Toxicology Reports 10 (2023) 690–705

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Pre-puberty cannabichromene exposure modulates reproductive function via alteration of spermatogenesis, steroidogenesis, and eNOS pathway metabolites

Odunayo Anthonia Taiwo [a,b], Oluwatosin Adebisi Dosumu [b,*], Adewale Segun James [c], Emmanuel Ifeanyichukwu Ugwor [b], Oluwafemi Adeleke Ojo [d,*], Gabriel Adewunmi Dedeke [e], Oladipo Ademuyiwa [b]

[a] Department of Biochemistry, College of Natural and Applied Sciences, Chrisland University, Ajebo Abeokuta, Ogun-state, Nigeria
[b] Department of Biochemistry, College of Biosciences, Federal University of Agriculture Abeokuta, Nigeria
[c] Faculty of Science, Department of Chemical Sciences (Biochemistry Programme), Augustine University, Ilara-Epe, Nigeria
[d] Phytomedicine, Molecular Toxicology, and Computational Biochemistry Research Laboratory (PMTCB-RL) Department of Biochemistry, Bowen University, 232101 Iwo, Nigeria
[e] Department of Pure and Applied Zoology, College of Biosciences, Federal University of Agriculture Abeokuta, Nigeria

ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
Cannabis
Cannabichromene
Reproduction
Steroidogenesis
Erectile dysfunction

ABSTRACT

Background: Cannabis and cannabinoids affect almost every system of the body and exert systemic effects such as alterations in memory and cognitive functions, neurotransmission impediment, as well as obstruction of endocrine and reproductive system functions. Reproduction is a complicated phenomenon that integrates biological, psychological and behavioural aspects, hence susceptible to intracellular and extracellular modulations by numerous chemicals and toxicants like cannabis.

Aim: The effects of early-life exposure to cannabis on reproductive function biomarkers and genes were investigated in male and female Wistar rats in this study.

Method: An initial computational analysis (molecular docking and induced fit docking) of some cannabinoids with reproductive enzymes; androgen and follicle stimulating hormone receptors was conducted. Overall, cannabichromene (CBC) had the best IFD scores and binding free energies for the two proteins studied and it interacted with notable amino acids within their active sites. Subsequently, forty (40) Wistar rats, 20 male and 20 female (24–28 days old, weighing 20–28 ± 2 g) were divided into two groups each and orally administered CBC for 21 days. Penile tissues, testes and ovaries, were collected for biochemical analysis (hormonal assays, enzyme activities, and metabolite concentrations), gene expressions, and histological evaluations.

Results: Activities of arginase and phosphodiesterase-5 in the penile tissue were significantly increased, while nitric oxide and calcium levels were significantly ($p < 0.05$) decreased in the CBC-exposed groups relative to the control group. Semen analysis showed significantly more abnormalities and decreased concentration of spermatozoa in the CBC-exposed group compared to the control. Activities of 17β-hydroxysteroid dehydrogenase and cholesterol level were decreased in both testes and ovaries of CBC-exposed groups. Furthermore, levels of testosterone, progesterone, luteinizing, and follicle-stimulating hormones were reduced in the serum of CBC rats. Moreover, relative expressions of androgen receptor and follicle-stimulating hormone receptor genes were significantly downregulated in the CBC-exposed groups. Histological evaluations revealed lesions, tubular necrosis, and cellular congestions in both the testes and ovaries.

Conclusion: This study suggests that pre-puberty exposure to cannabis modulates reproductive functions via cannabichromene inhibition of steroidogenesis, stimulation of erectile dysfunction (modulation of intermediates and enzymes of the endothelial nitric oxide synthase (eNOS) pathway in the penile tissue), and downregulation of the expressions of genes associated with reproduction.

* Corresponding authors.
E-mail addresses: dosunmuoa@funaab.edu.ng (O.A. Dosumu), oluwafemi.ojo@bowen.edu.ng (O.A. Ojo).

https://doi.org/10.1016/j.toxrep.2023.06.001
Received 12 April 2023; Received in revised form 19 May 2023; Accepted 2 June 2023
Available online 3 June 2023

1. Introduction

Cannabis is mostly used recreationally or as a medicinal drug and its use is increasing at an exponential rate in recent years among the young population [1]. According to Hall and Pacula, [2], cannabis is the most illicit recreational drug used around the world. It contains unique phytochemicals that have been implicated in various diseases and disorders, with more than 500 detected to date [3].

Cannabis use is a significant problem in the society, since it induces both psychological and physiological disturbances on the consumer and combines many of the properties of alcohol, tranquillizers, opiates and hallucinogens [4].

Cannabinoids are the most abundant phytochemicals in the cannabis plant, through which cannabis exert its inhibitory or excitatory effects by interacting with specific endogenous cannabinoid receptors CB_1 and CB_2 [5].

Several aspects of the endocannabinoid system have been shown to play different roles in male and female reproductive function [4]. Reproduction, is a delicate but complicated biological process governed by an interconnected hormonal system, a vital component of which is the production and regulation of gonadotropins with balanced elements of sexual function and fertility [6]. It is a complicated phenomenon that integrates biological, psychological, interpersonal and behavioural aspects; hence it is susceptible to intracellular and extracellular modulations [6].

Various endocrine disrupting chemicals (EDCs) like phthalates, dioxins, bisphenols and heavy metals have been intrinsically involved in modulation of sexual reproduction [7,8]. These chemicals are components of most illicit drugs like tobacco, cannabis, cocaine, and acts via inhibition of spermatogenesis and steroidogenesis, reduced sexual arousal, initiation of anovulation, implantation, erectile and ejaculatory dysfunction [9].

The female reproductive system is also as complex as the male system [10]. It is a multi-organ system that involves the hypothalamus, pituitary gland, ovaries, uterus (endometrium and cervix), and vagina, supported by ligaments, fasciae, and muscles [11]. Luteinizing hormone, oestrogen, progesterone and follicle stimulating hormone all work interdependently to facilitate the reproductive process and functions through puberty, conception and gestation phases [12].

Cannabis, an established neurotoxicant, could mediate neurological disturbances prodromal to sexual dysfunction (SD) in male and female by directly inhibiting the neurological pathways for genital sensations [13].

Cannabis or cannabinoid usage in adolescence is a subject of particular concern and a challenge in the fields of science and medicine due to changes in the social and political attitudes toward the drug [14]. As a result, there is a greater need to educate the public regarding the recognized dangers and potential risks associated with early life cannabis use as it relates to reproductive toxicity. Current reports reveal that cannabis and its constituents, as well as the intrinsic endocannabinoid pathway and its modifiers, represent a versatile research store, with effects spanning the entire human body, and various pathological entities [15].

Therefore, this research employed *in silico* and in vivo experimental procedures to determine the cannabinoid with the best binding interaction with key reproductive receptors; Androgen and follicle stimulating hormone receptors and evaluate its effects on key male and female reproductive enzyme biomarkers, hormones, metabolites as well as physical sexual function indices.

2. Method

2.1. Aqueous extraction of cannabis constituents and GC-MS analysis

Cannabis sativa was obtained from and identified by the National Drug Law Enforcement Agency (NDLEA); the apex drug regulatory agency in Nigeria. It was air-dried at room temperature and pulverized using a clean, dry electric blender. Two hundred and fifty grams (250 g) of milled cannabis was soaked in 1 litre of distilled water in a round bottom flask and placed on a shaker for 24 h, and decanted. The filtrate was kept and fresh 500 ml of distilled water was added to the residue. This was repeated until the filtrate became colourless. The filtrate was concentrated using a rotary evaporator and the concentrated extract was dissolved in olive oil and used for GC-MS analysis.

2.1.1. GC-MS ANalysis of Constituents of Cannabis Extract

The constituents of the concentrated extract were determined using GS-MS (Agilent Technologies 6890 N Network System). A mass spectrophotometer with an ion trap detector in full scan mode under electron impact ionization (70 eV), HP-5 capillary column (30 m x 0.32 mm, film thickness 0.25 mm) was used. Helium was used as the carrier gas at a flow rate of 1 ml/min. The injections were performed at spitless mode at 230 ℃. One microliter of the oil solution was injected and analysed with the column held initially at 60 ℃ for 2 min and then increased to 260 ℃ with a 5 ℃/min heating ramp and subsequently kept at 260 ℃ for 13 min

Table 1
Gene Primer sequences of AR, FSHR and Beta actin.

AR	Forward: GGTCTTTCCCTGGACGAAGG	55.88
	Reverse: TAGGCTAGCCAAGTCCCCAT	53.83
FSHR	Forward: ATTCTTGGGCACGGGATCTG	53.83
	Reverse: CGGTCGGAATCTCTGTCACC	55.88
B-actin	Forward: CCCGCGAGTACAACCTTCTT	53.83
	Reverse: AACACAGCCTGGATGGCTAC	53.83

Table 2
Analysis of the molecular docking results of the understudied proteins.

Compounds name	Androgen receptor (AR)				Follicle stimulating hormone receptor (FSHR)			
	Docking score	Intermolecular interactions	Binding free energy	IFD score	Docking score	Intermolecular interactions	Binding free energy	IFD score
Delta 9 THC	-9.004	-	-16.35	-541.38	-4.889	**HID134**, LEU135	-28.37	-613.11
Cannabich- romene	-9.731	**LEU704**	-40.17	-542.16	-4.733	**HID134**, **TYR110**, HID134	-37.45	-613.59
Cannabicou-maronone	-8.134	-	-23.04	-539.22	-4.274	LEU135, VAL138	-23.76	-609.85
Dronabinol	-7.755	ASN705	26.14	-541.30	-4.262	ILE111	-35.25	-614.02
Cannabidiol	-8.786	**Trp741**, leu704	-25.74	-541.49	-4.094	LEU135	-29.83	-607.96
Cannabinol	-8.299	**PHE764**, LEU704	-10.01	-540.88	-3.973	**HID134, TYR110**	-40.82	-611.09
Cannabicyclol	-10.657	LEU704	-32.46	-539.47	-3.961	HID134, ILE111	-33.84	-609.66
Reference	-12.924	ASN705, THR877, ARG752, MET745	-76.89	-535.84	-	-	-	-

2.2. Computational analysis

All computational tools used for this study were embedded in Schrodinger suite 2020–1 on maestro molecular interface (version 12.5).

2.2.1. Retrieval and preparation of ligands

The structures of the cannabinoids obtained from the GC-MS of the cannabis extract (delta 9 THC, cannabichromene, cannabicoumaronone, dronabinol, cannabidiol, cannabinol and cannabicyclol) were downloaded from PubChem. The compounds were uploaded on maestro for preparation and conversion to three-dimensional (3D) structure. The ligands were geometrically minimized using the software Ligprep module according to the protocol described by Elekofehinti [16] and Iwaloye [17].

2.2.2. Collection and minimization of target proteins

For this study, two proteins were used for the *in silico* analysis, the crystal structure of androgen receptor (AR) (PDB ID: 2AM9) and follicle stimulating hormone receptor (FSHR) (PDB ID: 4A19) were downloaded from http://www.RCSG.org, and prepared by protein preparation wizard to remove missing hydrogen and fix the missing side chain. This protocol has been described in detail by Iwaloye et al. [18,19].

2.2.3. Grid file generation

The active site of AR was initially predefined by the ligand co-crystalized with the proteins. Therefore, the grid file for defining the amino acid residues within the active site was generated by picking the co-ligand. After visualizing the crystal structure of the FSHR, it was conspicuous that the structure was complexed with follicle stimulating hormone (FSH). The active site of the receptor was predicted using SiteMap, the module predicted five possible active sites and the site with biggest size (0.946), highest site score (0.946) and D-score (0.951) was used to define the residues necessary for ligand interactions and non-covalent binding.

2.2.4. Molecular docking and post docking studies

Docking was carried out to predict the orientation of the ligands within the binding pocket of the preferred proteins (AR and FSHR) using glide algorithm as scoring function. The complexes of resulting ligand-protein interaction were analysed for stability using post docking analysis called Prime MM-GBSA. Again, downstream of molecular docking study called induced fit docking was also adopted to improve the binding affinities and scores of the compounds with the targets [20].

