is further associated with carcinogenesis [22]. Cytochrome p450 plays a role in steroidogenesis [34], and it is an established biomarker of drug metabolism and carcinogenesis [29]. Caspase-3 plays regulatory roles in spermatogenesis [36], and it is a biomarker of apoptosis and carcinogenesis [3]. In addition, sVEGFR is opined to be a biomarker of testicular inflammation and toxicity [14], and it is an established biomarker of angiogenesis [23] while MBP is a biomarker of myelination [3]. Furthermore, Dopamine and Glutamate are neurotransmitters of importance in evaluation of neuro-cancer interactions [19], Cadmium exists as a divalent cation, complexed with other elements, such as cadmium chloride ($CdCl_2$) [7, 8, 16, 37]. Therefore, in-order to answer stated research questions, this study examined the mechanisms underlying *in vivo* $CdCl_2$-induced testicular toxicity and the ameliorative potentials of MO11 (isolated from *Moringa oleifera* leaves) and MS06 (isolated from *Musa sapientum* suckers) on tissue levels of Cytochrome-p450, Caspase-3, sVEGFR, MBP, Dopamine and Glutamate in the testes of adult male rats in $CdCl_2$-induced testicular toxicity.

Materials and Methods

Ethics approval

Ethical approval for this study was sought and received from the Ethical Review Committee of the University of Ilorin, Nigeria. Appropriate measures were observed to ensure minimal pain or discomfort of rats used in this study. The ethical approval number is UERC/ASN/2018/1161. Furthermore, this research study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as provided in the European Community guidelines (EEC Directive of 1986; 86/609/EEC), the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the Guidelines of the U.S. Public Health Service and NIH regarding the care and use of animals for experimentation (NIH publication #85-23, revised in 985).

Authentication and deposition of MO leaves and MS suckers

Freshly cut MO leaves and MS suckers were obtained from forest reserves in Ilorin, Kwara State of Nigeria. The plants' samples were authenticated and assigned Herbarium Identification Numbers: UILH/001/1249 and UILH/002/1182 respectively at the Department of Botany of study institution.

Evaluations of antioxidant and antimicrobial activities of MO and MS fractions

Antioxidant activities of plants' extracts were evaluated using modified 2,2- diphenyl-1-picrylhydrazyl method as previously described [9], Antimicrobial activities of plants' extracts were evaluated by testing cyto-toxic potentials of each fraction against growths of *Escherichia coli* and *Salmonella tiphimurium* as previously described [12].

Extractions of MO11 and MS06 from MO leaves and MS suckers

MO11 and MS06 were extracted as final therapeutic isolates from MO leaves and MS suckers following series of antioxidant analyses, antimicrobial cyto-toxicity potentials, column chromatography and Liquid chromatography-mass spectrometry as previously reported [5, 6].

Animals

Twenty-four (24) adult male Wistar rats (average weight of 155 g and 2 months of age) were purchased from a colony breed at Badagry in Lagos state, Nigeria. The rats were acclimatized for a week, and randomly divided into 6 groups with 4 rats per group. The rats were kept under standard conditions. The body weights of rats in grams were computed on daily bases using electronic SF-400C compact weighing scale (Valid Enterprise, Mumbai, India).

Experimental design

MO11 and MS11 were dissolved in Olive Oil (vehicle). Rats of Control Group 1 received physiological saline only for 17 Days (Days 1 – 17). The dose of 1.5 mg/kg body weight $CdCl_2$ was used to induce testicular toxicity as determined from a previous study which investigated the effects of low dose of $CdCl_2$ on the testis [23].

Each rat of Experimental Groups 2 – 4 and 6 received single intra-peritoneal administration of 1.5 mg/kg body weight $CdCl_2$ (Sigma-Aldrich, Japan Co.) on Day 1. Rats of Group 2 (Toxic Control) were left untreated throughout experimental procedure for 17 Days (Days 1 – 17). Thereafter, rats of Group 3 were post-treated with oral administration of 15 mg/kg body weight of MO11 for 17 Days (Days 1 – 17). Rats of Group 4 were post-treated with oral administration of combined mixture of 15 mg/kg body weight of MO11 and 7 mg/kg body weight of MS06 for 17 Days (Days 1 – 17). Rats of Group 5 received only oral administration of 1 ml/kg body weight of Olive Oil (vehicle) for 17 Days (Days 1 – 17), and were not exposed to administration of $CdCl_2$. Rats of Group 6 were post-treated with oral administration of 3.35 mg/kg body weight of Doxorubicin (standard anticancer drug – Positive Control) for 17 Days (Days 1 – 17).

