

Original Research Article

Contraceptive effect of the ethanol leaf extract of *Hibiscus rosa-sinensis* via *in-silico* virtual screening and pharmacological activity

Krishnamoorthy Venkateskumar^{1*}, Veerasamy Ravichandran², Geethaa Sahgal³, Selvaraja Shobhaharan¹, Thambiraja Sanchithaa¹, Sukumaran Manickam⁴, Surash Ramanathan⁵, Jayaprakash Venkatesan⁶, Subramani Parasuraman^{3,7}

¹Unit of Pharmaceutical Technology, Faculty of Pharmacy, AIMST University, Semeling 08100, Kedah Darul Aman, ²Unit of Pharmaceutical Chemistry, Faculty of Pharmacy, AIMST University, Semeling 08100, Kedah Darul Aman, ³Unit of Pharmacology, Toxicology and Basic Health Sciences, Faculty of Pharmacy, AIMST University, Semeling 08100, Kedah, Darul Aman, ⁴Rimba Ilmu Botanic Garden, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, ⁵Centre for Drug Research, Universiti Sains Malaysia, 11800 Penang, Malaysia, ⁶Department of Pharmaceutical Sciences, Birla Institute of Technology & Sciences (BITS), Ranchi, Jharkhand, ⁷Centre for Global Health Research, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India

*For correspondence: **Email:** venkateskumar@aimst.edu.my; **Tel:** +60 16-4542991

Sent for review: 15 February 2025

Revised accepted: 20 October 2025

Abstract

Purpose: To investigate the male contraceptive potential of the ethanol leaf extract of *Hibiscus rosa-sinensis* (EEHR) in male Sprague Dawley rats.

Methods: The extracts of *Hibiscus rosa-sinensis* (HR) leaves were prepared by maceration using distilled water, ethanol, and methanol as solvents. Phytochemical analysis and antioxidant potential were assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydrogen peroxide scavenging assay. Sprague Dawley rats were administered EEHR daily and its effect on sperm count, sperm motility, biochemical parameters, organ weight analysis and histopathological changes was assessed after 21 days of treatment. Molecular docking studies of target proteins (1D2S, 1E3G) with phytochemical constituents reported in leaves of HR were also carried out.

Results: Phytochemical analysis revealed the presence of phenol, tannin, flavonoids, terpenoids, triterpenes and diterpenes. Antioxidant effect of EEHR was significantly higher in aqueous and methanol extracts compared to ethanol extract ($p < 0.05$). Sperm count, sperm motility, and weight reduction of epididymal caput/corpus, and absence of changes in biochemical content were significantly reduced in EEHR compared to control ($p < 0.05$). Histopathological analysis revealed alterations in seminiferous tubules and intraepithelial vacuolation in EEHR-treated rats. Molecular docking results showed that quercitrin, (-)-epicatechin, and quercetin showed the most effective binding with the target protein (1D2S), whereas quercetin, rhamnetin, and quercitrin showed effective binding with the target protein (1E3G).

Conclusion: Ethanolic extract of leaves of *Hibiscus rosa-sinensis* (EEHR) significantly reduces sperm count, sperm motility, epididymal caput/corpus weight, and induces alterations of seminiferous tubules. Furthermore, docking study results clearly support the contraceptive potential of EEHR.

Keywords: Antioxidant, Contraceptive agent, Fertility, Free radicals

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Scopus, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Population growth is one of the major problems faced by developing countries with low-resource settings. It needs to be controlled to have a significant effect on global growth and women's health. Family planning delays pregnancies in young women, prevents unintended pregnancies, reduces risks related to pregnancy in older women and limits family size. Such initiatives prevent population surge and alleviate sexual and reproductive risks (SRH) in women. Reports suggest that 214 million women of reproductive age in developing countries are not using modern contraceptive methods, and 80 – 90 million unintended pregnancies occur annually, which are preventable [1].

Although the United Nations Population Fund Annual Report focuses on the achievement of Millennium Development Goals (MDG) on areas such as poverty, primary education, women empowerment, child and maternal health, more progress is needed in sexual and reproductive health. Diversified family planning approaches are currently available, but they are more skewed toward women than men [2]. Men could play a greater role in contraception and current trends also necessitate the need for the development of effective contraceptive agents that are useful. Such an approach will reduce the burden carried by women, support women to improve their sexual and reproductive health and significantly contribute to women's empowerment.