2.3. Chemicals

Cannabichromene (CBC), (the cannabinoid with the best binding properties from *in silico* analysis) was purchased from Sichuan BioCrick Biotech Limited, Wuhou District, Chengdu China (CAS NUMBER: 20675–51–8). Other chemicals used in this study were of pure and analytical grades. ELISA kits for testosterone, follicle stimulating

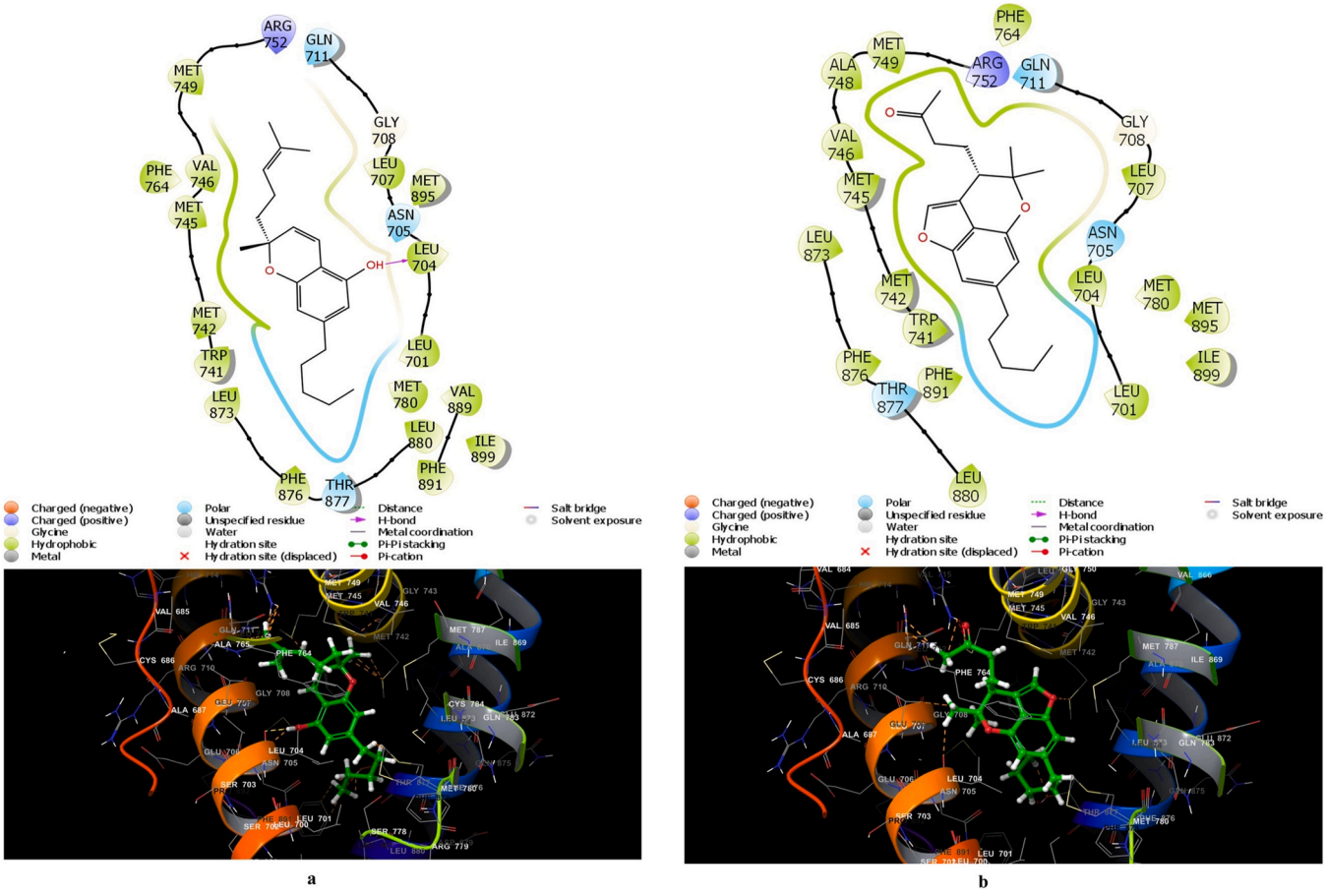

Fig. 1. a: 2D and 3D Interactions of cannabichromene within the active site of AR. b: 2D and 3D Interactions of cannabicoumaronone within the active site of AR. c: 2D and 3D Interactions of cannabicyclol within the active site of AR. d: 2D and 3D Interactions of cannabidiol **within** the active site of AR. e: 2D and 3D Interactions of cannabinol **within** the active site of AR. f: 2D and 3D Interactions of delta 9 THC **within** the active site of AR. g: 2D and 3D interactions of dronabinol **within** the active site of AR. h: 2D and 3D interactions of testosterone **within** the active site of AR.

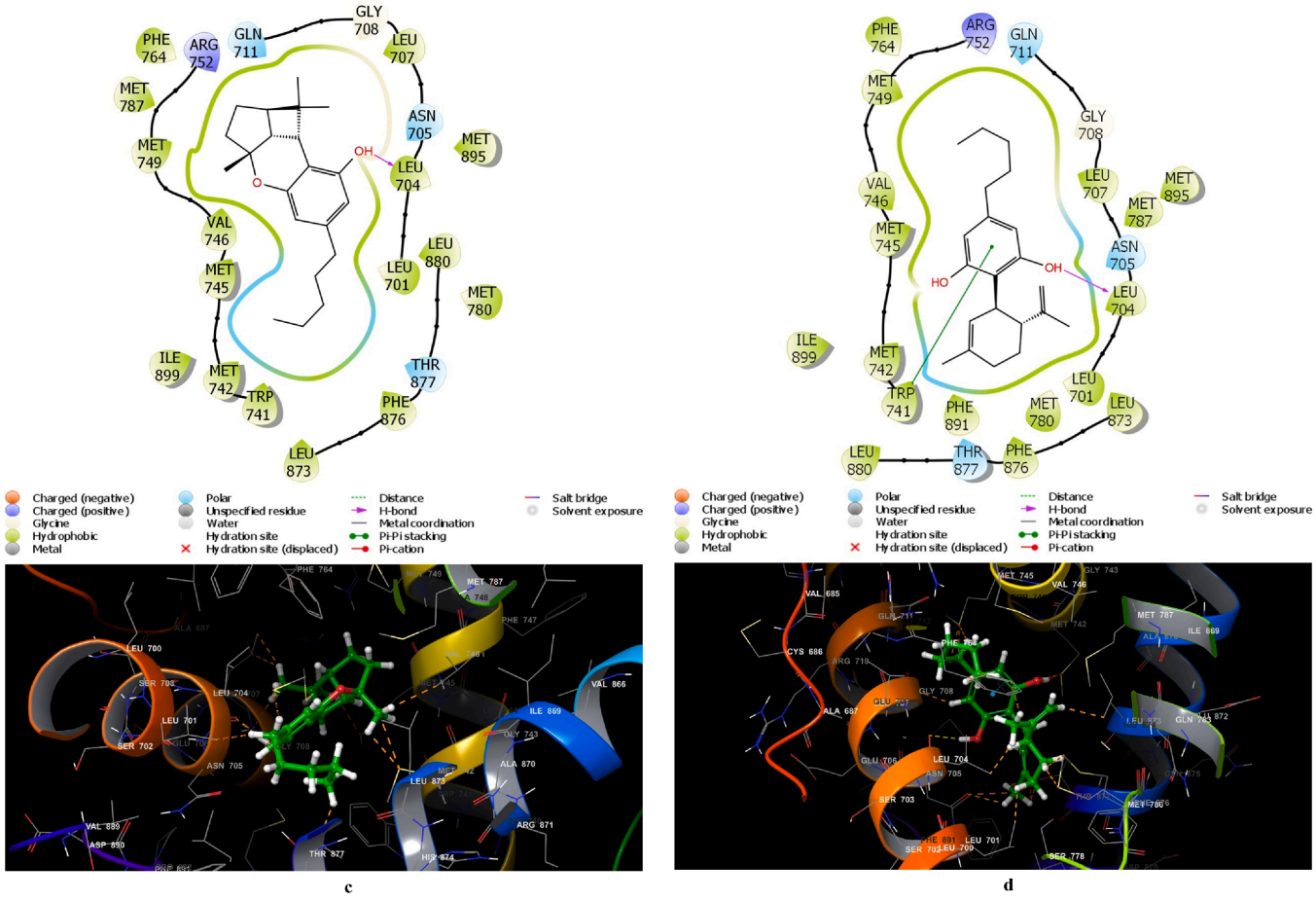

Fig. 1. (*continued*).

hormone, progesterone and oestrogen were obtained from Shanghai Institute of Measurement and Testing Technology, Shangai, China; Total cholesterol kit was a product of Labkits, Barcelona, Spain. Primer sequence was designed by ShineGene Corporation (Shanghai, China).

2.4. Acquisition of the rats

Forty [40] rats apparently healthy male and female Wistar rats, 24–28 days old, weighing 20–30 g were purchased from Redox animal house, Abeokuta. They were kept in animal housing unit of the Department of Biochemistry, Federal University of Agriculture, Abeokuta. The rats were acclimatized for 21 days. The research was approved by the committee on research, Biochemistry department, Federal University of Agriculture (PG15/0103[5]).

2.5. Experimental design

The rats were separated into 2 groups per sex each of 10 rats (n = 10) in plastic cages (55 cm × 40 cm) with good ventilation and supplied with standard pellet and clean water ad libitum, they were identified by tail-coloured marks and allowed to acclimatize for 21 days.

Group 1: Control male (Olive oil).
Group 2: Exposed male (10 mg/kg CBC).
Group 3: Control female (Olive oil).
Group 4: Exposed female (10 mg/kg CBC).

Orally administered 10 mg/kg body weight dose of cannabichromene (CBC) daily for 21 days.

2.5.1. Animal sacrifice and tissues preparation

After 21 days of CBC administration, the rats were used for:

i. **Biochemical assays**: The rats were fasted overnight. They were anaesthetize using ketamin/xylazine, then blood sample was collected via retro-orbital plexus into well labelled plain tubes, spun using a centrifuge for 10 min at 3500 rpm to obtain the serum. The rats were euthanized by cervical dislocation. Testes and penile tissues in males as well as ovaries in female rats were excised, blotted, weighed for the computation of organ-body weight ratio and preserved at frozen temperature for biochemical analyses. The Gidez et al. [21] approach was used to isolate the lipids from the organs. The organ homogenates for cholesterol analysis (10% w/v) were made in a chloroform-methanol (2:1 v/v) solution. All hormonal assays (testosterone, progesterone, luteinizing, progesterone and follicle stimulating hormone) were evaluated using ELISA while spectrophotometric principles were used for other biochemical parameters.

ii. **Semen analysis**: semen samples from the testes was collected and used for sperm count and sperm cell morphology evaluation

iii. **Gene expression analyses**: a tiny section of the testes and ovaries were stored in 100 μl of TRIZOL reagent for gene expression analysis of androgen receptor (AR) and follicle stimulating hormone receptor (FSHR). Until needed, the gene expression samples were stored at − 20 ℃ temperature. Primer sequence was designed by ShineGene Corporation (Shanghai, China). The gene expression analysis was done at the Biotechnology Laboratory of Chrisland University, Abeokuta, Ogun