Completion of experimental procedures

No anesthesia were used for animal sacrifice as approved by the University Ethical Review Committee after evaluation of experimental protocol based on the fact that the biomarkers to be examined include enzymes such as Caspase-3 and metabolic agents such as Cytochrome p450, which may be endogenously altered by anesthetic agents requiring post-experimental control of confounding factors. Hence, the rats were sacrificed by cervical dislocation as previously applied [28].

Morphometric analyses of Gonado-Somatic Index (GSI)

The testes of each rat were excised and separately weighed in grams. GSI was computed for each rat using the formula: Gonads Weight (g)/Body weight (g) × 100 [32].

Tissue-ELISA analyses of levels of Dopamine, Glutamate, Myelin Basic Protein (MBP), Cytochrome p450, Caspase-3 and sVEGFR in the testes

The excised and isolated testes of rats of Groups 1–6 were thoroughly homogenized using porcelain mortar and pestle in ice-cold 0.25 M sucrose. 1 g of testicular tissue was homogenized in 4 ml of 0.25 M sucrose solution. The tissue homogenates were additionally filled up to 5 ml with sucrose in a 5 ml serum bottle. Testicular homogenates were consequently centrifuged at 3000 revolution per minute for 15 minutes using a centrifuge (Model 90-1). The supernatant was collected with Pasteur pipettes and placed in a freezer at -20°C, and thereafter assayed for concentrations of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR in the testes of all rats of Control Group 1 and Experimental Groups 2 – 6 using ELISA technique as previously described [5]. ELISA kits were products of CUSABIO Technology LLC, Houston, USA). AgileReader™ ELISA plate reader was employed with absorbance read at the wavelength of 450 nm.

This Tissue-ELISA assay uses the quantitative sandwich enzyme immunoassay method. Dopamine, Glutamate and Myelin Basic Protein specific antibodies were pre-coated onto the microplate. Samples and Standards were tuneled into the wells while presenting Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR were bound by the immobilized antibody. A biotin-conjugated antibody specific for each of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR was added to the wells following removal of unbound substances. Thereafter, Avidin conjugated Horseradish Peroxidase was transferred into the wells after washing. A substrate solution was introduced into the wells resulting in colour development in relationship with the quantity of each of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR bound in the initial step following removal of any unbound avidin-enzyme reagent via washing. Finally, colour intensity was measured and the development of colour was stopped. The mean absorbance against the protein concentration was plotted and the curve best fitting the standard result was drawn, and the absorbance of samples were interpolated to the curve to calculate the concentration for each biomarker.

Data analysis

Computed data of concentrations of each biomarker was expressed as arithmetic means ± standard error of mean. Mann-Whitney U test (Wilcoxon-Mann-Whitney Test, 2016) was used for statistical comparison of the concentration of each biomarker between two groups. Significant difference was confirmed at 95% confidence interval with associated p value of less than 0.05 ($p \leq 0.05$).

Results

GSI: Group 2 versus Groups 1 and 3 – 6

Results showed significant lower ($p \leq 0.05$) levels of GSI in rats of Group 2, when compared with Group 1. In addition, results showed statistically non-significant lower ($p \geq 0.05$) levels of GSI in rats of Group 2, when compared with Groups 3 – 6 (**Table 1**).

Concentrations of Dopamine, Glutamate and MBP in testes of rats

Results showed statistically significant lower ($p \leq 0.05$) levels of Dopamine and Glutamate, but statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Control Group 1 (**Table 1**). Furthermore, results showed statistically non-significant lower ($p \geq 0.05$) levels of Dopamine, but statistically significant lower ($p \leq 0.05$) levels of Glutamate in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. In addition, results showed statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. Results showed statistically non-significant lower ($p \geq 0.05$) levels of Dopamine, but statistically significant lower ($p \leq 0.05$) levels of Glutamate in homogenates of testes of rats of Group 2, when compared with Group 6. In addition, results showed statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Group 6.