Medicinal plants have been used to treat many diseases. Traditional medicine practices carry a strong platform relying on various principles and methods based on multiple cultures' theories, beliefs and experiences. World Health Organization (WHO) emphasizes phytochemicals as rich sources of various drugs that may be explored further. Over 80,000 plants are used as medicinal plants worldwide, and a significant number have been passed down from one generation to another. In many countries, medicinal plants are used in longstanding traditional medicine practices [3]. Reports suggest that reproductive rights, along with family planning, are major challenges faced by Malaysian women [4]. The Contraceptive Prevalence Rate (CPR) in Malaysia has been capped at 55 % for all contraceptive methods, while modern contraceptive methods usage rate is at a low 35 % rate compared to other neighbouring countries. This scenario is attributed to factors such as cultural attitudes, misconceptions, fear of contraceptive use, negative responses from healthcare workers and lack of male contraceptives [5].

Malaysia is a country with rich biodiversity and abundant natural resources that are used for treating various health disorders. Use of plant resources to treat various disorders is widely practised in traditional medicine in Malaysia [6]. One of such plant species that is traditionally used is the Hibiscus plant, reported to possess a wide variety of pharmacological activities such as antidiabetic, cytotoxicity, anti-parasitic, wound healing properties and much more. Consumption of flowers of *Hibiscus rosa-sinensis* crushed with sour gruel and jaggery has been used as a contraceptive in traditional medicine; the juice of crushed flowers is mixed with sugar to treat hypermenorrhea and flower buds minced with milk are used to treat various gynaecological problems [7-9]. Therefore, this study investigated the effect of *Hibiscus rosa-sinensis* leaves extract on spermatogenesis in male rats. The outcome of the study would provide valuable insight and create newer approaches for male contraception. Further studies are expected to develop a reversible, safe, and effective modern contraceptive that is used in males to prevent unintended pregnancies, share the contraception burden, and empower millions of women.

EXPERIMENTAL

Collection of plant leaves

The leaves of *Hibiscus rosa-sinensis* were identified and collected from the garden area of AIMST University student residences. The plant parts were authenticated by USM Herbarium, Pusat Pengajian Sains Kaji-hayat, Universiti Sains Malaysia, Pulau Pinang (herbarium voucher number 11838). The collected leaves were washed thoroughly under running water to remove any impurities present on the leaves' surface. The leaves were dried in a hot air oven at 20 ± 2 °C for 3 days. After drying, the leaves were crushed into a coarse powder using an electrical grinder. The ground powder was then stored in airtight containers until further use.

Extraction

Powdered leaves were extracted with ethanol, methanol, and distilled water by maceration for 3 days with intermittent shaking at normal room temperature. Later, the extract was filtered using a muslin cloth, and the ethanol and methanol extracts were concentrated using a rotary evaporator (Yamamoto, Japan). Aqueous extract was prepared by freeze-drying. After evaporation, the extracts were kept in a refrigerator at 4 °C until further use.

Preliminary phytochemical screening

The crude methanol extract was assessed for secondary metabolites such as phenol, tannin, flavonoids, saponins, terpenoids, triterpenes, diterpenes, glycosides, anthraquinones, alkaloids and steroids using methods adapted from Begashaw *et al* [10].

Phytochemical analysis

Total phenolic content (TPC) present in the plant extract was determined by Follin-Ciocalteu method [11]. Gallic acid was used as a standard. This was done in triplicate to obtain an average value. The value obtained was compared to the calibration curve and total phenolic content was calculated and expressed as mg of gallic acid equivalent (mg GAE) per g dry weight.

Total flavonoid content in the plant extracts was determined using the aluminum chloride colourimetric method [11]. In this method, quercetin was used as standard. This was done in triplicate to obtain an average value. The value obtained was compared to the calibration curve and total flavonoid content was calculated and expressed as mg of quercetin equivalent (mg QE) per gram dry weight.

Antioxidant assay

The free radical scavenging properties of the ethanolic, methanolic, and aqueous extracts of leaves of *Hibiscus rosa-sinensis* were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide methods [12].

Animals

Healthy, male *Sprague-Dawley* rats (250 - 300 g) were used in the animal study. The rats were obtained from the Centralized Animal House of AIMST University. The animals were placed in a polycarbonate cage under standard laboratory environmental conditions in a 12-hour light and dark cycle. The animals were given *ad libitum* access to food and water. Ethical approval was obtained from the AIMST University Human and Animals Ethics Committee (approval no. AUAEC/FOP/2021/18).

