Phytochemical Profiling, Antioxidant Capacity, Cytotoxicity, and *In silico* Androgen Receptor Binding Interactions of the Aqueous–Ethanol Root Extract of *Cnestis ferruginea* Vahl ex DC. (Connaraceae)

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DOI: https://doi.org/10.51584/IJRIAS.2026.11060317

Received: 01 July 2026; Accepted: 06 July 2026; Published: 21 July 2026

ABSTRACT

Male infertility affects an estimated 55 million men worldwide, with prevalence rising across sub-Saharan Africa where affordable interventions remain limited. Oxidative stress is a key driver of male reproductive dysfunction, disrupting spermatogenesis via lipid peroxidation, DNA damage, and impaired androgen receptor (AR) signalling. Medicinal plants with antioxidant and AR-interacting phytoconstituents offer an underexplored strategy for fertility-modulating lead discovery. *Cnestis ferruginea* Vahl ex DC. (Connaraceae), a West African shrub used ethnomedicinally for male sexual dysfunction, lacks molecular-level pharmacological evidence. This study reports the phytochemical profile, GC–MS composition, DPPH antioxidant activity, brine shrimp lethality, and *in silico* AR binding of *C. ferruginea* aqueous–ethanol root extract (AECF). Cold maceration (65:35 v/v ethanol–water) yielded 4.70% (w/w) extract containing flavonoids, phenols, terpenoids, steroids, saponins, tannins, phlobatannins, and reducing sugars. GC–MS identified 17 compounds (89.27% of the profile), dominated by methyl benzoate (67.12%). Docking against the AR ligand-binding domain (PDB: 2AM9) showed methyl benzoate scored −2.632 and −2.921 kcal/mol at AR Sites 1 and 2, comparable to testosterone (−2.539 and −2.926 kcal/mol), with slightly superior binding. All key constituents satisfied Lipinski's Rule of Five, supporting oral bioavailability. DPPH assay gave an IC_{50} of 97.94 µg/mL (vs 34.60 µg/mL for ascorbic acid), indicating moderate antioxidant activity; brine shrimp lethality gave an LC_{50} of 347.80 ppm, indicating moderate cytotoxicity. These findings provide the first computational evidence for AR-mediated androgenic potential of *C. ferruginea*, supporting its traditional use in male reproductive health and identifying methyl benzoate as a priority candidate for isolation and *in vivo* validation.

Keywords: *Cnestis ferruginea*; androgen receptor; methyl benzoate; antioxidant; brine shrimp lethality; GC–MS; male reproductive health

INTRODUCTION

The global burden of male infertility has increased substantially over the past three decades. In 2021, an estimated 55 million men of reproductive age were living with infertility worldwide, corresponding to approximately 1,820 cases per 100,000 population, with age-standardised prevalence rates increasing by an average of 0.49% annually between 1990 and 2021.[1] The global number of male infertility cases increased by

over 74% between 1990 and 2021, with the highest burden concentrated in middle socio-demographic index regions and a disproportionate rise recorded in low- and low-middle income countries.[2] Sub-Saharan Africa bears a particularly significant proportion of this burden, compounded by limited access to assisted reproductive technologies and the high cost of synthetic pharmacotherapies.[5]

Oxidative stress has emerged as one of the most clinically significant aetiological factors in male infertility. During the final stages of sperm maturation, the majority of cytoplasm is discarded, leaving spermatozoa with a markedly diminished antioxidant defence system that renders them highly susceptible to the damaging effects of reactive oxygen species (ROS).[7] Oxidative stress disrupts the balance between ROS and antioxidants, detrimentally affecting sperm function and viability through molecular changes including DNA damage, lipid peroxidation, and alterations in protein expression.[3,4] In parallel, disruption of androgen receptor (AR) signalling; whether through insufficient ligand availability or competitive receptor antagonism impairs spermatogenesis, testosterone-regulated gene expression, and the development of secondary sexual characteristics essential for male reproductive competence.[6,7]

The AR is a member of the nuclear receptor superfamily encoded by a gene on the X chromosome at locus Xq11–Xq12, consisting of four functional domains: the N-terminal domain responsible for transcriptional activation, the DNA-binding domain, the hinge region regulating nuclear localisation, and the ligand-binding domain (LBD) which binds androgens including testosterone and dihydrotestosterone.[28] Recent studies have shed new light on the broader implications of AR in male fertility, demonstrating that alterations in AR signalling can influence spermatogenesis, impacting both the quantity and quality of sperm production.[6] The AR-LBD therefore represents a validated and mechanistically relevant molecular target for the identification of phytochemical lead compounds with androgenic or fertility-modulating activity.

Medicinal plants constitute the primary healthcare resource for an estimated 80% of the population in sub-Saharan Africa, and a significant proportion of traditional formulations in this region address male sexual dysfunction and infertility.[8] However, the molecular mechanisms underlying the claimed fertility-enhancing effects of most African medicinal plants remain poorly characterised, and systematic phytochemical and pharmacological validation studies are limited. *Cnestis ferruginea* Vahl ex DC. (Connaraceae), known in Yoruba as *Ègún-ègún* or *Gbóyìn-gbóyìn*, and distributed from Senegal to Cameroon is among the most widely cited plants in West African ethnomedicine for the management of male reproductive disorders including sexual dysfunction, oligospermia, and infertility.[9,10]

Different parts of *C. ferruginea* are used in traditional African medicine for treating infectious diseases such as dysentery, bronchitis, gonorrhoea, and syphilis, among other conditions.[31] Its roots are specifically employed in Yoruba traditional medicine as decoctions for enhancing male sexual vigour, and the plant has been reported to exhibit antioxidant, anti-inflammatory, analgesic, antimicrobial, laxative, anticonvulsant, and hypoglycaemic activities.[10,11] Recent bioassay-guided studies have further established that *C. ferruginea* constituents including hydroquinone and benzoquinone are responsible for documented antibacterial activity.[27] Notwithstanding these advances, a systematic investigation integrating GC–MS chemical profiling with computational AR docking and bioactivity evaluation of the root extract has not previously been reported.