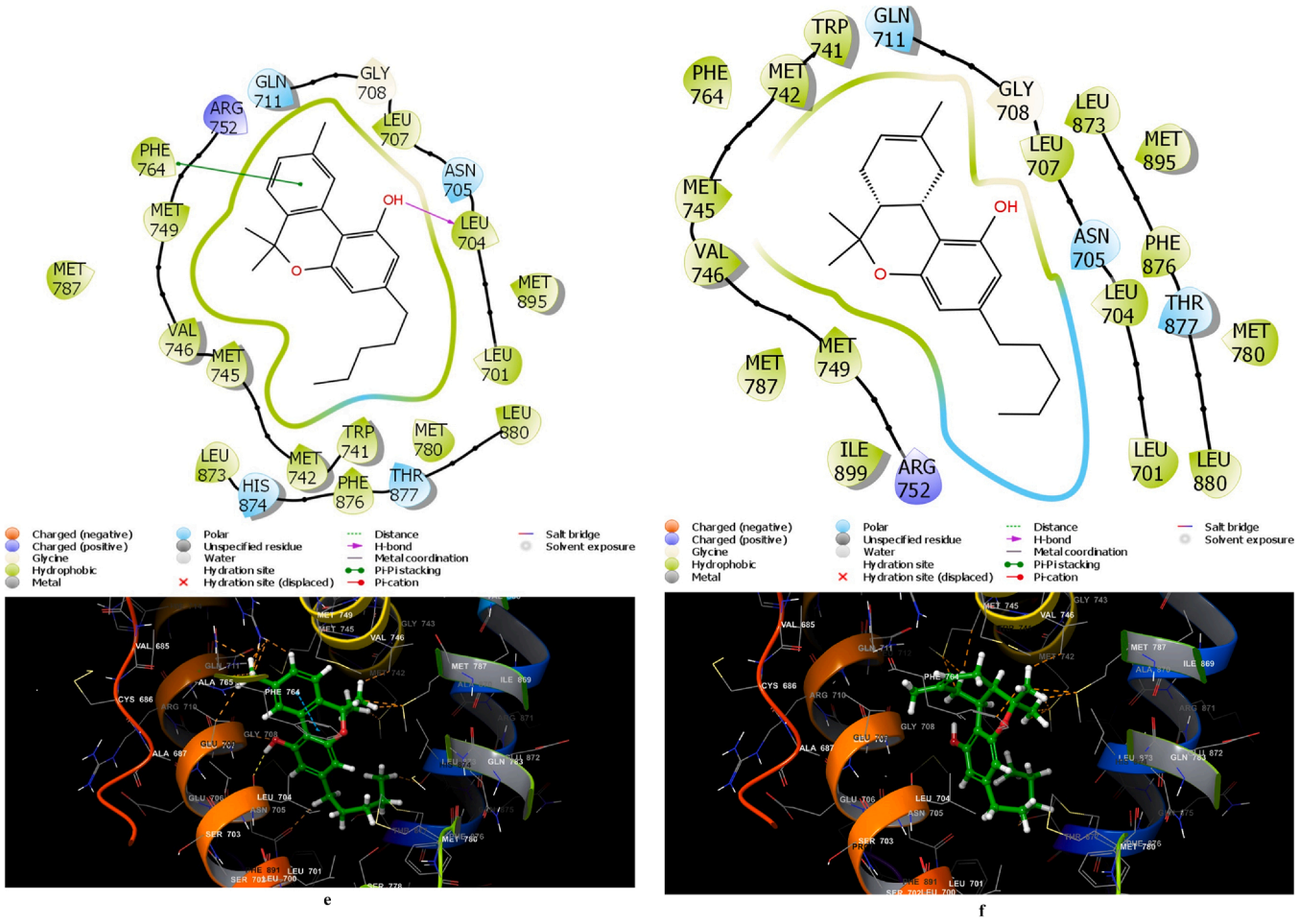

Fig. 1. (*continued*).

State, Nigeria. Using Image-J software in accordance with Schneider et al. (2012), the snapshot of the migrating bands was submitted to densitometry scanning, and the band intensity of each gene's cDNA fragment was standardized against the band intensity of the house-keeping control gene (beta actin).

iv.Histology: a section of testes and ovaries was fixed in 10% formalin for histological evaluation. The tissues were cleaned in xylene and embedded in paraffin wax (melting point 56 ℃) [22]. Tissue sections were prepared as described by [23] and stained with haematoxylin / eosin (H&E). The processed histology slide was read under a light microscope. Photomicrographs of the testes and ovaries were captured at a magnification Mg. × 400.

2.6. Biochemical assays

2.6.1. Penile function markers

2.6.1.1. Arginase activity. Penile Arginase activity was determined using the method of Zhang et al. [23]. Briefly, Tris-HCl, pH 7.5 was added to 0.05 ml Penile tissue homogenate and incubated at 37^0 C for 10 mins to activate the enzymes. The hydrolysis reaction was performed by incubating the mixture containing arginase with 0.05 ml of L-arginine at 37 ^{0}C for 1 hr and was stopped by adding 0.4 ml of the acid solution mixture (H_2SO_4/ H_3PO_4/H_2O) 1:3:7 (v/v/v). The mixture was heated at 100 ^{0}C for 45 mins. Absorbance was read at 540 nm and expressed as arginase activity/mg protein.

2.6.1.2. Nitric oxide level. **Principle:** This method employs metallic cadmium for quantitative conversion of nitrate to nitrite prior to quantitation of nitrate using Griess reagent, thus providing for accurate determination of total NO production. Nitrites compound forms purple/magenta complex with N (1 Naphtyl) – diamine dihydrochloride (NED) at acidic pH.

Procedure: This was measured using the Griess reagent (one part of 0.1% N (1-naphthyl)-diamine dihydrochloride (NED) in distilled water and one part of 1% sulphanilamide in 5% concentrated H3PO4) and incubated for 10 min at room temperature, as reported by [24] with a few minor adjustments. Equal parts of 1% sulphanilamide and tissue homogenate (1 ml each) were combined and incubated for 15 min in the dark. The NED solution (1 ml) was then added, carefully mixed, and incubated for a further 10 min at room temperature in the dark. Within 15 min, the absorbance of the purple/magenta colour that had formed at 520 nm was measured.

2.6.1.3. Phosphodiesterase 5′ (PDE-5) activity. This was measured according to the method of Thompson and appleman. Briefly, penile tissue homogenate (50 μl) was measured into 400 μl of Tris-base buffer (pH 7.4) and incubated at 37^0 C for 10 mins. Then, 50 μl of para nitrophenyl phosphate (PNPP) as substrate was added to the reaction mixture. The resulting mixture was read at 400 nm. The basis for the assay is the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase to form 5′-AMP or 5′-GMP respectively. The 5′-nucleotide released is further cleaved into the nucleoside and phosphate by the enzyme 5′-nucleotidase. The phosphate released due to enzymatic cleavage is

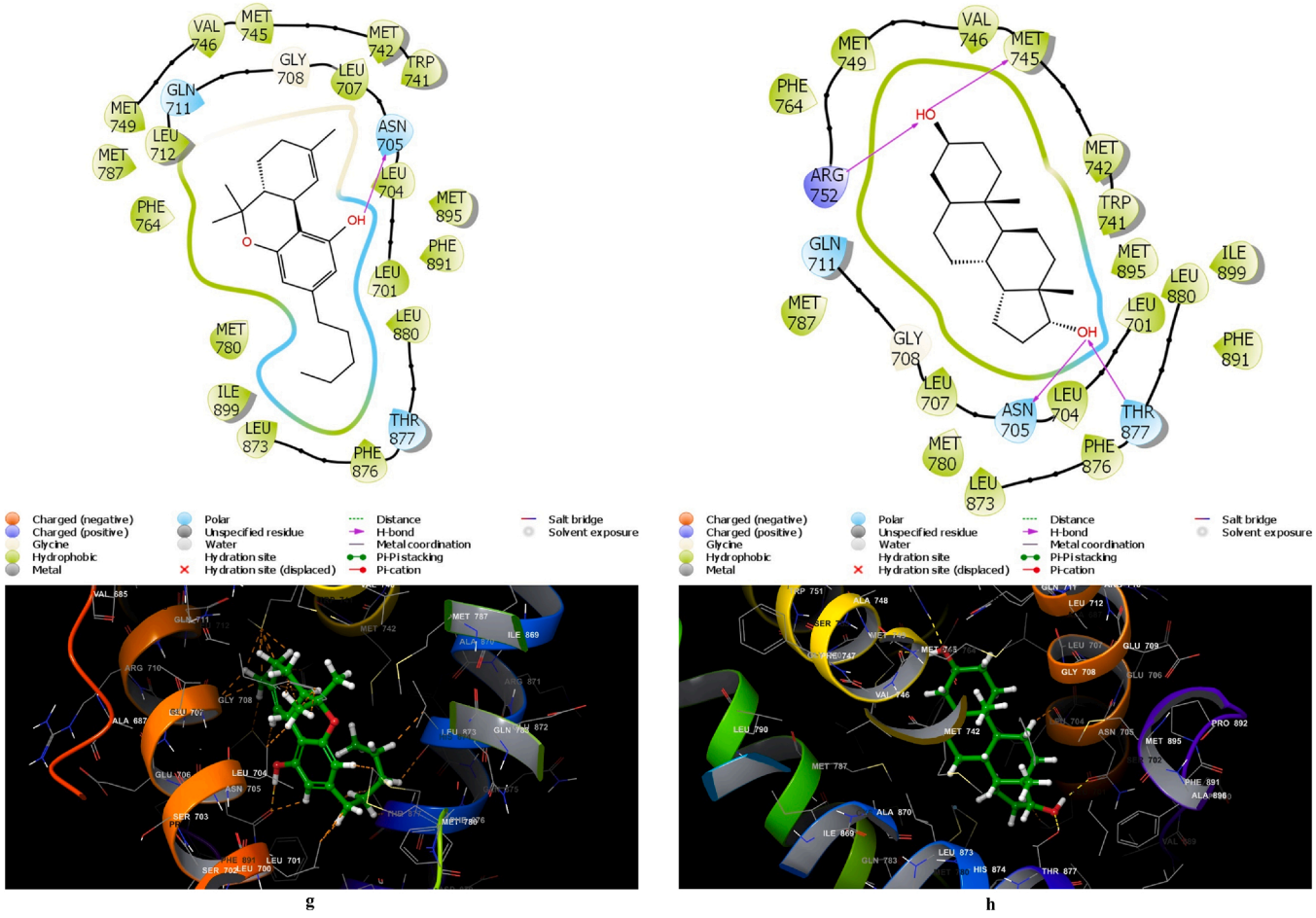

Fig. 1. (*continued*).

quantified in the presence of a highly sensitive phosphate detection solution (green assay reagent) which gives a green colour that is measured.

2.6.1.4. Calcium concentration determination. **Principle:** At neutral pH, calcium reacts with Arsenazo III (1,8-dihydroxy-3,6-disulpho-2,7-naphthalene-bis (azo)-dibenzenearsonic acid) to produce a complex that is blue in color and whose intensity is proportional to the calcium content.

Procedure: The calcium concentration was determined spectrophotometrically in the penile homogenate as described in Randox diagnostic kit manual. Concisely, three Eppendorf tubes were labelled blank, standard and sample. Reagent standard (10 µl) was pipetted into standard tube, 10 µl of working sample was added into sample tube, and 1.0 ml of working reagent was pipetted into the three tubes. The tubes were incubated for 2 min at 25 °C. Absorbance of standard and sample was read against blank at 578 nm.

2.6.2. Testicular and Ovarian function markers

2.6.2.1. Cholesterol level determination. The level of cholesterol in the testes and ovaries was determined as described by [25] in the Labkits cholesterol manual. Concisely, 1 ml of cholesterol working reagent was pipetted into clean Eppendorf tubes labeled blank (B), standard (S), and test (T). After, 10 µl of sample and cholesterol standard was added into the tubes labeled T and S respectively. The tubes were mixed well and allowed to incubate for 10 min, at room temperature, the absorbance of the samples and standard were taken at 505 nm against the blank. The cholesterol esters present in the sample is hydrolysed to form cholesterol which is then oxidised to produce 4-Cholestenone and hydrogen peroxide. The reaction of H_2O_2, phenol and 4-aminophenazone produces a coloured comples, the intensity of which is proportional to the cholesterol concentration in the sample. originates a coloured complex.

2.6.2.2. 17β-Hydroxysteroid dehydrogenase. **Principle:** The reaction velocity is measured as an increase in absorbance at 340 nm resulting from the reduction of NAD. One unit reduces one micromole NAD per min at 25 ^{0}C and pH 9.0, using androsterone or testosterone as a substrate under the specified conditions.

Procedure: The activity of 17β-hydroxysteroid dehydrogenase was determined in the testes and ovaries according to the method modified by [26]. The following reagents were pipetted into each cuvette:

sodium pyrophosphate (0.6 ml), NAD (0.2 ml), Reagent grade water (2.0 ml) and tissue homogenate (0.1 ml). It was incubated in spectrophotometer at 340 nm and 25 ^{0}C for 3–4 mins to achieve temperature equilibrium and establish blank rate if any. At zero-time, 0.01 ml (10 µl) of testosterone solution was added. Abs_{340} was recorded for 3 min.

2.6.3. Serum reproduction biomarkers

2.6.3.1. Testosterone level. **Principle***:* The principle is based on competitive binding between testosterone in the test specimen and testosterone enzyme conjugate for a constant amount of anti- testosterone polyclonal antibody.