Concentrations of Cytochrome p450, Caspase-3 and sVEGFR in testes of rats

Results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450, but statistically significant higher ($p \leq 0.05$) levels of Caspase-3 and sVEGFR in homogenates of testes of rats of Group 2, when compared with Control Group 1 (**Table 2**). Furthermore, results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450 in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. In addition, results showed statistically significant higher ($p \leq 0.05$) levels of Caspase-3 and sVEGFR in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. Results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450 in homogenates of testes of rats of Group 2, when compared with Group 6. In addition, results showed statistically significant higher ($p \leq 0.05$) levels of Caspase-3, but insignificantly higher ($p \geq 0.05$) levels of sVEGFR in homogenates of testes of rats of Group 2, when compared with Group 6.

Discussion

GSI is used in the assessment of increase or decrease of testicular weight. Results of this study showed significant reduction of GSI in rats of $CdCl_2$-treated only Group 2, when compared with Normal Saline-only treated Control Group 1 and Groups 3, 4 and 6 (**Table 1**). This observation suggests that $CdCl_2$-exposure resulted in shrinkage of the testes with associated significant reduction of GSI and testicular weight in rats. The findings of this study are in agreement with those of previous studies which reported that Cd-exposure resulted in decreased testicular weight in animal models [1, 33].

Post-treatments with MO11, MS06 and Doxorubicin resulted in non-significant increase of GSI in rats of $CdCl_2$-exposure + MO11 post-treated Group 3, $CdCl_2$-exposure + MO11 + MS06 post-treated Group 4 and $CdCl_2$-exposure + Doxorubicin post-treated Group 6, when compared with $CdCl_2$-only treated Group 2 (**Table 1**). These observations suggests that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced decreased weight of the testes.

Dopamine is involved in regulations of arousal, motor control, motivation, reinforcement, reward, sexual gratification, nausea and lactation [27]. Dopamine-induction of apoptosis occurred via Cytochrome C/Caspase-dependent pathway and Dopamine possessed the capability to inhibit tumour growth [19]. However, Dopamine levels are downregulated in tumours [17, 37]. In addition, Cd-exposure resulted in decreased Dopamine levels in rats [13, 15] with accompanied motor dysfunctions [15], low energy, lack of motivation and depression [13].

Furthermore, Glutamate is the major excitatory neurotransmitter of the central nervous system, and it is at the cross-road of several metabolic pathways and could cause excito-toxicity when excessively excited. Hence, too little or too much Glutamate is harmful to the body system requiring cells to have the right glutamate-sensitivity, withstand normal glutamate-stimulation and remove Glutamate at normal rates from the right places [38]. Metabolism-related genes are mutated in cancers, making cancers to be glutamate-dependent. Hence, dysregulation of glutamate levels promotes tumour growth [39].

The findings of this study showed significant downregulations of Dopamine and Glutamate in Group 2, when compared with Control Group 1 (**Table 1**). These observations are in agreement with previously reported Cd-induced downregulations of Dopamine [15] and Glutamate [18] in rats. Both Dopamine [29] and Glutamate [35] are expressed in the testis. Hence, Cd-induced downregulations of Dopamine and Glutamate in the testes of rats of Group 2 possibly implied adverse effects on neurotransmitter levels required for normal innervation of the testes.

Post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted in upregulations of Dopamine and Glutamate, when compared with Group 2 (**Table 1**). Hence, these observations suggest that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced dysregulations of Dopamine and Glutamate in the testes, and possess testicular-protective and neuro-protective potentials.

MBP is a membrane actin-binding protein and the second most abundant protein of myelin after proteolipid protein. It transmits extracellular signals to tight junctions of myelin and to the cytoskeleton of oligodendrocytes [26]. Astrocytes' depletion result in breach of the glial-limiting membrane, Schwann cells' invasion for myelin sheath repair, dissociation of MBP from the plasma membrane and consequent loss of myelin sheath (de-myelination) in response to axonal degeneration and consequent oxidative stress [2]. Therefore, MBP-upregulation is associated with demyelination [16].