In silico molecular docking has become an indispensable tool in natural product drug discovery, enabling the rapid and cost-effective identification of phytochemical constituents capable of interacting with validated biological targets prior to resource-intensive *in vitro* and *in vivo* experimentation.[13,14] Applied to the AR-LBD, this approach has yielded mechanistically credible predictions of androgenic and anti-androgenic activity for diverse classes of natural compounds, providing a rational basis for prioritising candidates for further pharmacological development.[29]

The present study was therefore designed to: (i) characterise the qualitative phytochemical composition and GC–MS volatile profile of the aqueous–ethanol root extract of *C. ferruginea*; (ii) evaluate its antioxidant capacity by DPPH radical scavenging assay and cytotoxicity by brine shrimp lethality assay; and (iii) perform *in silico* molecular docking of identified GC–MS constituents against the human AR-LBD to computationally evaluate their androgenic potential as a mechanistic basis for the plant's traditional use in male reproductive health.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume XI Issue VI June 2026

MATERIALS AND METHODS

Plant Material Collection, Authentication, and Preparation

Fresh roots of *Cnestis ferruginea* Vahl ex DC. (Connaraceae) were collected from the Botanical Garden of the University of Ibadan, Ibadan, Oyo State, Nigeria. Taxonomic identification and authentication were carried out by a plant taxonomist at the University of Ibadan Herbarium, where a voucher specimen was deposited under accession number UIH-23515. The roots were thoroughly washed with clean running water, air-dried under shade at ambient temperature to prevent photodegradation of thermolabile constituents, and pulverised to a fine granule-size using a clean mechanical grinder. The pulverised plant material was stored in an airtight container at room temperature until extraction.

Preparation of Extract and Determination of Extraction Yield

One kilogram (1.0 kg) of powdered *C. ferruginea* roots was subjected to cold maceration in 65:35 (v/v) ethanol–distilled water at ambient temperature for 72 hours with intermittent manual agitation. The resulting mixture was filtered sequentially through muslin cloth followed by Whatman No. 1 filter paper. The combined filtrates were concentrated under reduced pressure at 40 °C using a rotary evaporator (Büchi R-210, Switzerland), and the resulting concentrate was freeze-dried to obtain the crude aqueous–ethanol extract of *C. ferruginea* roots (AECF). The dried extract was weighed, and percentage yield was calculated gravimetrically as: % yield = (mass of dried extract / mass of starting material) × 100. The extract was stored in a sealed amber vial at −20 °C until use.

Qualitative Phytochemical Screening

Preliminary phytochemical screening of AECF was performed using standard reagent-based methods described by Evans[11] and Sasidharan *et al*.[12] The extract was tested for alkaloids (Dragendorff's and Mayer's reagents), flavonoids (alkaline reagent test), steroids and terpenoids (Salkowski and Liebermann–Burchard tests), phenols (ferric chloride test), saponins (froth test), tannins (ferric chloride), phlobatannins (vanillin-HCl), cardiac glycosides (Keller–Kiliani test), and reducing sugars (Benedict's test). Positive results were confirmed by characteristic colour changes as described in the standard protocols.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Volatile chemical profiling of AECF was performed using an Agilent Technologies 7890A Gas Chromatograph interfaced with a 5975C Mass Selective Detector (Agilent Technologies, USA). Chromatographic separation was achieved on an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). High-purity helium (99.999%) served as the carrier gas at 1.0 mL/min with a split ratio of 1:10. The GC oven temperature was programmed as follows: initial temperature of 70 °C held for 2 minutes, ramped at 12 °C/min to 280 °C, and held for 6 minutes. The injector temperature was maintained at 250 °C. The mass spectrometer operated in electron ionisation (EI) mode at 70 eV, with ion source and quadrupole temperatures at 230 °C and 150 °C, respectively. A 1.0 µL aliquot was injected in split mode. Compound identification was based on comparison of mass spectral fragmentation patterns against the NIST 11.L library database (match threshold ≥ 80%). Relative percentage abundances were calculated from peak area integration relative to the total ion chromatogram.

In silico Molecular Docking Studies

In silico molecular docking was performed to predict the binding interactions of major GC–MS-identified constituents of AECF with the human androgen receptor ligand-binding domain. The AR crystal structure was retrieved from the RCSB Protein Data Bank (PDB ID: 2AM9; resolution: 1.64 Å; human AR-LBD in complex with testosterone). Protein preparation was performed using the Protein Preparation Wizard module of the Schrödinger Maestro Suite (Schrödinger LLC, New York, USA, version 2023), which included removal of co-crystallised ligands and water molecules, addition of hydrogen atoms, assignment of bond orders, and restrained energy minimisation using the OPLS3e force field. Active binding pockets were identified using the SiteMap

module. Ligand structures were retrieved from PubChem, geometry-optimised using LigPrep with OPLS3e parameterisation, and subjected to ionisation state generation at pH 7.0 ± 2.0. Molecular docking was performed using the Glide Standard Precision (SP) scoring module.[13,14] Drug-likeness was evaluated against Lipinski's Rule of Five.[15]

DPPH Free Radical Scavenging Assay

Antioxidant activity of AECF was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay following the modified protocols of Onocha *et al*.[16] and Aiyedun *et al*.[17] Serial dilutions of AECF and ascorbic acid were prepared in methanol at final concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/mL. Reactions were conducted in triplicate in 96-well microplates with 50 µL sample and 150 µL of freshly prepared 0.004% (w/v) DPPH methanolic solution (ascorbic acid applied at 10 µL/well owing to its substantially higher potency). Absorbance was measured at 517 nm after 30 minutes of dark incubation at 27°C using a Spectrumlab 752S UV–Visible spectrophotometer. Percentage inhibition was calculated as: % inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. IC_{50} values were determined by linear regression of percentage inhibition against $\log_{10}$-transformed concentration.