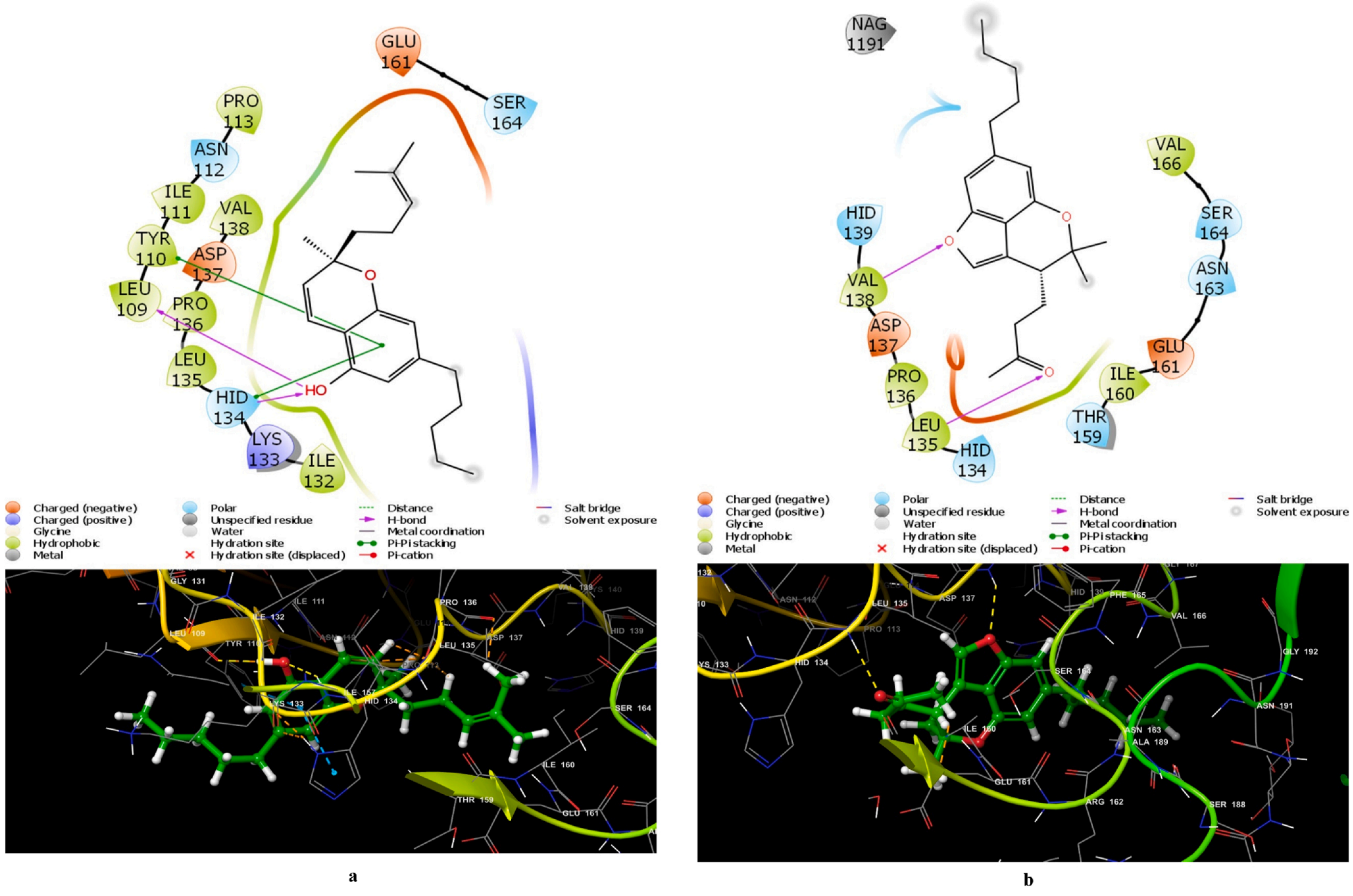

Fig. 2. a: 2D and 3D interactions of cannabichromene within the active site of FSHR. b: 2D and 3D interactions of cannabicoumaronone within the active site of FSHR. c: 2D and 3D interactions of cannabicyclol within the active site of FSHR. d: 2D and 3D interactions of cannabidiol within the active site of FSHR. e: 2D and 3D interactions of cannabinol within the active site of FSHR. f: 2D and 3D interactions of delta 9 THC within the active site of FSHR. g: 2D and 3D interactions of dronabinol within the active site of FSHR.

Procedure: Testosterone concentration was determined in the serum of male rats according to the method of [27]. Briefly, all reagents were first brought to 25 ^{0}C. Standards (25 µl) and sample (25 µl) were pipetted into appropriate wells and 100 µl of working solution of testosterone-enzyme conjugate added into each well. The contents of the wells were mixed well on a shaker for 20 s and then incubated at 25 ^{0}C for 60 min. The liquid in each well was decanted, wells washed three times with 300 µl of 1X wash buffer and blotted on absorbent paper towels. TMB reagent (100 µl) was added into each well, the plate covered and incubated at 25 ^{0}C for 15 min and the reaction stopped by adding 50 µl of stop solution to each well. After 20 s of gentle mixing, the plate's absorbance was measured at 450 nm after 15 min.

2.6.3.2. Follicle stimulating hormone (FSH) level. **Principle**: The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for FSH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of FSH is conjugated to horse radish peroxidase (HRP). The intensity of the colour formed by the enzymatic reaction is directly proportional to the concentration of FSH in the sample.

Procedure: Follicle stimulating hormone (FSH) concentration was determined in the serum of male and female rats by following the method of [28]. Firstly, all reagents were taken to 25 ^{0}C after which 25 µl of each of calibrator, control and specimen sample was pipetted into correspondingly labeled wells. Assay buffer (100 µl) was added to each well and the plate incubated on a plate shaker (200 rpm) for 30 min at 25 ^{0}C. The wells were then washed three times with 300 µl of diluted wash buffer and the plate tapped firmly against absorbent paper to ensure that it is dry. Conjugate working solution (100 µl) was added to each well and the plate incubated again on a plate shaker (200 rpm) for 30 min at 25 ^{0}C. The wells were then washed again as previously described. 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (100 µl) substrate was added to each well and the plate incubated for 20 min at 25 ^{0}C. Stop solution (50 µl) was added to each well and absorbance read at 450 nm within 20 min after addition of the stop solution.

2.6.3.3. Luteinizing hormone (LH) level. **Principle:** The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for LH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of LH is conjugated to horse radish peroxidase (HRP).

Procedure: Luteinizing hormone (LH) concentration was determined in the serum of male and female rats by following the method of [29]. Here, all reagents were taken to 25 ^{0}C after which 25 µl of each of calibrator, control and specimen sample was pipetted into correspondingly labeled wells. Assay buffer (100 µl) was added to each well and the plate incubated on a plate shaker (200 rpm) for 30 min at 25 ^{0}C. The wells were then washed three times with 300 µl of diluted wash buffer and the plate tapped firmly against absorbent paper to ensure that it is dry. Conjugate working solution (100 µl) was added to each well and the plate incubated again on a plate shaker (200 rpm) for 30 min at 25 ^{0}C. The wells were then washed again as previously described. 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (100 µl) substrate was added to each well and the plate incubated for 20 min at 25 ^{0}C. Stop solution (50 µl)

was added to each well and absorbance read at 450 nm within 20 min after addition of the stop solution.

2.6.3.4. Progesterone level. The serum progesterone concentration was quantitatively determined using a solid phase competitive ELISA kit as described by [27] in the manufacturer's protocol version. Briefly, to 2.5 μl of each calibrator, control and serum sample in the microplate wells, 0.10 ml of the conjugate was dispensed into each well. After gently mixing the microplate for 30 s, it was incubated at 25 °C for 60 min. After the microplate's contents were decanted, 0.3 ml of the washing solution was gradually added over the course of four additions. TMB-substrate (0.1 ml) was added 94 and incubated at room temperature for 25 min in the dark after which 0.15 ml of stopping reagent was dispensed into each well. Within 20 min after introducing the stopping reagent, the absorbance was measured using a microplate reader at 450 nm. The principle is same with LH.

2.6.3.5. Estrogen level. The serum estrogen concentration was quantitatively determined by adopting the procedures described by [30]. Estrogen was assayed by placing the desired number of coated strips into the holder followed by the addition of 50 ml of total oestrogens standards, controls, and samples into designated microwells. A known amount (100 ml) of the working solution of total estrogens enzyme conjugate reagent was then added into each well followed by the addition of 50 ml of anti-total estrogens antibody reagent into each well. The plate was then covered and incubated for 120 min at 25 °C with shaking (600 rpm). The liquid was later removed from all the wells. The wells washed three times with 350 ml of 1x Wash Buffer thereafter blotting on absorbent paper towels. Exactly 100 ml of 95 TMB substrate was then added into each well. The plate was covered, incubated at 25 °C for 30 min with shaking (600 rpm). Stop solution (50 ml) was added into each well and mixed gently for 10 s. The absorbance was then read at 450 nm within 10 min using a microplate reader.

2.6.4. Semen analysis

2.6.4.1. Sperm count. The method described by [31] was adopted for the evaluation of sperm count. The testis and epididymis were taken out and dried. They were then gently cut into slices and submerged in 5 ml of ordinary saline. Sperm cells swam equally into the solution when the tube was shaken by inversion. A quantity of the solution (1 ml) was pipetted into a better Neubauer haemocytometer using a Pasteur pipette. After the solution had been in place for a short while, the spermatozoa were counted under a microscope with a 10x objective. A 2 square mm region was used for the counting of spermatozoa. The number counted was multiplied by 100,000 to get the quantity of spermatozoa present in 1 ml of fluid.

2.6.4.2. Sperm morphology. Sperm morphology was determined by following the method described by [32]. The semen was placed on a prewarmed slide, two drops of warm eosin-nigrosin stain were added, a uniform smear was created, and the slide was air dried. The stained slide was then viewed under an x400 magnification microscope. The types and numbers of abnormalities found in sperm cells were assessed from the total number of sperm cells in the five randomly chosen fields of the microscope, and the results were expressed as a proportion of aberrant spermatozoa to the total number of spermatozoa.

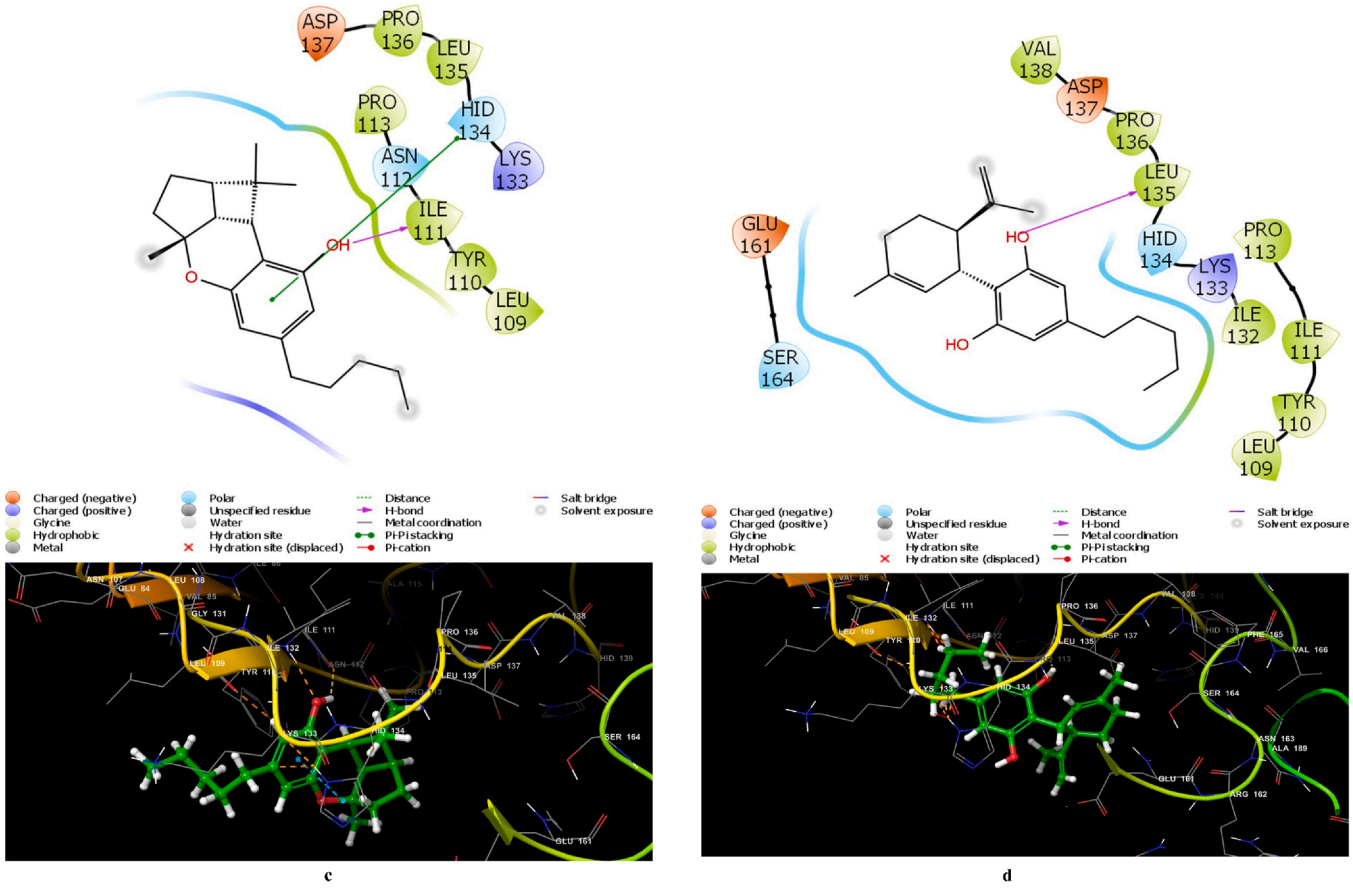

Fig. 2. (*continued*).