Will $CdCl_2$-induced testicular toxicity be associated with demyelination via increased MBP levels of the testis? Results showed non-significant upregulation of MBP levels in the testes of Group 2, when compared with Control Group 1 (**Table 1**). This observation suggests that Cd-induced toxicity did not result in evident de-myelination of nerve fibres supplying the testes of rats of Group 2. In addition, post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted

in non-significant decrease of MBP levels, when compared with Group 2 (**Table 1**). These observations suggest no significant changes in MBP levels either with $CdCl_2$-exposure or further post-treatments with MO11, MO11+MS06 and Doxorubicin.

Cytochrome p450 (CYPs) are monooxygenases that oxidize fatty acids, steroids and xenobiotics thereby enhancing the water-solubility and expulsion of foreign compounds. Cytochrome p450 thus plays regulatory roles in the clearance of drugs and compounds, detoxification of drugs and xenobiotics, vitamin D metabolism, synthesis of cholesterols and hormones, cellular metabolism and homeostasis [25, 31]. Cytochrome p450 is involved in activation/inactivation of carcinogen as well as activation/inactivation of anticancer drugs, and clearly plays strong roles in cancer therapy [25]. Furthermore, Cytochrome p450 promotes steroidogenesis [34].

Will $CdCl_2$-induced testicular toxicity result increase or decrease Cytochrome p450 levels in the testes? Results showed significant downregulations of Cytochromes p450 levels in the testes of Group 2, when compared with Control Group 1 (**Table 2**). This observation suggests that the previously reported Cd-inductions of depletion of germ cells, testicular necrosis and carcinogenesis [7, 30, 33] in *in vivo* models as well as significant decreases of sperm count, sperm motility, testosterone synthesis, spermatogonia, Sertoli cells and Leydig cells in rat testis [23, 40] may involve adverse effects on testicular levels of Cytochrome p450.

Post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted in significant increase of Cytochrome p450 levels, when compared with Group 2 (**Table 2**). These observations suggest that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced decreased levels of Cytochrome p450 in rat testes.

Caspase-3 is the major executioner protease amongst the reported 14 caspases implicated in the human apoptotic pathway mechanism [36]. Hence, the resolution of cytotoxicity via apoptosis involves the activation of Caspase-3 in both the intrinsic mitochondrial pathway and the extrinsic death-receptor pathway of apoptosis [16, 22]. Furthermore, the several mitotic divisions and clonal expansion of germ cells in spermatogenesis require apoptotic control mechanism to match the number of germs cells with functional capacity of available number of nursing Sertoli cells [36]. Hence, over-activation of Caspase-3 in the testis may drive depletion of germ cells.

Will $CdCl_2$-induced testicular toxicity result in upregulation of Caspase-3 levels in the testis of rats? Results of this study showed significant upregulations of Caspase-3 in the testes of Group 2, when compared with Control Group 1 (**Table 2**). These observations suggest that previously reported Cd-induction of depletion of testicular germ cells, Leydig cells and Sertoli cells [7, 23, 30, 33] may involve over-activation of Caspase-3 levels in the rat testis.

In addition, Cd is an established carcinogen [1, 8, 30, 33], while angiogenesis is a significant component of carcinogenesis and consequent associated metastasis [7, 37]. VEGF is an established angiogenic factor, and abnormal VEGF upregulation is associated with increased angiogenesis [25]. Furthermore, VEGF upregulation is a promising biomarker of testicular inflammation [14].

Will $CdCl_2$-induced testicular toxicity result in upregulation of sVEGFR levels in the testis of rats? Results of this study showed significant upregulation of sVEGFR in the testes of Group 2, when compared with Control Group 1 (**Table 2**). These observations suggest that Cd-induction of testicular toxicity is possibly associated with increased angiogenesis and inflammation in the testes of rats of Group 2.

Post-treatments with MO11 and MO11+MS06 in Groups 3 and 4 respectively resulted in significant downregulation of sVEGFR levels, when compared with Group 2 (**Table 2**). These observations suggest that MO11 and MS06 ameliorated $CdCl_2$-induced testicular associated angiogenesis and inflammation, and possess anticancer potentials.