Brine Shrimp Lethality Assay (BSLA)

Cytotoxicity of AECF was assessed using the Brine Shrimp Lethality Assay as described by Meyer *et al*.[18] *Artemia salina* Leach eggs were hatched in artificial seawater (3.8% NaCl, w/v) for 48 hours at 28 ± 1 °C. Serial dilutions of AECF were prepared at 1000, 100, and 10 ppm. DMSO-only controls were prepared at identical solvent concentrations. Ten nauplii per vial were exposed in triplicate and mortality assessed after 24 hours. Observed mortality was corrected for background mortality in the corresponding DMSO vehicle control using Abbott's formula: corrected mortality (%) = $[(T - C) / (100 - C)] \times 100$, where T is the observed percentage mortality in the AECF-treated group and C is the percentage mortality in the vehicle control at the corresponding concentration.[32] LC_{50} values were determined by Probit analysis[21] of the Abbott-corrected mortality data, and toxicity was classified by Meyer criteria.[18]

Statistical Analysis

All quantitative data are expressed as mean ± standard deviation (SD) of triplicate determinations. Statistical comparisons among treatment groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. Statistical significance was accepted at $p \leq 0.05$. IC_{50} and LC_{50} values were calculated by linear regression and Probit regression analysis, respectively. All statistical computations were performed using GraphPad Prism (version 9.0, GraphPad Software Inc., San Diego, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS AND DISCUSSION

Extraction Yield and Qualitative Phytochemical Composition

Cold maceration of 1.0 kg of pulverised *C. ferruginea* roots in 65:35 (v/v) ethanol–water yielded 46.99 g of dry crude extract, corresponding to a gravimetric percentage yield of 4.70% (w/w) (Table 1). This is consistent with extraction yields reported for other West African medicinal plants subjected to hydroethanolic maceration, typically ranging between 3% and 8% (w/w), and reflects the capacity of the binary solvent system to solubilise a broad spectrum of semi-polar bioactive metabolites.[19]

Qualitative phytochemical screening of AECF revealed the presence of flavonoids, phenols, steroids, terpenoids, saponins, tannins, phlobatannins, and reducing sugars, while alkaloids and cardiac glycosides were absent (Table 2). This metabolite profile is consistent with previously reported phytochemical compositions of *C. ferruginea* extracts, and is pharmacologically significant. Phenolic compounds and flavonoids are well-established free radical scavengers directly relevant to the oxidative stress-driven impairment of male reproductive function. Plant steroids and terpenoids, structurally related to the isoprenoid biosynthetic pathway, have been shown to

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume XI Issue VI June 2026

interact with mammalian steroid hormone receptors and modulate endocrine-related pathways, providing a plausible biochemical basis for the traditional androgenic claims associated with this plant.

Table 1. Extraction Yield of AECF from *C. ferruginea* Roots

Parameter	Value
Mass of starting material (g)	1000.00
Mass of crude dried extract (g)	46.99
Percentage yield (% w/w)	4.70
Solvent system	Ethanol–Water 65:35 (v/v)
Extraction method	Cold maceration, 72 h

Table 2. Qualitative Phytochemical Composition of AECF

Phytochemical Class	Reagent/Test	Result
Flavonoids	Alkaline reagent	+
Phenols	Ferric chloride	+
Steroids	Liebermann–Burchard	+
Terpenoids	Salkowski	+
Saponins	Froth test	+
Tannins	Ferric chloride	+
Phlobatannins	Vanillin-HCl	+
Reducing Sugars	Benedict's test	+
Alkaloids	Dragendorff's / Mayer's	−
Cardiac Glycosides	Keller–Kiliani	−

+ = present; − = absent

GC–MS Chemical Profiling

GC–MS analysis of AECF identified 17 volatile and semi-volatile chemical constituents, collectively accounting for 89.27% of the total chromatographic profile (Table 3). The identified compounds span six broad chemical classes, with the aromatic ester methyl benzoate constituting the dominant single constituent at 67.12% total abundance, followed by nitroaromatic and nitroso derivatives (4.54%), nitrogen-containing heterocycles of the pyrimidine/diazine class (4.27%), aliphatic fatty acid esters (3.02%), a terpenoid derivative (1.29%), and a group of minor aliphatic and alicyclic constituents comprising alkyne esters, unsaturated alcohols, an acyl chloride, an aromatic carboxylic acid, an alkynol, a macrocyclic lactone, an epoxide, and a saturated hydrocarbon (collectively 9.03%).

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Methyl benzoate (RT 4.735 min; $C_6H_5COOCH_3$; 67.12%) was identified as the overwhelmingly dominant constituent of AECF, contributing nearly two-thirds of the total detected volatile fraction.

This aromatic ester has been reported to possess documented antimicrobial efficacy,[24] insecticidal activity,[25] and antioxidant properties in structurally related benzoate ester series.[26] Its predominance in AECF is a particularly noteworthy finding given its favourable drug-likeness profile and the *in silico* AR binding results discussed in Section 3.3. Although the chemical impossibility of methyl ester formation from the ethanol–water extraction system argues against solvent-mediated artifact origin, the absence of a concurrent solvent blank GC–MS run is a methodological limitation; confirmation via solvent blank analysis is recommended in future investigations.