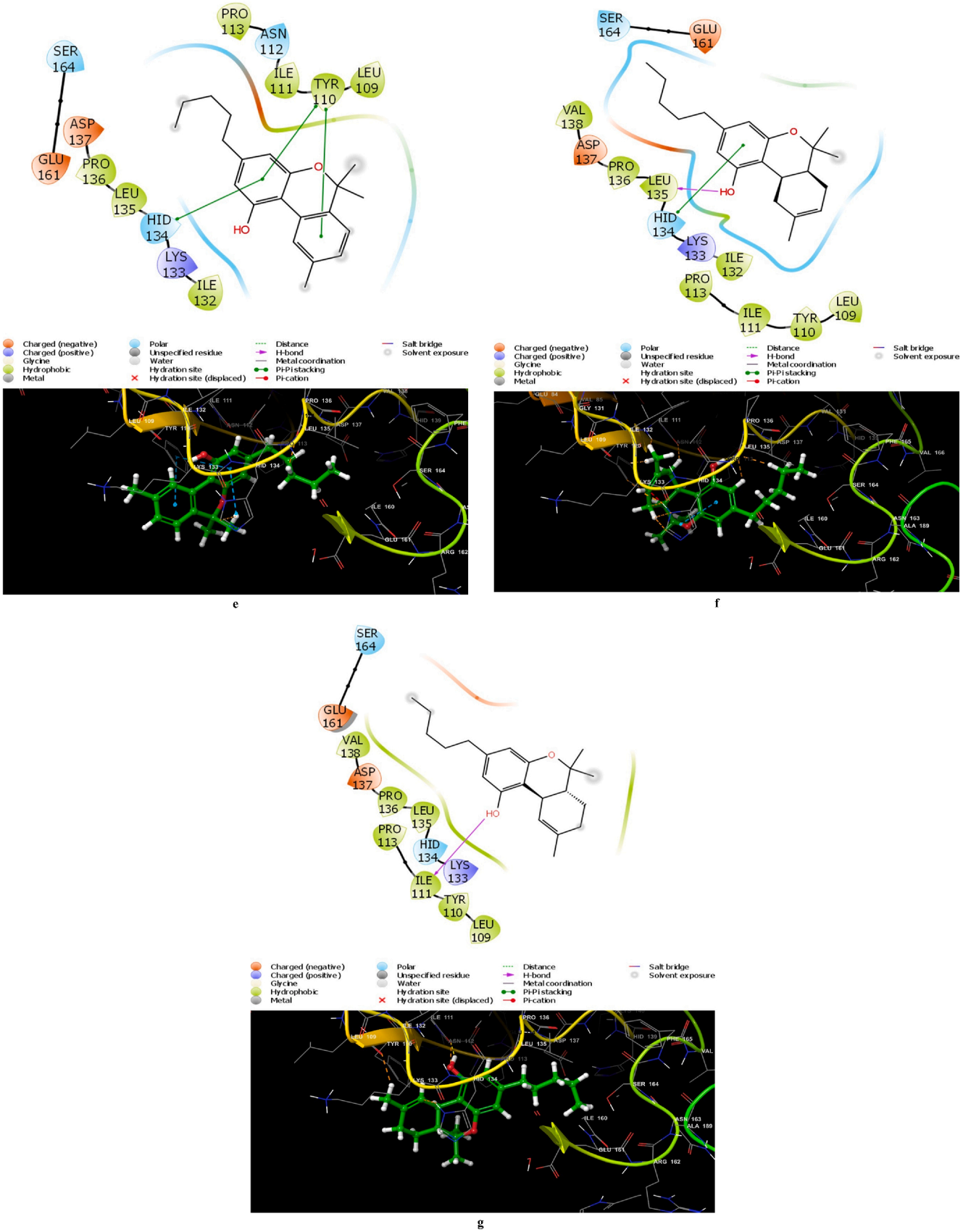

Fig. 2. (*continued*).

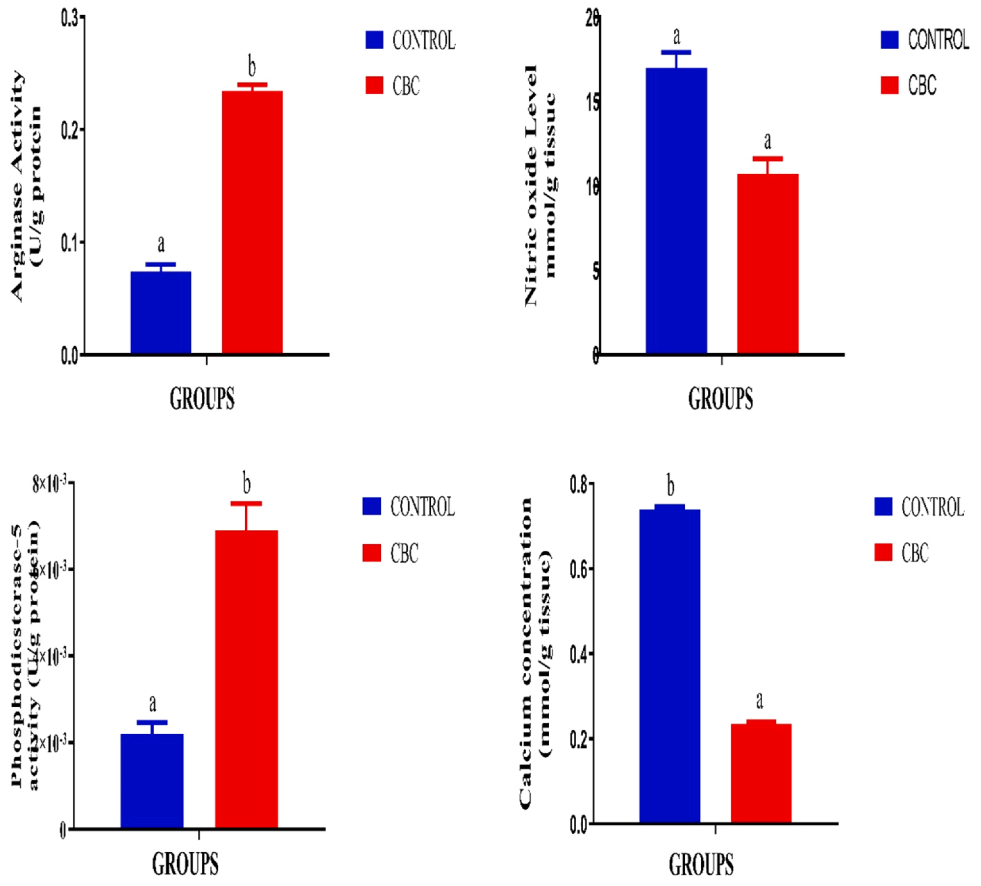

Fig. 3. Penile function biomarkers (Arginase activity, nitric oxide level, phosphodiesterase −5 activity and calcium concentration). Data are presented as mean ± SEM (n = 6). Bars with different letters are statistically distinct at $p < 0.05$.

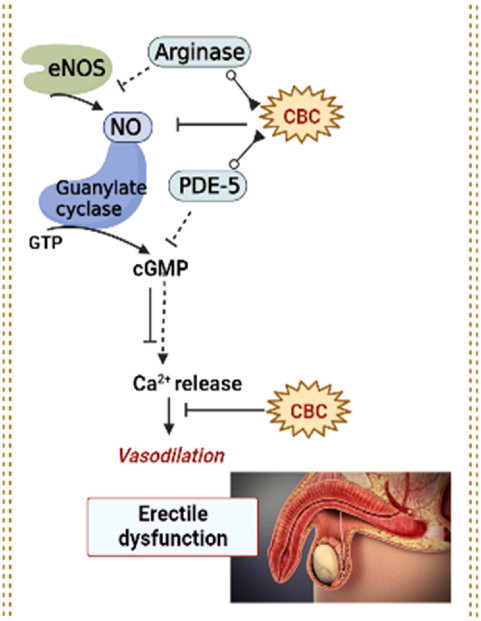

Fig. 4. Graphical illustration of the effects of cannabichromene (CBC) on erectile function markers.

2.6.5. Evaluation of male sexual behavior

Male rats were paired with female in ratio 1:1. Sexual behaviour parameter was monitored according to the method of [33]:

- Mount frequency (MF): the number of mounts by the male rats, with intromission from the time of introduction of the female rats until ejaculation. Mounting of the female rats from incorrect orientations like the flanks and head were not scored.

2.7. Statistical analysis

Students t-test was used and values expressed as mean ± standard error mean (S.E.M) with $p < 0.05$ considered as significant value. All analyses were done using Statistical Package for Social Sciences (SPSS) version 20.0. The band densities (from Image-J) are plotted as a bar graph (mean ± SEM, n = 3), using Graph Pad Prism (version 8.0).

3. Results

The molecular docking analysis of the seven compounds against the two understudied proteins AR and FSHR is given in Table 2. The docking result showed that delta 9 THC, cannabichromene, cannabicoumaronone, dronabinol, cannabidiol, cannabinol, cannabicyclol achieved glide XP docking of − 9.00 kcal/mol, − 9.731 kcal/mol, − 8.134 kcal/mol, − 7.755 kcal/mol, − 8.786 kcal/mol, − 8.299 kcal/mol and − 10.657 kcal/mol with AR protein. The docking score of the seven compounds with FSHR were low but still favorable. The IFD score for the

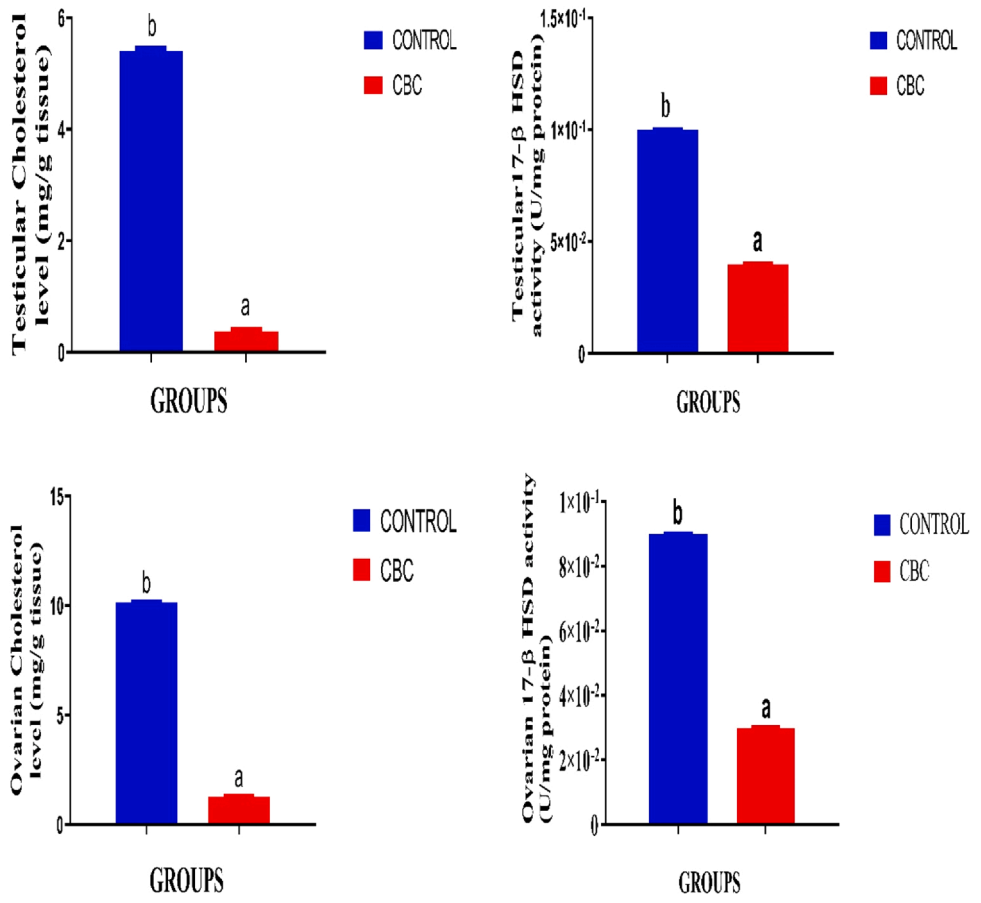

Fig. 5. Testicular and Ovarian function markers (cholesterol level and 17-hydroxysteroid dehydrogenase activity). Data are presented as mean ± SEM (n = 6). Bars with different letters are statistically distinct at $p < 0.05$.

two proteins showed an improved binding affinity of the compounds (Table 2). The two- and three-dimensional interaction of the compounds with the respective protein crystal structure are given in Figs. 1a-2g.