Contrari-wise post-treatments with Doxorubicin in Group 6 resulted in non-significant downregulations of Caspase-3 and sVEGFR levels, when compared with Group 2 (**Table 2**). These observations suggest that Doxorubicin ameliorated $CdCl_2$-induced testicular angiogenesis. However, Doxorubicin possesses lesser anti-angiogenesis potentials when compared with MO11 and MS06. In addition, the findings of this study showed no adverse effects of Olive Oil (the vehicle used to dissolve MO11 and MS06) on weight of the testis and levels of biomarkers of drug metabolism, carcinogenesis, angiogenesis and neuro-cancer interactions, when compared with $CdCl_2$-only treated group.

Which factor underlies the repro-protective potentials of MO11 and MS06 as observed in this study? Spectroscopic analyses showed the presence of Glutamic acid, Guanine, Phenylalanine, Leucine, and other anticancer compounds in MO11 and MS06 isolates [6]. Glutamic acid [11], Guanine [10], Phenylalanine [21, 41], and Leucine [20] are established anti-inflammatory, anti-cancer and antioxidant compounds. Therefore, Leucine, Glutamic acid, Guanine, Phenylalanine and other anticancer compounds in MO11 and MS06 isolates could have been responsible for their observed repro-protective and anticancer potentials.

Conclusion

Overall, the findings of this study suggest that $CdCl_2$-induced testicular toxicity resulted in downregulation of Cytochrome p450 which may possibly be associated with inhibition of steroidogenesis. In addition, $CdCl_2$-induced testicular toxicity resulted in upregulations of Caspase-3 and sVEGFR which may possibly be associated with increased testicular angiogenesis and inflammation as well as depletion of germ cells. Furthermore, study findings showed that $CdCl_2$-induced testicular dysregulation of neurotransmitters occurred via downregulations of levels of Dopamine and Glutamate in the testes of rats.

However, post-treatments of $CdCl_2$-induced testicular toxicity with MO11, MS06 and Doxorubicin conferred neuro-protection and repro-protection against $CdCl_2$-induced testicular damage via upregulations of Dopamine, Glutamate and Cytochrome p450, but downregulations of Caspase-3 and sVEGFR in the testes of rats. These observations indicate that MO11, MS06 and Doxorubicin possess neuro-protective, repro-protective, anti-angiogenesis and anticancer potentials. Hence, MO11 and MS06, are recommended for further evaluations as potential drug candidates for the treatments of $CdCl_2$-induced repro-toxicity and angiogenesis.

Acknowledgements: The Authors acknowledge the technical support of Laboratory staff members of the Departments of Anatomy, Olabisi Onabanjo University, Ogun State, Nigeria and the Department of Chemistry, University of Ilorin, Nigeria, who assisted with taking care of laboratory animals and extraction of plants materials.