Table 3. GC–MS Chemical Constituents Identified in AECF

No.	Compound	RT (min)	Molecular Formula	% Area	Chemical Class
1.	5-Methyl-pyrimidine	3.476	$C_5H_6N_2$	1.30	Diazine
2.	Methyl benzoate	4.735	$C_8H_8O_2$	67.12	Aromatic ester
3.	1-(4-nitrosophenyl)ethanone	6.429	$C_8H_7NO_2$	1.29	Aromatic nitroso
4.	1-Ethenyl-3-nitrobenzene	6.543	$C_8H_7NO_2$	1.30	Aromatic nitro
5.	Benzyl propiolate	6.589	$C_{10}H_8O_2$	1.13	Alkyne ester
6.	1-Cyclohexene-1-methanol	6.629	$C_7H_{12}O$	1.40	Unsaturated alcohol
7.	(5-Carbamoyl-2,4-dioxo-3H-pyrimidin-1-yl)acetic acid	6.767	$C_7H_7N_3O_5$	2.97	Pyrimidine derivative
8.	5,6,7-Trinitro-1,4-benzodioxane	8.123	$C_8H_5N_3O_8$	1.01	Nitro aromatic
9.	1,3,3-trimethyl-2-(1-methylbut-1-en-3-on-1-yl)-1-cyclohexene	9.645	$C_{15}H_{24}O$	1.29	Terpenoid
10	2-exo-chloro-bicyclo[2.2.1]heptane-1-carbonyl chloride	9.834	$C_8H_{10}Cl_2O$	1.58	Acyl chloride
11	2-Acetylbenzoic acid	10.326	$C_9H_8O_3$	0.89	Aromatic carboxylic acid
12	2-Nitro-benzenesulfinic acid	11.361	$C_6H_5NO_4S$	0.94	Aromatic nitro
13	2-Hexyn-1-ol	13.170	$C_6H_{10}O$	1.09	Alkynol
14	Methyl dec-4-enoate	13.501	$C_{11}H_{20}O_2$	3.02	Fatty acid ester
15	Heptadecanolide	14.932	$C_{17}H_{32}O_2$	1.03	Macrocyclic lactone

16	2,2-Dimethyl-5-(3-methyloxiranyl)cyclohexanone	14.955	$C_{12}H_{20}O_2$	0.90	Epoxide
17	Eicosane	19.332	$C_{20}H_{42}$	1.01	Saturated hydrocarbon

RT = retention time; % Area = relative percentage abundance based on total ion chromatogram peak area integration.

Figure 1. GC–MS spectrum of aqueous–ethanol root extract of *C. ferruginea* (AECF).

In silico Androgen Receptor Docking and Drug-Likeness

Molecular docking of the two principal AECF constituents against the human AR-LBD (PDB ID: 2AM9, 1.64 Å resolution) using Glide SP scoring revealed differential binding behaviour across the three SiteMap-predicted active pockets (Tables 4–6). SiteMap analysis identified three candidate binding pockets with SiteScores of 0.902, 0.837, and 0.811 for Sites 1, 2, and 3, respectively. Site 1, characterised by the highest SiteScore and largest volume (205.1 $Å^3$), encompasses key canonical AR-LBD residues including Gln711, Arg752, Tyr763, and Met745.[28]

Methyl benzoate achieved Glide SP docking scores of −2.632, −2.921, and −1.364 kcal/mol at Sites 1, 2, and 3, respectively. The reference ligand testosterone scored −2.539, −2.926, and −1.866 kcal/mol at the corresponding sites. These results demonstrate that methyl benzoate binds comparably or marginally superiorly to testosterone at the two highest-ranked and most pharmacologically relevant pockets, while testosterone exhibits stronger binding at Site 3. It is important to contextualise these findings accurately: the score differences at Sites 1 and 2 are marginal (0.093 and 0.005 kcal/mol, respectively), representing computational predictions that require validation by in vitro binding assays before mechanistic conclusions can be drawn.

Methyl dec-4-enoate (cis) achieved substantially lower docking scores of −1.419, −1.599, and −0.597 kcal/mol at Sites 1, 2, and 3, respectively, indicating significantly inferior AR binding affinity and precluding it as a priority androgenic candidate.

Table 4. SiteMap-Predicted Androgen Receptor Binding Pockets

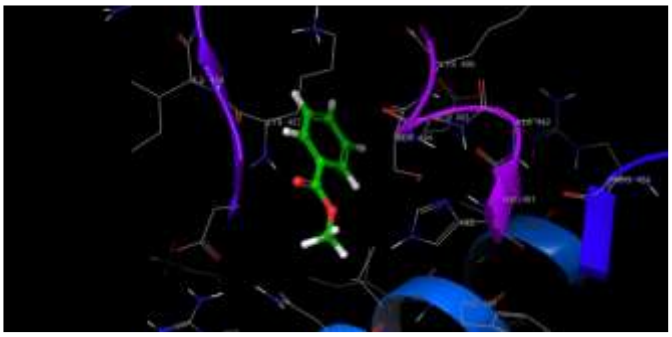
Site	SiteScore	Volume (Å³)	Phobic Score	Philic Score	Key Residues
1	0.902	205.1	0.302	1.171	Gln711, Arg752, Tyr763, Met745
2	0.837	142.3	0.733	0.718	Leu704, Trp741, Met895
3	0.811	61.0	0.284	0.993	Asn705, Thr877

Table 5. Glide SP Docking Scores (kcal/mol) of AECF Constituents and Testosterone against AR-LBD (PDB: 2AM9)