Fig. 3 depicts the effect of CBC on penile eNOS pathway enzymes and metabolites. Significant increases ($p < 0.05$) were observed in the activities of the enzymes while the concentrations of the intermediate metabolites were significantly decreased in the CBC-exposed groups relative to the control. .

Also, the concentrations of testicular and ovarian function markers were significantly reduced after CBC administration (Fig. 5). A similar trend was observed for the hormone levels in the serum of both male and female test rats as shown in Fig. 6. Semen analysis illustrated in Figs. 7 and 8 revealed reduced spermatozoa concentrations with significant abnormalities in the CBC group. .

Relative expressions of genes in the testes and ovaries (Fig. 10) were downregulated in the test groups while lesions and atrophies were observed in the histological evaluations of the CBC-tissues (Figs. 11 and 12).

Docking score represents the binding affinity of the compound with protein; IFD score is the flexible docking results of the compound upon binding to the protein; binding free energy is a measure of the stability of compound bound protein; the bolded amino acid are the residues that formed π-π interactions with the atom of the compound.

4. Discussion

Molecular docking scores provide possible mechanism of interactions between the compounds or ligands and proteins or enzymes. It predicts the affinity for binding of the ligands with the target macromolecules while taking into account the type of non-covalent interactions that foster the favourability of the reactions. The non-covalent interactions may include hydrogen bond acceptor or donor, hydrophobic interactions, hydrophilic interactions and pi-pi interactions (aromatic interaction) [34].

For the purpose of comparison, reference compounds were included, in the case of AR crystal structure, the co-crystalized ligand testosterone served as reference structure. Despite the favorable binding affinity exhibited by the compounds, the docking scores of testosterone was comparably better. The same trend was followed by induced fit docking (IFD) scores where testosterone also recorded most favorable binding among the investigated compounds (−542.84 kcal/mol). The high docking score recorded for all the seven understudied compounds and testosterone may signify their modulatory prowess on AR crystal protein structure. The docking scores of the seven compounds with FSHR were low but still favourable, however the IFD score showed improved binding affinities of the compounds with FSHR.

The residues within the binding pocket of the proteins interacting with the compounds were mapped to understand the possible modulatory mechanism exhibited by the ligands. Studies have shown that hydrogen bond is one of the major intermolecular interaction necessary for stabilizing ligand-bound protein [35,36]. In the active site of AR, the residues MET749, VAL746, PHE764, MET742, TRP741, LEU873, PHE876, THR877, PHE891, ILE899, VAL889, MET780, LEU704, LEU701, ASN705, MET895, LEU707, GLY708, GLN711 and ARG752 were found to contribute to the docking scores of the investigated compounds. However, the seven compounds are shown to only make

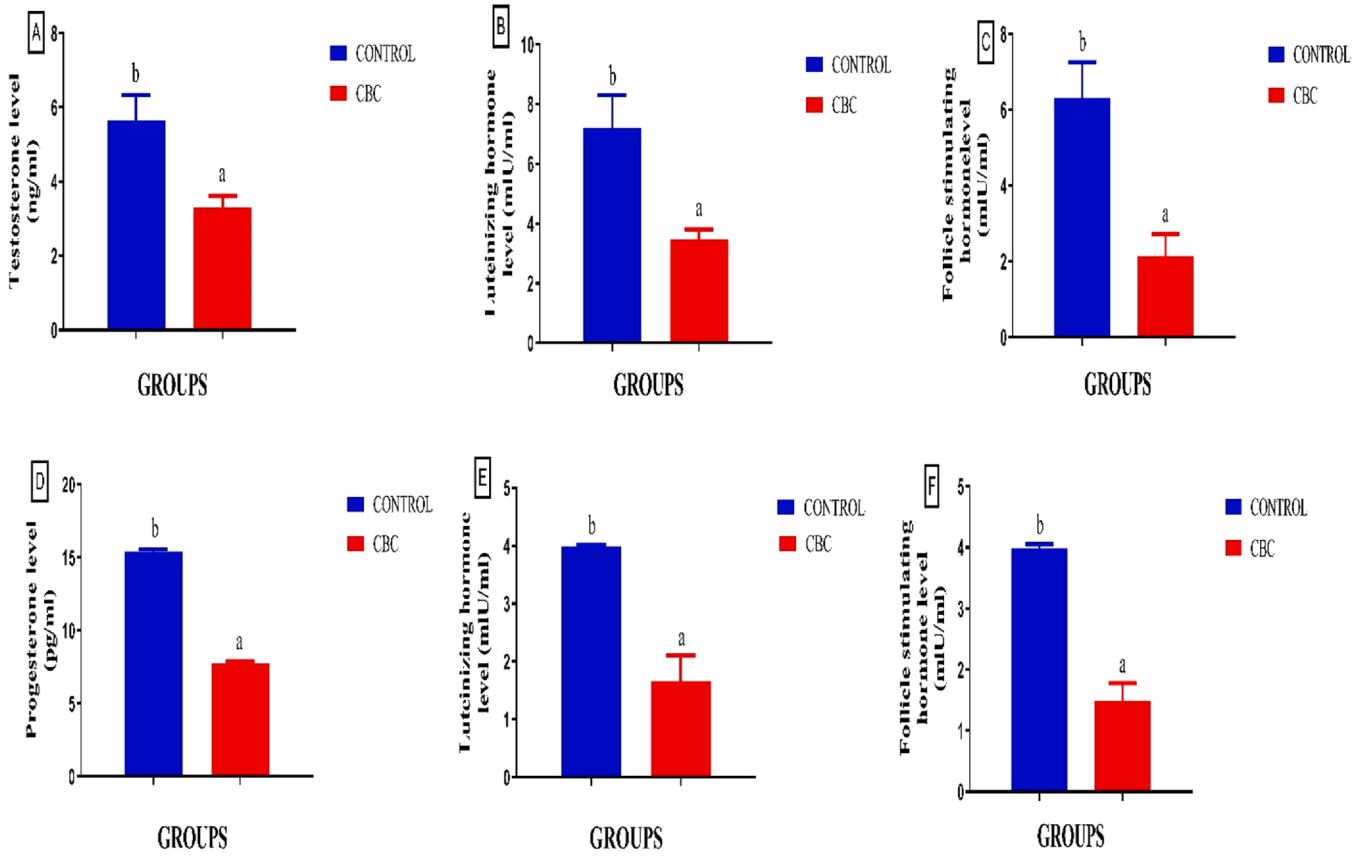

Fig. 6. Serum hormone levels (Testosterone, progesterone, Luteinizing and Follicle stimulating hormone). Legends: A: male testosterone level; B: male luteinizing hormone; C: male follicle stimulating hormone; D: female progesterone level; E: female luteinizing hormone; F: female follicle stimulating hormone. Data are presented as mean ± SEM (n = 6). Bars with different letters are statistically distinct at $p < 0.05$.

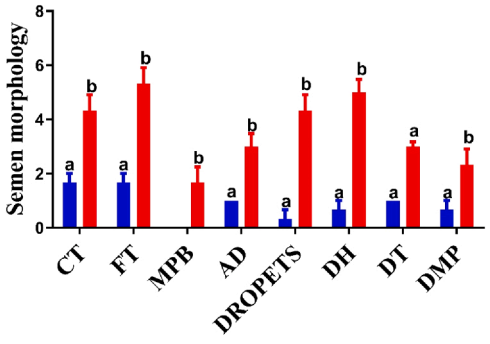

Fig. 7. Spermatozoa morphology in the semen of male parent Wistar rats. Data are presented as mean ± SEM (n = 3). Bars with different letters are statistically distinct at $p < 0.05$. CT – Coiled Tail, FT – Free Tail, MPB – Mid piece bent, AD – Acrosomal Defect, DH – Detached Head, DT – Dwarf Tail, DMP – Detached mid piece.

H-bond or pi-pi interactions with ASN705, LEU704 and TRP741. Compounds like Delta 9 THC and cannabicoumaronone did not contribute to any form of intermolecular interactions with the residues. The reference compound (testosterone) formed the highest number of hydrogen bond with the residues; their rich intermolecular interactions may be responsible for the high favourability in term of docking score.

The seven investigated compounds also displayed varying degree of intermolecular interactions with residues within the active site by forming hydrogen bond with one or more of the following residues: GLU634, GLU604 and MET636. However, the interactions of cannabinol and Delta 9 THC with residues, did not promote any form of intermolecular interactions.

Validation of the feasibility of binding affinity of a ligand in complex with a receptor is crucial, hence the need to consider the stability of the complex through post docking analysis [37]. The binding free energy of the docked complexes were visualized and calculated via Prime MM-GBSA. The result showed that the ligand bound protein were stable, which signifies that the result of the docking score is reliable. Overall, CBC had the best IFD scores and binding free energies across the three proteins studied and it interacted with notable amino acids within their active sites.

In reality, a number of elements must coexist in order to preserve normal sexual function. When these parameters are changed, male sexual dysfunction (MSD) occurs. Intra cavernosal nitric oxide system, androgen functions, vascular and neuronal activity, are a few examples of such factors [12]. An increase in NO (vasodilator) level in the trabecular arteries and smooth muscle of the penis caused arterial dilatation which allowed the corpora cavernosa and corpora spongiosum of the penis to be filled with blood, leading to penile rigidity [38]. Through the endothelial nitric oxide synthase (eNOS) pathway, which is activated by oxygen, the cofactors Ca^{2+} and calmodulin, and guanylyl cyclase, nitric oxide reduces the tone of many types of smooth muscles, including blood vessels [39].

The reduction in penile NO, parameters of penile erection, can be attributed to CBC induction of cavarnosal vasoconstriction through cyclic adenosine monophosphate (cAMP) depletion. Another likely mechanism could also be the diversion of arginine, a substrate for NO synthesis to the production of urea, evident through the elevation of

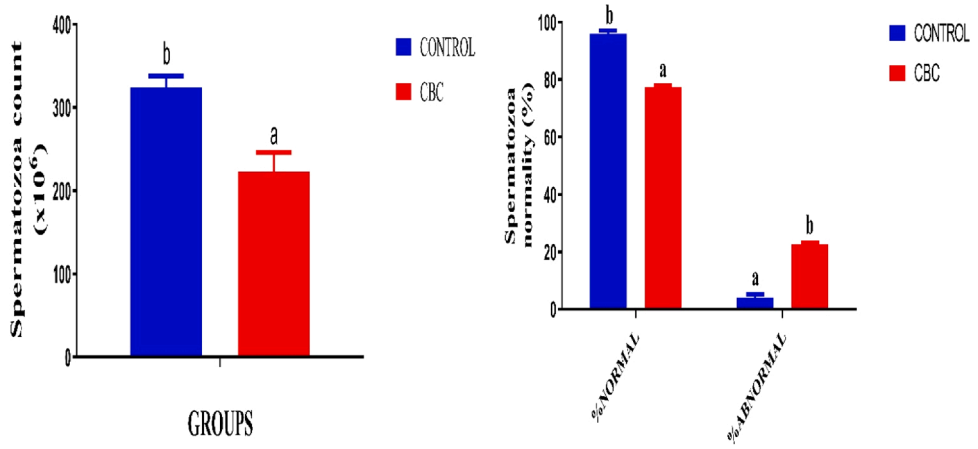

Fig. 8. Spermatozoa concentration and % normality in the semen Data are presented as mean ± . SEM (n = 3). Bars with different letters are statistically distinct at $p < 0.05$.