References

1. **Abd Elhafeez, E. M., A. H. Amal, F. H. Mohamed, M. A. Mamdouh.** Effects of Cadmium and/or Chromium on reproductive organs and semen profiles of male albino rats. – *Mansoura veterinary medical journal,* **20**, 2019, 3, 14-18.
2. **Afifi, O. K., A. S. Embaby.** Histological study on the protective role of Ascorbic acid on Cadmium induced cerebral cortical neurotoxicity in adult male albino rats. – *Journal of microscopy and ultrastructure*, **4**, 2016, 36-45.
3. **Akinlolu, A. A., F. A. Sulaiman, S. Tajudeen, S. K. Suleiman, A. A. Abdulsalam, N. T. Asogwa.** Cajanus cajan drives apoptosis via activation of caspase3/p53 pathway and possesses re-myelination and anti-gliosis potentials in Ethidium Bromide-induced neurotoxicity in rats. – *Nigerian journal of scientific research,* **19**, 2020a, 4, 286-293.
4. **Akinlolu, A. A., M. Ameen, T. Quadri, O. Odubela, G. Omotoso, G., R. Yahya, S. Biliaminu, M. Adeyanju, G. Ebito, J. Otulana.** Extraction, isolation and evaluation of anti-toxic principles from Moringa oleifera (MOF_6) and Myristica fragrans (Trimyristin) upregulated Acetylcholinesterase concentrations in Sodium arsenite-induced neurotoxicity in rats. – *Journal of phytomedicine and therapeutics*, **19**, 2020b, 2, 503-519.
5. **Akinlolu, A. A., A. O. Oyewopo, R. E. Kadir, A. Lawal, J. Ademiloye, A. Jubril, M. O. Ameen, G. E. Ebito.** Moringa oleifera **and** Musa sapientum **ameliorated 7,12** Dimethylbenz[a]anthracene-induced upregulations of Ki67 and Multidrug resistance1 genes in rats. – *International journal of health sciences*, **15**, 2021, 3, 26-33.
6. **Ameen, M., A. A. Akinlolu.** Chromatography-Spectroscopic isolated MO11 (M. oleifera) and MS06 (M. sapientum) positively immunomodulated ACE2 levels in blood, kidney and liver of rats. – *Malaysian journal of pharmaceutical sciences*, **21**, 2023, 1, 15-30.
7. **Andjelkovic, M., D. A. Buha, E. Antonijevic, B. Antonijevic, M. Stanic, J. Kotur-Stevuljevic, V. Spasojevic-Kalimanovska, M. Jovanovic, N. Boricic, D. Wallace, Z. Bulat.** Toxic effect of acute Cadmium and Lead exposure in rat blood, liver, and kidney. – *International journal of environmental research and public health*, **16**, 2019, 2, 274.
8. **Bernhoft, R. A.** Cadmium toxicity and treatment. – *Scientific world journal*, Article ID394652, 2013, 7.
9. **Chaves, N., S. Antonio, C. A. Juan.** Quantification of the antioxidant activity of plant extracts: analysis of sensitivity and hierarchization based on the method used. – *Antioxidants*, **9**, 2020, 1, 76.
10. **Dasari, S., P. B. Tchounwou.** Cisplatin in cancer therapy: molecular mechanisms of action. – *European journal of pharmacology*, **740**, 2014, 364-378.
11. **Dutta, S., S. Ray, K. Nagaran.** Glutamic acid as anticancer agent: An overview. – *Saudi pharmaceutical journal*, **21**, 2013, 4, 337-343.
12. **Elisha, I. L., F. S. Botha, L. J. McGaw, J. N. Eloff.** The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts. – *BMC Complementary and alternative medicine*, **17**, 2017, 1, 133.
13. **El-Tarras, A. E., H. F. Attia, M. M. Soliman, M. A. El Awady, A. A. Amin.** Neuroprotective effect of grape seed extract against cadmium toxicity in male albino rat. – *International journal of immunopathology and pharmacology*, **29**, 2016, 3, 398–407.
14. **Gualdoni, G. S., P. V. Jacobo, C. M. Sobarzo, C. V. Pérez, L. A. H. Durand., M. S. Theas, L. Lustig, V. A. Guazzone.** Relevance of angiogenesis in autoimmune testis inflammation. – *Molecular human reproduction*, **27**, 2021, 2.
15. **Gupta, R., R. K. Shukla, A. B. Pant, V. K. Khanna.** A Dopamine-dependent activity in controlling the motor functions in Cadmium-induced neurotoxicity: Neuroprotective potential of Quercetin. – *Parkinsonism related disorders*, **46**, 2018, 2.