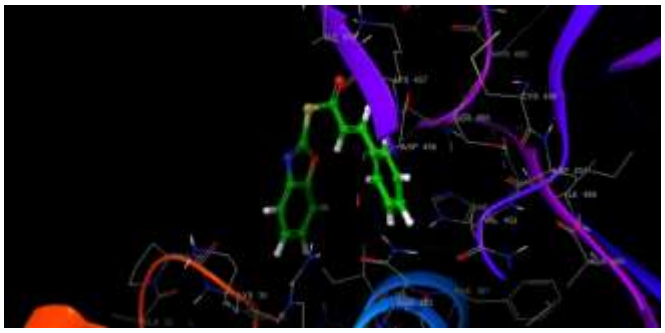
Ligand	Site 1 Score	Site 2 Score	Site 3 Score	Best Site
Methyl benzoate	−2.632	−2.921	−1.364	Site 2

Methyl dec-4-enoate (cis)	−1.419	−1.599	−0.597	Site 2
Testosterone (reference)	−2.539	−2.926	−1.866	Site 2

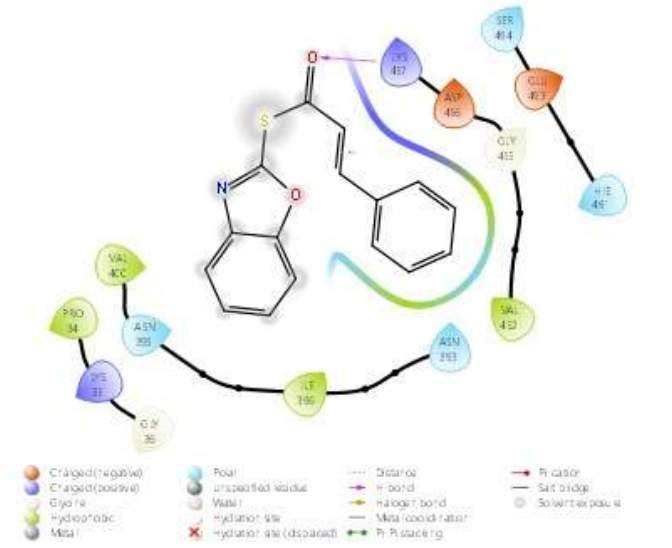
A: Methyl benzoate docked at AR Active Site 1



B: Methyl benzoate docked at AR Active Site 2



C: Methyl dec-4-enoate (cis) docked at AR Active Site 1



D: Methyl dec-4-enoate (cis) docked at AR Active Site 2

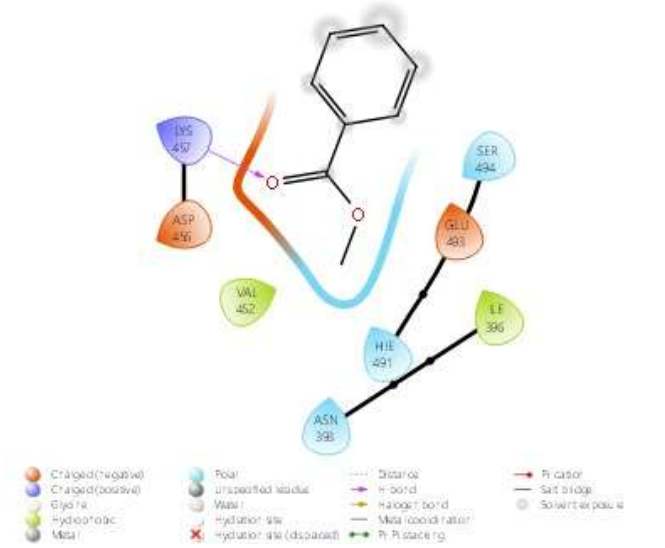


Figure 2. Molecular docking binding poses of AECF principal constituents at human androgen receptor (AR) active sites. (A) Methyl benzoate at AR Site 1 (GScore = −2.63 kcal/mol); (B) Methyl benzoate at AR Site 2 (GScore = −2.92 kcal/mol); (C) Methyl dec-4-enoate (cis) at AR Site 1 (GScore = −1.42kcal/mol); (D) Methyl dec-4-enoate (cis) at AR Site 2 (GScore = −1.60 kcal/mol). Ligands shown in stick representation; receptor binding pocket shown as transparent surface. Dashed lines indicate hydrogen bond interactions. Key interacting residues are labelled by amino acid code and position number. Docking performed using the Glide Standard Precision (SP) module of the Schrödinger Maestro Suite.

Table 6. Lipinski Rule of Five Drug-Likeness Parameters

Parameter	**Limit**	**Methyl Benzoate**	**Methyl Dec-4-enoate**	**Testosterone**
MW (Da)	≤ 500	136.2	184.3	288.4
HBD	≤ 5	0	0	1

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume XI Issue VI June 2026

HBA	≤ 10	2	2	2
QlogPo/w	≤ 5	2.1	3.3	3.3
PSA (Å²)	≤ 140	36.1	52.6	74.6
%HOA	≥ 80	100	100	100
RoF Violations	0	0	0	0

MW = molecular weight; HBD = hydrogen bond donors; HBA = hydrogen bond acceptors; QlogPo/w = calculated octanol–water partition coefficient; PSA = polar surface area; HOA = human oral absorption; RoF = Rule of Five.

DPPH Free Radical Scavenging Activity

AECF demonstrated concentration-dependent DPPH free radical scavenging activity across all tested concentrations (31.25–1000 µg/mL), yielding an IC_{50} value of 97.94 µg/mL compared to 34.60 µg/mL for the ascorbic acid standard (Tables 7–8). The approximately 2.83-fold lower potency of AECF relative to ascorbic acid indicates moderate antioxidant capacity, consistent with the absence of alkaloids from the phytochemical profile. Nonetheless, an IC_{50} below 100 µg/mL is generally considered indicative of biologically relevant radical scavenging capacity for crude plant extracts.[30] The antioxidant activity of AECF is pharmacologically significant in the context of male reproductive health, as oxidative stress-driven depletion of spermatozoal antioxidant defences constitutes a primary mechanistic pathway in male infertility.[3,4]