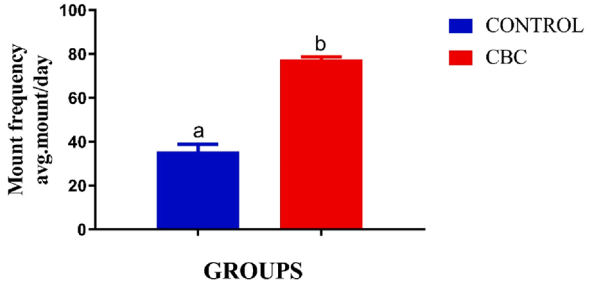

Fig. 9. Mount frequency in rats. Data are presented as mean ± SEM (n = 6). Bars with different letters are statistically distinct at $p < 0.05$.

arginase activity, an enzyme expressed throughout the body in endothelial and muscle cells [40]. Furthermore, increase in the activity of PDE5 which is responsible for rapid decyclization of cyclic adenosine monophosphate (cAMP) and guanosine monophosphate (cGMP) is also another possible mechanism by which CBC could induce erectile dysfunction.

The importance of cholesterol for normal testicular as well as ovulation and folliculogenesis activities has been well documented [41, 42]. Together with 17β-hydroxysteroid dehydrogenase (17β-HSDs), they play essential roles in steroidogenesis of testicular and ovarian endocrine tissues [43]. Cholesterol is a precursor for steroidogenesis while 17β-HSDs catalyse the final- step in the synthesis of active steroids [44]. In this study, CBC inhibited the activities of 17β-HSD and cholesterogenesis significantly in the testes and ovaries of rats administered 10 mg/kg dose of the cannabinoid, these results are consistent with the finding of [45]. It is therefore not surprising to observe a notable decrease in the concentrations of all endocrine sexual hormones evaluated.

The determination of serum concentrations of testosterone, progesterone, luteinizing, and follicle- stimulating hormone have been used for the assessment of sexual function by many researchers [46, 47, 48, 41]. An essential intermediate for the synthesis of oestradiol, dihydrotestosterone, and other androgens necessary for healthy male and female sexual function and fertility is testosterone, which induces androgenicity [49]. Follicle stimulating hormone (FSH) is necessary for gamete generation, gonadal development, and maturation at puberty [50]. While luteinizing hormone stimulates the secretion of steroid hormones from gonads [51,52]. We can therefore postulate that the reduction of endocrine hormone levels observed in this work, is unarguably linked to CBC inhibition of cholesterogenesis and 17β-HSD activities.

Key indicators of male fertility include variables like sperm count, sperm motility, sperm viability, and sperm morphology because these are the key indicators of testicular spermatogenesis and epididymal maturation [53,54]. The substantial drop in sperm count could be the result of androgen deprivation, which may have decreased spermatogenesis due to the reduced testosterone levels. The spermatozoa's ability to mature and survive physiologically may have been hampered by CBC, which may have impaired sperm motility and produced a large number of aberrant cells [55]. Carvalho et al. [56] reported similar result after oral administration of CBD; A decreased total circulating testosterone by 76% and significantly increased abnormalities in spermiation, 38% reduction in spermatozoa in the epididymis tail and more head abnormalities in the sperm as well as cytoplasmic droplets in the flagella medial region using mice model.

The recorded increase in Mount Frequency (MF) is an indication that the animals were sexually invigorated and desirous. This is however surprising, as penile function parameters points towards induction of erectile dysfunction. A plausible explanation could be that CBC initiated sexual motivation as indicated by the MF but this does not translate to the ability to sustain penile erection.

Indeed, downregulation of expressions of androgen receptor (AR) and follicle stimulating hormone receptor (FSHR), as well as severe congestion of cortical capillaries and lesions in the testes seen in histological evaluations might also be a cue to invoke impairments on reproductive functions by CBC in this study.

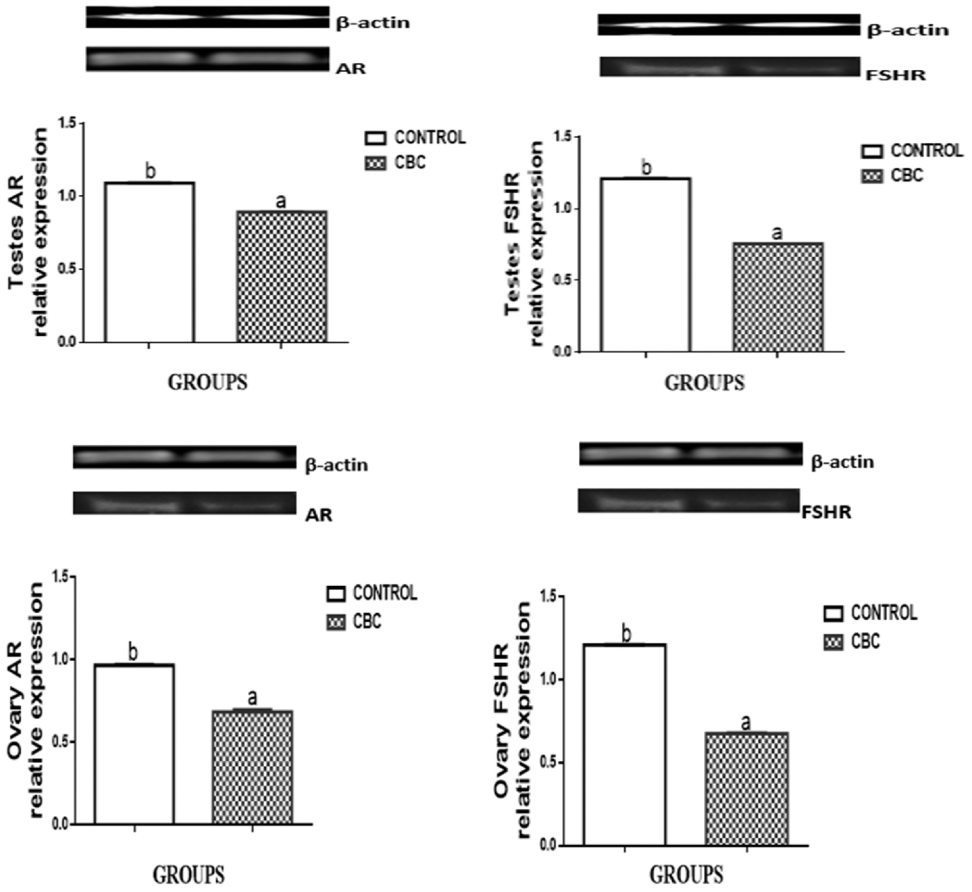

Fig. 10. Relative expression of testicular and ovarian androgen receptor (AR) and follicle stimulating hormone. Values are expressed as mean ± SEM (n = 3), bars with different letters are statistically distinct at $p < 0.05$.

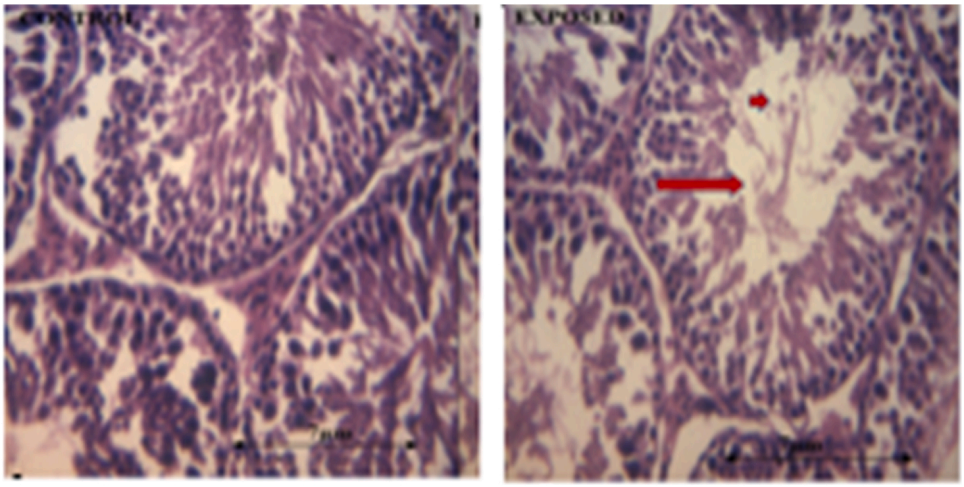

Fig. 11. Photomicrographs of the parent testes shows normal testicular cells in the control group with a severe lesion and congestion of neutrophils in the CBC-exposed group (Red arrow) H&E X 400.

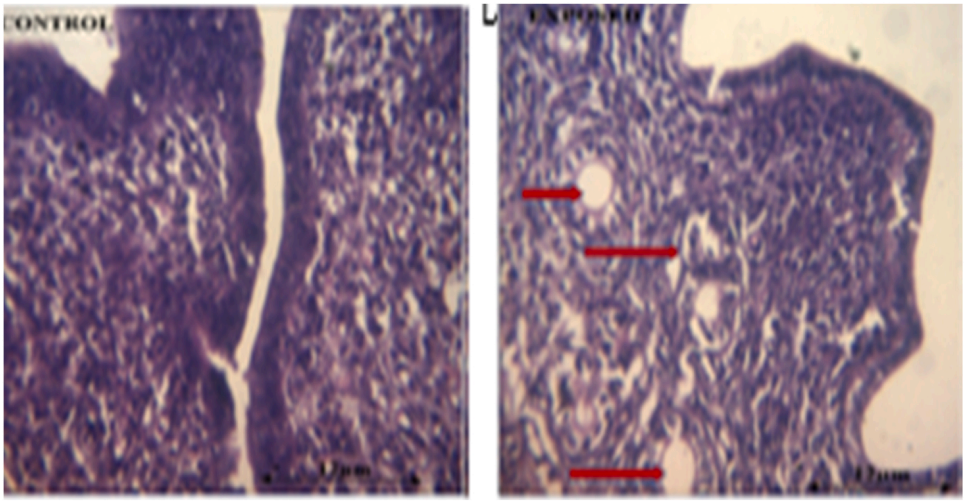

Fig. 12. Photomicrographs shows normal uterine cell in the control, and the exposed group with a prominent glandular investment in the endometrium in the exposed group (Red arrows). H&E X 400.

Conclusion

Early life exposure to cannabis modulates the morphology of reproductive organs and their functions in male and female Wistar rats via cannabichromene inhibition of steroidogenesis, erectile function and gene expressions.

Ethical approval

The research was approved by the supervisory committee on research, Biochemistry department, Federal University of Agriculture (PG15/0103(5)).

Funding

This research received no external funding.

CRediT authorship contribution statement

O.A.D. and O.A.T conceptualized and designed the research study, O. A.T, E.I.U. A.S.J. and O.A.O performed the study. O.A.T., E.I.U. and A.S. J. generated and curated the data. O.A.T., O.A.D., and O.A.O wrote the first draft of the manuscript; O.A.D., G.A.D. and O.A. supervised the study; O.A.D., O.A.T., G.A.D. and O.A. revised the manuscript for intellectual content. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