Effect of ethanol extract of EEHR on spermatogenesis

Ethanol extract (EEHR) contains a higher amount of TPC and exhibits more antioxidant effect compared to methanolic and aqueous extracts. Hence, EEHR was used for pharmacological studies. The animals were

divided into four groups comprising 6 animals each. Group 1 was the control; groups 2, 3, and 4 received 400, 800 and 1600 mg/kg, respectively, of EEHR. The EEHR were suspended in 0.5 % w/v Carboxymethyl cellulose (CMC) and administered once daily through oral gavage for 21 consecutive days. Changes in body weight were monitored at regular intervals. At the end of the study, blood samples were collected from the animals for serum biochemical analysis. Animal semen was collected from the cauda epididymis for microscopic analysis of the sperm. Thereafter, the animals were sacrificed to harvest organs for histopathological analysis.

Sperm motility

Sperm samples were collected from the cauda epididymis at the end of the study. The cauda epididymis was isolated and placed in a petri dish containing Biggers, Whitten, and Whittingham (BWW) medium to allow the sperm to swim (swim-up technique). An appropriate volume of spermatozoa was transferred to a preheated cell count plate. From a total of 200 spermatozoa, the active ones were observed using a microscope to calculate sperm motility.

Sperm count

After the motility test, the rest of the sperm suspension was quickly transferred to a water bath (60 °C) for 5 to 10 min to induce inactivation. The sperm suspension was then placed on a Neabeur haemocytometer and the cells were counted microscopically. Number of spermatozoa was calculated using the red blood cell counting method [13].

Biochemical analysis

Blood samples were collected from the animals through the orbital sinus under diethyl ether anesthesia. The samples were centrifuged at 3000 rpm for 20 min and serum was collected. The serum sample was used to estimate glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, creatinine and urea using Reflotron kit and Reflotron Plus biochemical analyzer (Roche Diagnostics, Germany).

Histopathology of the testis

The testes of the animals were dissected, weighed and recorded. The testes were then placed in a container containing 10 % formalin solution and kept till further use. The testes were then fixed in Bouin's fluid and dehydrated in a

series of graded ethanol. The sample was cleared in benzene and embedded in paraffin. Testicular tissue was sectioned at 5 μ m and the sections were stained with Eosin and Haematoxylin, and examined microscopically [14].

Molecular docking studies

(+)-Catechin, (-)-epicatechin, rhamnetin, quercetin 3,4'-diglucoside, quercetin 7,4'-diglucoside, quercetin 3,7-diglucoside, hyperoside, isoquercitrin, kaempferol 3-o-glucoside, kaempferol 7-o-glucoside, quercitrin and quercetin are the few phytoconstituents of *Hibiscus rosa-sinensis* plants [7,15,16]. Chemical constituents present in *Hibiscus rosa-sinensis* species and their structure were used for molecular docking studies to ascertain possible role of interaction between the *Hibiscus rosa-sinensis* constituents and targeted proteins (1D2S and 1E3G proteins).

Hardware and software specifications

All simulation experiments were executed using a DELL workstation with Windows 10 (64-bit as OS, Intel® Core™ i7-11,800 CPU@2.30 GHz processor, 16 GB RAM, 4 GB GPU). Docking studies were performed using AutoDock 4.2.6 and MGLTools 1.5.6. The Protein Data Bank was used to get all co-crystallized protein structures. ChemOffice Suite 2015 by PerkinElmer Informatics was used to generate all ligand structures. Ligplot⁺ was used to depict 2D protein-ligand interactions. PyMol was used to display 3D protein-ligand interactions.

Protein and ligand structure preparation

The proteins employed in this study were: (i) Crystal structure of the N-terminal Laminin G-like Domain of SHBG in complex with dihydrotestosterone (PDB ID: 1D2S), and (ii) Human Androgen Receptor Ligand binding in complex with the ligand metribolone (R1881; PDB ID: 1E3G). The proteins were prepared using AutoDock 4.2.6 program, which involved removing water molecules, adding polar hydrogen, defining the AD4 atom type, and

adding Gasteiger charges to the protein system. Additionally, selective flips to residues were used, and all-atom interactions were investigated. The ligands were prepared using ChemOffice Suite 2015 and AutoDock 4.2.6 modules in individual structures in ChemDraw 19.1, followed by energy minimization using the MM₂ module. The final protein and ligand structures were saved in .pdb format for protein–ligand docking studies.

Docking validation and identification of active site region

The purpose of the re-docking was to assess the quality of the docking process and the effectiveness of the docking methods. The DHT (co-crystallized ligand) from 1D2S protein and R18 (co-crystallized ligand) from 1E3G protein were extracted with UCSF Chimera 1.16 and redocked into the active site using AutoDock 4.2.6. The root mean square deviation (RMSD) was then computed by superimposing the redocked complex onto the reference co-crystallized complex in AutoDock 4.2.6. The deviation must be less. The 3D grid box for both proteins was found by wrapping all the macromolecular residues that interact with the ligand around the internal ligand as a center. This guarantees that all potential ligand conformations are contained within the grid box (active site; Table 1).