Table 7. DPPH Radical Scavenging Activity of AECF and Ascorbic Acid

Concentration (µg/mL)	**AECF % Inhibition (Mean ± SD)**	**Ascorbic Acid % Inhibition (Mean ± SD) †**
31.25	30.96 ± 1.00^{a}	30.75 ± 4.20^{a}*
62.5	52.62 ± 5.42^{a}	38.40 ± 1.44^{a}*
125	56.17 ± 0.42^{a}	45.26 ± 3.67^{ab}*
250	62.33 ± 2.11^{a}	71.58 ± 2.86^{a}*
500	64.57 ± 1.16^{a}	78.85 ± 0.90^{a}*
1000	67.44 ± 3.70^{a}	81.30 ± 0.69^{a}*

*Values expressed as mean ± SD (n = 3). Superscript (^{a}) within rows denote homogeneous subsets (Tukey HSD, $p < 0.05$). * = significantly higher than AECF at corresponding concentration. † Ascorbic acid applied at one-fifth the test sample volume. AECF = Aqueous–Ethanol Extract of Cnestis ferruginea Root.*

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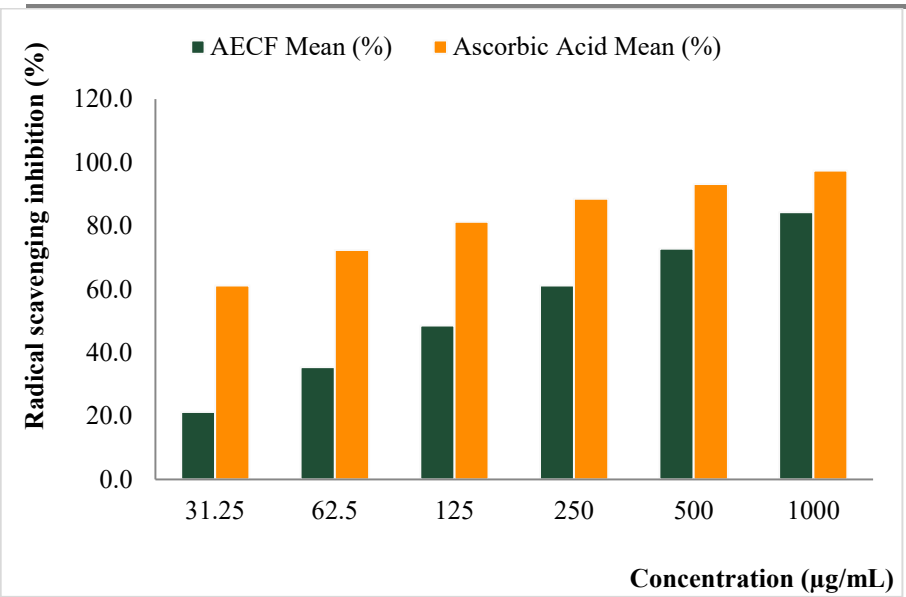

Figure 3. DPPH free radical scavenging activity (% inhibition) of AECF and ascorbic acid at graded concentrations.

Table 8. IC_{50} Values for DPPH Radical Scavenging Activity

Sample	IC_{50} (µg/mL)	Interpretation
AECF	97.94	Moderate antioxidant activity
Ascorbic acid (standard)	34.60	Strong antioxidant activity (reference standard)

IC_{50} = concentration required to inhibit 50% of DPPH radicals. Lower IC_{50} = higher potency.

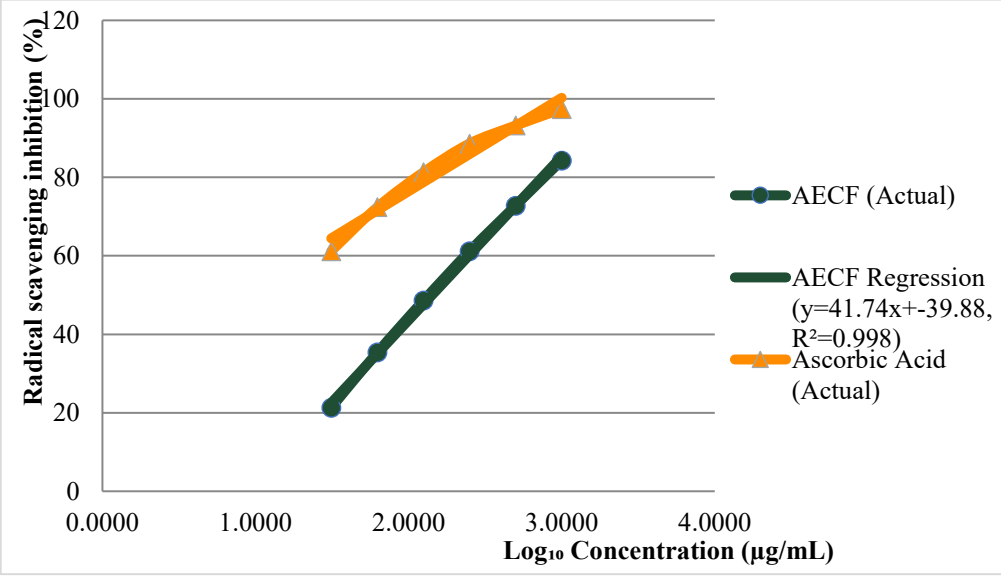

Figure 4. IC_{50} determination: % inhibition vs. log concentration (DPPH assay). AECF: IC_{50} = 97.94 µg/mL (y = 21.30x + 7.81, R^2 = 0.815); Ascorbic acid: IC_{50} = 34.60 µg/mL (y = 34.88x − 3.73, R^2 = 0.985).