References

[1] R.J. Bloomer, M. Butawan, N. Smith, Chronic marijuana smoking does not negatively impact select blood oxidative stress biomarkers in young, physically active men and women, Health 10 (1) (2018) 960–970.
[2] W. Hall, R.L. Pacula, Cannabis Use and Dependence: Public Health and Public Policy, Cambridge University Press, 2003, p. 38.
[3] D. Namdar, M. Mazuz, A. Ion, H. Koltai, Variation in the compositions of cannabinoid and terpenoids in *Cannabis sativa* derived from inflorescence position alongthe steam and extraction, Ind. Crops Prod. 113 (6) (2018) 376–382.
[4] O.A. Dosumu, O.A. Taiwo, O.A. Akinloye, A.O. Obadina, S.O. Rotimi, O. P. Owolabi, O.A. Ojo, Implications of Cannabis sativa serotonin receptors 1B (HTR1B) and 7 (HTR7) genes in modulation of aggression and depression, Vegetos 35 (1) (2021) 19–25.
[5] W.A. Devane, L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, Isolation and structure of a brain constituent that binds to the cannabinoid receptor, Science 258 (23) (1992) 1946–1949.
[6] A. Abdul Salam, I. Elsegaey, R. Khraif, A. AlMutairi, A. Aldosari, Components and public health impact of population growth in the arab world, PLoS ONE 10 (5) (2015), e0124944.
[7] K.M. Menon, B. Menon, Structure, function and regulation of gonadotropin receptors - a perspective, Mol. Cell. Endocrinol. 356 (1–2) (2012) 88–97.
[8] M. Jain, M. Singh, Environmental toxins and infertility. StatPearls. Treasure Island (FL), StatPearls Publishing, 2022.
[9] S.S. du Plessis, A. Agarwal, A. Syriac, Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility, J. Assist. Reprod. Genet. 32 (11) (2015) 1575–1588.
[10] O.S. Walker, A.C. Holloway, S. Raha, The role of the endocannabinoid system in female reproductive tissues, J. Ovarian Res. 12 (1) (2019) 3–10.
[11] a) V. Tech, J. Mcguire, T. Tan, R. Vita, Mechanical properties of female reproductive organs and supporting connective tissues: a review of the current state of knowledge, Appl. Mech. Rev. 68 (6) (2017) 1–12;
b) A.H. Clayton, S.A. Kingsberg, I. Goldstein, Evaluation and management of hypoactive sexual desire disorder, Sex. Med. 6 (2) (2018) 59–74.
[12] A.H. Clayton, S.A. Kingsberg, I. Goldstein, Evaluation and management of hypoactive sexual desire disorder, Sex. Med. 6 (2) (2018) 59–74.
[13] R. Mazzilli, J. Elia, M. Delfino, F. Benedetti, G. Scordovillo, F. Mazzilli, Prevalence of diabetes mellitus (DM) in a population of men affected by Erectile Dysfunction (ED), ClinicalTherapeutics 166 (1) (2015) 317–320.
[14] P.F. Whiting, R.F. Wolff, S. Desphande, M. Di Nisio, S. Duffy, A.V. Hernandez, J. C. Keurentjes, S. Lang, M. Westwood, J. Kleijmen, Cannabinoids for medical use: a systematic review and meta-analysis, J. Am. Med. Assoc. 313 (24) (2015) 2456–2473.
[15] G. Tarantino, V. Citro, C. Finelli, Recreational drugs: a new health hazard for patients with concomitant chronic liver diseases, J. Gastrointest. Liver Disord. 23 (2014) 79–84.
[16] O.O. Elekofehinti, O. Iwaloye, C.D. Famusiwa, O. Akinseye, J.B. Rocha, Identification of main protease of coronavirus SARS-CoV-2 (M^{pro}) inhibitors from melissa officinalis, Curr. Drug Discov. Technol. 18 (5) (2021), e17092020186048.
[17] O. Iwaloye, O.O. Elekofehinti, B. Kikiowo, T.M. Fadipe, M.O. Akinjiyan, E.O. Ariyo, O.O. Aiyeku, N.A. Adewumi, Discovery of traditional chinese medicine derived compounds as wild type and mutant plasmodium falciparum dihydrofolate reductase inhibitors: induced fit docking and ADME studies, Curr. Drug Discov. Technol. 18 (4) (2021) 554–569.
[18] O. Iwaloye, O.O. Elekofehinti, E.A. Oluwarotimi, K. Babatomiwa, I.A. Momoh, E. O. Ariyo, In silico molecular studies of natural compounds as possible anti-Alzheimer's agents: ligand-based design, Netw. Model. Anal. Health Inform. Bioinforma. 9 (1) (2020) 1–4.
[19] O. Iwaloye, O.O. Elekofehinti, E.A. Oluwarotimi, K. Babatomiwa, T.M. Fadipe, Insight into Glycogen synthase kinase-3β inhibitory activity of Phyto-constituents from *Melissa officinalis*: *In silico* studies, Silico Pharmacol. 8 (1) (2020) 2–15.
[20] O.O. Elekofehinti, O. Iwaloye, S.S. Josiah, A.O. Lawal, M.O. Akinjiyan, E.O. Ariyo, Molecular docking studies, molecular dynamics and ADME/tox reveal therapeutic

potentials of STOCK1N-69160 against papain-like protease of SARS-CoV-2, Mol. Divers. 25 (3) (2021) 1761–1773.
[21] L.I. Gidez, G.J. Miller, M. Burstein, S. Slagle, H.A. Eder, Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure, J. Lipid Res. 23 (8) (1982) 1206–1223.
[22] W.J. Krause, The art of examining and interpreting histologic preparations. A student handbook, Partheton Publishing Group, UK, 2001, pp. 9–10.
[23] R.A. Drury, E.A.C. Wallington. Coreleton's Histological technique, 4th ed., Oxford University Press, Oxford, 1980.
[24] M.N.A. Rao, Nitric oxide scavenging by curcuminoids, J. Pharm. Pharmacol. 49 (1) (1997) 105–107.
[25] C.C. Allain, L.S. Poon, C.S. Chan, W.F.P.C. Richmond, P.C. Fu, Enzymatic determination of total serum cholesterol, Clin. Chem. 20 (4) (1974) 470–475.
[26] S. Marchais-Oberwinkler, C. Henn, G. Möller, T. Klein, M. Negri, A. Oster, A. Spadaro, R. Werth, M. Wetzel, K. Xu, M. Frotscher, R.W. Hartmann, J. Adamski, 17β-Hydroxysteroid dehydrogenases (17β-HSDs) as therapeutic targets: protein structures, functions, and recent progress in inhibitor development, J. Steroid Biochem. Mol. Biol. 125 (1–2) (2011) 66–82.
[27] N.W. Tietz. Clinical Guide to Laboratory Tests, 3rd ed., W. B. Saunders Company, Philadelphia, 1995.
[28] J.M. Kjeld, P. Harsoulis, S.F. Kuku, I.C. Marshall, B. Kaufman, T.R. Fraser, Infusions of hFSH and hLH in normal men, Acta Endocrinol. 81 (2) (1976) 225–233.
[29] D.C. Cumming, M.W. Vickovic, S.R. Wall, M.R. Fluker, Defects in pulsatile LH release in normally menstruating runners, J. Clin. Endocrinol. Metab. 60 (1) (1985) 810–812.
[30] G.E. Abraham, W.D. Odell, R.S. Swerdloff, K. Hopper, Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17-hydroxyprogesterone and estradiol-17β during the menstrual cycle, J. Clin. Endocrinol. Metab. 34 (1) (1972) 312–318.
[31] M. Cheesbrough, Part 2. District laboratory practice in tropical countries, 2nd edition.,, Cambridge University Press, 2006, p. 130. Part 2.
[32] K.O. Oyedeji, A.F. Bolarinwa, A.K. Adigun, Effect of aspirin on reproductive functions in male albino rats, IOSR J. Pharm. Biol. Sci. 4 (6) (2013) 49–54.
[33] A. Agmo, Male rat sexual behavior, Brain Res. Protoc. 1 (2) (1997) 203–209.
[34] A.O. Ojo, A.B. Ojo, A.B. Taiwo, O. Oluba, Novel coronavirus (sars-cov-2) main protease: molecular docking of puerarin as a potential inhibitor, Malays. J. Biochem. Mol. Biol. 10 (1) (2021) 108–114.
[35] B. Kikiowo, A.J. Ogunleye, O. Iwaloye, T.T. Ijatuyi, N.S. Adelakun, W.O. Alashe, Induced fit docking and automated QSAR studies reveal the ER-α inhibitory activity of cannabis sativa in breast cancer, Recent Pat. Anti-Cancer Drug Discov. 16 (2) (2021) 273–284.
[36] A.O. Fadaka, O.A. Taiwo, O.A. Dosumu, O.P. Owolabi, A.B. Ojo, N.R. Sibuyi, A. Ashwil Klein, A.M. Madiehe, M. Meyer, O.A. Ojo, Computational prediction of potential drug-like compounds from *Cannabis sativa* plant extracts targeted towards Alzheimer therapy, J. Mol. Liq. 360 (4) (2021), 119393.
[37] O.A. Taiwo, O.A. Dosumu, E.O. Oni, V.O. Akomolafe, S.T. Elazab, S. Qusti, E. M. Alshammari, G.E. Batiha, O.A. Ojo, Preclinical prediction of phytochemicals identified from cannabis as novel inhibitors of TEX 11, DHCR24, and CatSper 1 in fertility drug design, Inform. Med. Unlocked 26 (100747) (2021) 2352–2363.
[38] Y.A. Lu, J.G. Je, J. Hwang, Y.J. Jeon, B. Ryu, *Ecklonia cava* extract and its derivative dieckol promote vasodilation by modulating calcium signaling and PI3K/AKT/eNOS pathway in In Vitro and In Vivo models, Biomedicines 9 (4) (2021) 438–450.
[39] C.K. Patel, N. Bennett, Advances in the treatment of erectile dysfunction: what's new and upcoming? F1000 Res. 5 (1) (2016) 369–375.
[40] T.O. Kayode, M.T. Yakubu, *Parquet inanigrescens* leaves: chemical profile and influence on the physical and biochemical indices of sexual activity of male wistar rats, J. Integr. Med. 15 (1) (2017) 64–76.
[41] M.T. Yakubu, A.R. Atoyebi, Brysocarpus coccineus (Schum & Thonn) root reinstates sexual competenceand testicular function in paroxetine-inducedSexual dysfunction in male Wistar rats, Andrologia 50 (4) (2018), e12980.
[42] M.T. Yakubu, T.O. Oyeyipo, A.L. Quadri, M.A. Akanji, Effects of aqueous extract of Musaparadisiacaroot on testicular function parameters of male rats, J. Basic Clin. Physiol. Pharmacol. 24 (2) (2013) 151–157.
[43] E. Hilborn, O. Stål, A. Jansson, Estrogen and androgen-converting enzymes 17β-hydroxysteroid dehydrogenase and their involvement in cancer: with a special focus on 17β-hydroxysteroid dehydrogenase type 1, 2, and breast cancer, Oncotarget 8 (18) (2017) 30552–30562.
[44] S.M. Kim, J.S. Kim, A review of mechanisms of implantation, Dev. Reprod. 21 (4) (2017) 351–359.
[45] D. Sarkar, A. Chakraborty, D. Mahapatra, A.K. Chandra, Morphological and functional alterations of female reproduction after regular exposure of bamboo shoots of North East India, Asian Pac. J. Reprod. 6 (1) (2017) 151–157.
[46] M.T. Yakubu, S.S. Salimon, R.O. Abdul-Rasheed, F.M. Opakunle, R.O. Jimoh, M. O. Nafiu, Effects of aqueous extract of *Cissus populnea* stem on function indices of the ovary and uterus of female wistar rats, Centrepoint J. 20 (1) (2014) 131–141.
[47] C. Kim, S.D. Harlow, H. Zheng, D.S. McConnell, J.F. Randolph, Changes in androstenedione, dehydroepiandrosterone, testosterone, estradiol, and estrone over the menopausal transition, Women's Midlife Health 3 (1) (2017) 9–23.
[48] Q.O. Nurudeen, T.O. Ajiboye, Aqueous root extract of *Lecaniodiscus cupanioides* restores the alterations in testicular parameters of sexually impaired male rats, Asian Pac. J. Reprod. 1 (2) (2012) 120–124.
[49] M. Schulster, A.M. Bernie, R. Ramasamy, The role of oestradiol in male reproductive function, Asian J. Androl. 18 (3) (2016) 435–440.
[50] A. Al-Sinani, W. Mula-Abed, M. Al-Kindi, G. Al-Kusaibi, H. Al-Azkawi, N. Nahavandi, A novel mutation causing 17-β-hydroxysteroid dehydrogenase type 3 deficiency in an omani child: first case report and review of literature, Oman Med. J. 30 (2) (2015) 129–134.
[51] T.B. Mesen, S.L. Young, Progesterone and the luteal phase: a requisite to reproduction, Obstet. Gynecol. Clin. North Am. 42 (1) (2015) 135–151.
[52] M. Ayub, A.K. Ibrahim, A. Kausar, M. Fahad, Effective role of progesterone with proven evidence in threaten abortion at public healthcare sectors, J. Med. Res. 1 (4) (2015) 115–117.
[53] A.L. Quadri, M.T. Yakubu, Fertility enhancing activity and toxicity profile of aqueous extract of *Chasmanthera dependens* roots in male rats, Andrologia 49 (10) (2017), e12775.
[54] Y. Zhang, T. Ma, Z. Su, M. Ye, H. Tian, J. Li, J. Liu, Varicoceles affect semen quality of infertile men in Southern China, Medicine 96 (31) (2017), e7707.
[55] S.M. Kala, P.S. Tresina, V.R. Mohan, Antifertility investigations on the ethanol extract of *Eugenia singampattiana* Bedd leaf in Male Albino Rats, Int. J. Pharm. Sci. Res. 5 (7) (2014) 2991–2998.
[56] R.K. Carvalho, M.L. Santos, M.R. Souza, T.L. Rocha, F.S. Guimarães, J.A. Anselmo-Franci, R. Mazaro-Costa, Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss mice, J. Appl. Toxicol. 38 (1) (2018) 1215–1223.