16. Huff, J., R. M. Lunn, M. P. Waalkes, L. Tomatis, P. F. Infante. Cadmium-induced cancers in animals and in humans. – *International journal of occupation andenvironmental health*, **13**, 2007, 2, 202-212.
17. Kuol, N., L. Stojanovska, V. Apostolopoulos, K. Nurgali. Role of the nervous system in cancer metastasis. – *Journal of experimental clinical cancer research*, **37**, 2018, 5.
18. Lafuente, A., A. I. Esquifino. Possible role of Glutamate, Aspartate, Glutamine, GABA or Taurine on Cadmium toxicity on the hypothalamic pituitary axis activity in adult male rats. – *Biometals*, **15**, 2002, 2, 183-187.
19. Lan, Y. L., X. Wang, J. S. Xing, Z. L. Yu, J. C. Lou, X. C. Ma, B. Zhang. Anti- cancer effects of dopamine in human glioma: involvement of mitochondrial apoptotic and anti-inflammatory pathways. – *Oncotargets*, **6**, 2017, 51, 88488-88500.
20. Lee, J. H., E. Park, H. J. Jin, Y. Lee, S. J. Choi, G. W. Lee, P-S. Chang, A-D. Paik. Anti-inflammatory and anti-genotoxic activity of branched chain amino acids (BCAA in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophage. – *Food science and biotechnology*, **26**, 2017, 5, 1371-1377.
21. Lieu, E. L., T. Nguyen, S. Rhyne, J. Kim. Amino acids in cancer. – *Experimental molecular medicine*, **52**, 2020, 15-30.
22. Liu, Z. G., Jiao, D. Necroptosis, tumor necrosis and tumorigenesis. – *Cell Stress*, **4**, 2019, 1, 1-8.
23. Mahmoudi, R., A. Azizi, S. Abedini, J. V. Hemayatkhah, H. Abidi, B. M. Jafari. Green tea improves rat sperm quality and reduced cadmium chloride damage effect in spermatogenesis cycle. – *Journal of Medicine and Life*, **11**, 2018, 4, 371-380. DOI: 10.25122/jml-2018-0005.
24. Mahoney, E. R., L. Dumitrescu, A. M. Moore, F. E. Cambronero, P. L. De Jager, M. E. I. Koran, A. Vladislav, V. A. Petyuk, R. A. S. Robinson, S. Goyal, J. A. Schneider, D. A. Bennett, A. L. Jefferson, T. J. Hohman. Brain expression of the vascular endothelial growth factor gene family in cognitive aging and Alzheimer's disease. – *Molecular psychiatry*, **26**, 2021, 888-896.
25. Manikandan, P., S. Nagini. Cytochrome P450 structure, function and clinical significance: A review. – *Current drug target*, **19**, 2018, 1, 38-54.
26. Muller, C., N. Bauer, I. SchÃ¤fer, R. White. Making Myelin Basic Protein- from mRNA transport to localized translation. – *Frontiers of cellular neurosciences*, **7**, 2013, 169.
27. Noori, S., P. Friedlich, I. Seri. Pharmacology review: Developmentally regulated cardiovascular, renal, and neuroendocrine effects of Dopamine. – *Neoreviews*, **4**, 2015, 10, e283–e288.
28. Omotoso, G. O., E. R. Kadir, S. F. Lewu, I. T. Gbadamosi, A. A. Akinlolu, G. O. Adunmo, R. M. Kolo, M. O. Lawal, M. O. Ameen. Moringa oleifera ameliorates cuprizone-induced cerebellar damage in adult female rats. – *Research journal of health sciences*, **6**, 2018, 1, 13-25.
29. Otth, C., M. Torres, A. Ramírez, J. C. Fernandez, M. Castro, M. C. Rauch, M. Brito, A. J. Yañez, J. E. Rodríguez-Gil, J. C. Slebe, II. Concha. Novel identification of peripheral dopaminergic D2 receptor in male germ cells. – *Journal of cellular biochemistry*, **100**, 2007, 1, 141-150.
30. Patra, R. C., K. R. Amiya, D. Swarup. Oxidative stress in Lead and Cadmium toxicity and its amelioration. – *Veterinary medicine international*, Article ID: 457327, 2011.
31. Rodriguez-Antona, C., M. Ingelman-Sundberg. Cytochrome P450 pharmacogenetics and cancer. – *Oncogene*, **25**, 2006, 1679-1691.
32. Saalu, L. C., G. G. Akunna, A. O. Oyewopo. The histomorfometric evidences of *Vernonia amygdalina* leaf extract-induced testicular toxicity. – *International journal of morphology*, **31**, 2013, 2, 662-667.