Cytotoxicity: Brine Shrimp Lethality Assay

Exposure of *Artemia salina* nauplii to AECF produced dose-dependent mortality, with 100%, 30%, and 3.33% mortality recorded at 1000, 100, and 10 ppm, respectively. DMSO controls exhibited 0–10% mortality at

corresponding solvent concentrations. Probit analysis yielded an LC_{50} of 74.30 ppm for AECF[32], classifying the extract as strongly toxic by Meyer criteria[18] (Table 10). The DMSO vehicle control yielded an LC_{50} of 68.48 ppm, confirming that the vehicle itself contributes appreciable nauplii toxicity at the highest concentration tested and underscoring the importance of interpreting AECF toxicity relative to its vehicle-corrected contribution.

The strong cytotoxicity of AECF is consistent with the presence of bioactive secondary metabolites capable of disrupting fundamental cellular processes at elevated concentrations. The aromatic nitro and nitroso derivatives detected in the GC–MS profile represent structurally electrophilic constituents that may contribute disproportionately to the observed cytotoxicity relative to their combined abundance of 3.60%. These constituents would be important targets for removal during any future bioassay-guided fractionation aimed at isolating the androgenic principle of the extract.

Table 9. Brine Shrimp Lethality Assay Results for AECF

Conc. (ppm)	AECF % Mortality (T)	DMSO Control % Mortality (C)	Abbott-Corrected % Mortality
10	3.33	0.00	3.33
100	30.00	10.00	22.22
1000	100.00	40.00	99.99*

**Clipped to 99.99% for Probit regression (0% and 100% are undefined in the normal distribution). Corrected using Abbott's formula.[32]*

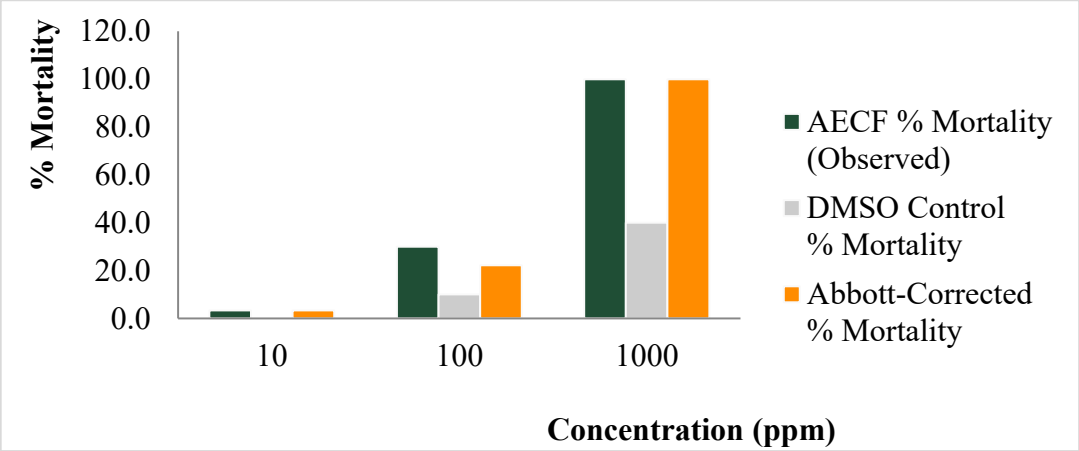

Figure 5. Percentage mortality of *Artemia salina* nauplii exposed to AECF and vehicle controls at 10, 100 and 1000 ppm.

Table 10. LC_{50} Values and Toxicity Classification

Sample	LC_{50} (ppm)	Toxicity Classification (Meyer *et al.*[18])
AECF	74.30	Strongly toxic (LC_{50} <100 ppm)
DMSO Control	68.48	Strongly toxic (LC_{50} <100 ppm)

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume XI Issue VI June 2026

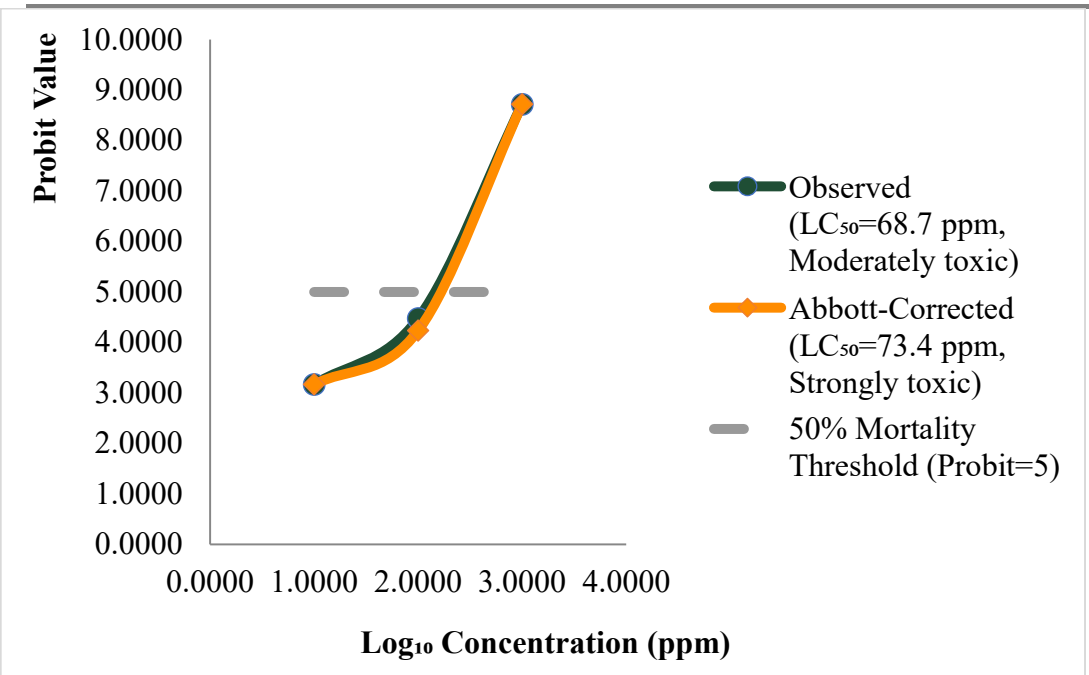

Figure 6. LC_{50} determination: probit regression of % mortality vs. log_{10} concentration (BSLA).

Integrated Interpretation and Ethnomedicinal Relevance

The collective findings of this study provide converging multi-level evidence for the pharmacological basis of the traditional use of *C. ferruginea* roots in West African ethnomedicine for male reproductive health. At the chemical level, GC–MS profiling identifies methyl benzoate as the overwhelmingly dominant volatile constituent of AECF. At the molecular level, *in silico* docking against the human AR-LBD demonstrates that methyl benzoate binds the receptor's two highest-ranked active pockets with affinity comparable to the endogenous ligand testosterone, satisfying all Lipinski drug-likeness criteria predictive of oral bioavailability. At the cellular protective level, AECF exhibits biologically relevant DPPH antioxidant activity pharmacologically meaningful in the context of oxidative stress-mediated male reproductive dysfunction.[22]

It is now well established that plants are capable of synthesising compounds structurally and functionally homologous to animal steroid hormones through the shared isoprenoid biosynthetic pathway.[22] The detection of plant steroids and terpenoids in the phytochemical screen is therefore consistent with a capacity to contribute steroid hormone precursors or receptor ligands complementing the dominant contribution of methyl benzoate. Future studies should proceed through: (i) bioassay-guided fractionation and isolation of methyl benzoate; (ii) *in vitro* AR competitive binding and androgen-responsive reporter gene assays to confirm receptor activation; (iii) *in vivo* evaluation in mammalian models at pharmacologically guided dose ranges; and (iv) mechanistic investigations of downstream AR signalling targets including spermatogenic gene expression and Leydig cell steroidogenesis.

CONCLUSION

This study reports the first integrated phytochemical, bioactivity, and *in silico* androgen receptor docking investigation of the aqueous–ethanol root extract of *Cnestis ferruginea* Vahl ex DC. (Connaraceae), providing multi-level pharmacological evidence that substantiates its traditional use in West African ethnomedicine for male reproductive health management. Cold maceration in 65:35 (v/v) ethanol–water yielded a crude extract (4.70% w/w) with a diverse phytochemical composition. GC–MS profiling identified 17 volatile constituents (89.27% of total chromatographic profile), with methyl benzoate (67.12%) as the dominant constituent. DPPH radical scavenging assay returned an IC_{50} of 97.94 µg/mL, indicating biologically relevant antioxidant capacity. Brine shrimp lethality assay yielded an LC_{50} of 74.30 ppm, classifying AECF as strongly cytotoxic at concentrations substantially exceeding predicted pharmacologically relevant ranges.

Critically, *in silico* molecular docking of methyl benzoate against the human AR-LBD (PDB ID: 2AM9) using Glide SP scoring demonstrated comparable binding affinity to testosterone at the two highest-ranked AR active pockets, with docking scores of −2.632 and −2.921 kcal/mol versus −2.539 and −2.926 kcal/mol for testosterone at Sites 1 and 2, respectively. Full compliance with Lipinski's Rule of Five confirmed favourable oral bioavailability. These findings collectively provide the first mechanistic computational rationale for the androgenic claims associated with *C. ferruginea* roots, identifying methyl benzoate as a priority lead compound for bioassay-guided isolation, *in vitro* AR binding confirmation, and *in vivo* pharmacological validation.

In particular, *in vitro* AR competitive binding assays and androgen-responsive reporter gene assays constitute the immediate and necessary next experimental step to confirm or refute the computational androgenic agonism predicted for methyl benzoate at the AR ligand-binding domain, prior to any *in vivo* validation programme.

DECLARATIONS

Author Contributions

Jamiu A. Akintola: Conceptualisation, methodology, investigation, formal analysis, data curation, writing; original draft, writing; review and editing, project administration, corresponding author. Patricia A. Onocha: Supervision, writing; review and editing, resources. Mubo A. Sonibare: Supervision, writing; review and editing, resources. Ganiyat K. Oloyede: Methodology, investigation, writing; review and editing. Isiaka Mohammed: Investigation, formal analysis. Raimot A. Fatai: Investigation, formal analysis. Bashirat A. Fatai: Investigation, formal analysis. All authors have read and approved the final version of the manuscript.

AI Assistance Disclosure: The authors disclose that Claude (Anthropic), an AI language model, was used to assist with manuscript preparation, literature search, structural organisation, and language editing during preparation of this article. All scientific content, experimental data, intellectual interpretation, and final editorial decisions remain solely the responsibility of the named authors. The AI tool did not generate, fabricate, or independently analyse any experimental data reported in this manuscript.

Funding

This research received no external funding. All costs associated with plant collection, extraction, phytochemical screening, GC–MS analysis, *in silico* docking studies, and bioassays were met by the authors personally.

Ethics Statement

This study did not involve the use of human participants, human tissue, vertebrate animals, or regulated invertebrates requiring institutional ethical approval. *Artemia salina* (brine shrimp) nauplii were used solely as a cytotoxicity screening tool in accordance with standard pharmacognostic practice. No formal ethics committee approval was required or sought for the reported experiments.

Data Availability Statement

All data supporting the findings of this study are contained within the manuscript and its supplementary materials. Raw numerical data are available from the corresponding author (jamiuakintola@gmail.com) upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge the technical support of the Departments of Chemistry and Pharmacognosy, University of Ibadan, Nigeria, for access to laboratory facilities and reagents. Taxonomic identification and voucher deposition services of the University of Ibadan Herbarium are gratefully acknowledged.

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