33. Siu, E. R., D. D. Mruk, C. S. Porto, C. Y. Cheng. Cadmium-induced testicular injury. – *Toxicology and applied pharmacology*, **238**, 2009, 3, 240-249.
34. Spinello, A., I. Ritacco, A. Magistrato. The catalytic mechanism of steroidogenic cytochromes p450 from all-atom simulations: entwinement with membrane environment, redox partners, and post-transcriptional regulation. – *Catalysts*, **9**, 2019, 1, 81.
35. Storto, M., M. Sallese, L. Salvatore, R. Poulet, D. F. Condorelli, P. Dell'Albani, M. F. Marcello, R. Romeo, P. Piomboni, N. Barone, F. Nicoletti, A. De Blasi. Expression of metabotropic glutamate receptors in the rat and human testis. – *Journal of endocrinology*, **170**, 2001, 1, 71-78.
36. Tamer, M. S., U. Paasch, H-J. Glander, A. Agarwal. Role of caspases in male infertility. – *Human reproduction update*, **10**, 2004, 1, 39-51.
37. Wang, B., Y. Du. Cadmium and its neurotoxic effects. – *Oxidative medicine and cellular longevity*, Article ID: 898034, 2013.
38. Yi, H., G. Talmon, J. Wang. Glutamate in cancers: from metabolism to signalling. – *Journal of biomedical research*, **34**, 2019, 4, 260-270.
39. Zhou, Y., N. C. Danbolt. Glutamate as a neurotransmitter in the healthy brain. – *Journal of neural transmission*, **121**, 2014, 8, 799-817.
40. Zhu, Q., Li, X., R-S Ge. Toxicological effects of cadmium on mammalian testis. – *Frontiers in genetics*, **11**, 2020, 527.
41. Ziyu, W., H. Q. Xie, H. Zhou, M. Zhang, J. Shen, D. Ju. Amino Acid degrading enzymes and autophagy in cancer therapy. – *Frontiers of pharmacology*, 2020, 11, 1-8.

Table 1. Gonado-Somatic Indices and Concentrations of Dopamine, Glutamate and MBP in testes of rats

Drug/Extract →	**Normal Saline only Group 1**	**$CdCl_2$ only Group 2**	**$CdCl_2$-exposure + MO11 post-treated Group 3**	**$CdCl_2$-exposure + MO11 + MS06 post-treated Group 4**	**Olive Oil only Group 5**	**$CdCl_2$-exposure + Doxorubicin post-treated Group 6**
Gonado-Somatic Index	**0.65±0.03	0.29±0.05	0.35±0.08	0.47±0.09	0.55±0.09	0.35±0.10
p–value	p<0.001		0.55	0.09	0.03	0.56
Dopamine (pg/ml)	**6.25±0.74	3.19±0.32	4.34±0.03	4.90±0.15	4.13±0.03	3.80±0.10
p-value	p<0.01		0.10	0.07	0.25	0.70
Glutamate (ng/ml)	**137.77±0.16	112.50±1.50	**137.22±0.39	**138.06±0.17	**130.27±1.04	**134.20±0.26
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
Myelin Basic Protein (ng/ml)	4.26±0.02	4.97±0.32	4.27±0.01	4.16±0.01	3.80±0.07	4.32±0.01
p–value	0.22		0.23	0.07	0.06	0.20

p – value at $p \leq 0.05$: Group 2 versus Groups 1 and 3 – 6
** – significant increase /$p \leq 0.05$/

Table 2. Concentrations of Cytochrome p450, Caspase-3 and sVEGFR in testes of rats

Drug/Extract →	**Normal Saline only Group 1**	**$CdCl_2$ only Group 2**	**$CdCl_2$-exposure + MO11 post-treated Group 3**	**$CdCl_2$-exposure + MO11 + MS06 post-treated Group 4**	**Olive Oil only Group 5**	**$CdCl_2$-exposure + Doxorubicin post-treated Group 6**
Cytochrome p450 (ng/ml)	**465.12±20.80	181.52±7.20	**378.45±1.76	**396.32±2.08	**349.12±7.77	**352.32±0.92
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
Caspase-3 (ng/ml)	*125.00±0.63	252.50±3.13	*137.50±0.72	*87.19±0.74	*176.67±6.14	*209.69±5.94
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
sVEGFR (ng/ml)	*28.75±0.42	49.72±2.65	*20.00±1.67	*15.00±0.59	*34.72±1.00	43.89±1.21
p–value	p<0.01		p<0.01	p<0.01	p<0.01	0.25

p – value at $p \leq 0.05$: Group 2 versus Groups 1 and 3 – 6
** – significant increase /$p \leq 0.05$/
* – significant decrease /$p \leq 0.05$/