Molecular docking

All molecular docking simulation studies were performed using AutoDock 4.2.6 program (ADP). After successful execution of AutoGrid 4.2, docking was initiated for all individual ligands using AutoDock 4.2 execution file. Before proceeding, the parameters were set as follows: number of genetic algorithm (GA) runs was set to 50, with 2,500,000 evaluations and population size of 150. The machine-generated .dlg file offers binding energies for every run and inhibition constant, along with a clustering histogram. From the histogram, low-energy conformer from the largest cluster was selected for analysis. Thereafter, the protein–ligand complex interactions were visualized.

Table 1: Tabular representation of the grid parameters used in the study

PDB ID	3D grid-box dimensions (Å)			Spacing (Å)	3D coordinates for center of the grid box		
	x-axis	y-axis	z-axis		x-axis	y-axis	z-axis
1D2S	40	40	40	0.375	21.806	10.811	32.304
1E3G	40	40	40	0.375	0.059	30.755	4.301

Statistical analysis

Results were presented in mean $\pm$ standard error of mean (SEM) and compared using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Yield

Percentage yield of ethanolic, methanolic, and aqueous extract of leaves of *Hibiscus rosa-sinensis* was 15.17, 8.23 and 13.17 % w/w, respectively. Both the ethanol and methanol extracts were brownish green in colour, while the aqueous extract was dark brown in colour.

Phytochemical screening

Total phenolic and flavonoid content was higher in ethanol extract compared to methanol and aqueous extracts (Table 2).

Table 2: Total phenolic content and total flavonoid content of leaves of *Hibiscus rosa-sinensis* (mean $\pm$ SEM; n = 3)

Extract	Total phenolic content (mg GAE)	Total flavonoid content (mg QE)
Ethanol	51.73 $\pm$ 20.87	94.71 $\pm$ 1.16
Methanol	29.90 $\pm$ 10.04	83.70 $\pm$ 13.43
Aqueous	24.57 $\pm$ 10.44	125.07 $\pm$ 9.70

Antioxidant activity

Ethanol extract exhibited free radical scavenging properties at low concentrations against DPPH and hydrogen peroxide radicals compared to methanol and aqueous extract (Table 3).

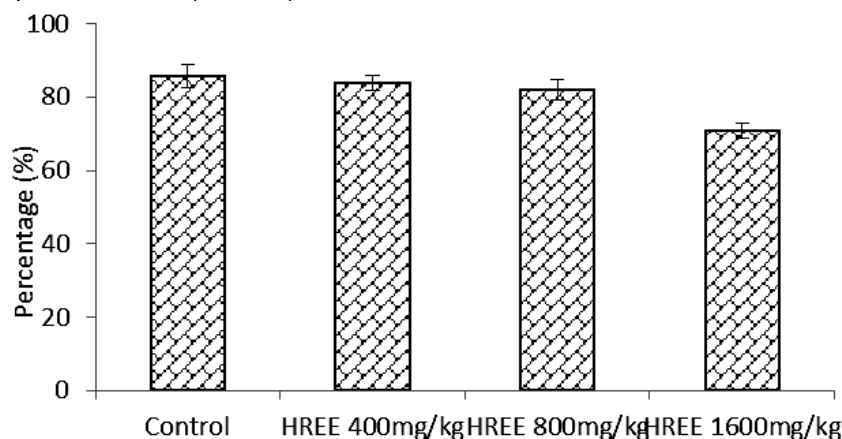


Figure 1: Effect of ethanolic extract of *Hibiscus rosa-sinensis* leaves (EEHR) on sperm motility. values are mean $\pm$ SEM (n =6). ** $P < 0.01$ compared to control

Table 3: Antioxidant effects of extracts of leaves of *Hibiscus rosa-sinensis* (IC₅₀ values)

Extract	DPPH method	Hydrogen peroxide method
Ethanolic	434.10	462.38
Methanolic	534.46	622.21
Aqueous	553.92	668.11
Ascorbic acid	52.82	464.89

Sperm motility

Treatment with EEHR at 1600 mg/kg demonstrated significant dose-dependent reduction in sperm motility (Figure 1) and sperm count (Table 4) compared to control ($p < 0.05$).

Table 4: Effect of ethanolic extract of *Hibiscus rosa-sinensis* leaves (EEHR) on sperm count (mean $\pm$ SEM; n = 6)

Group (mg/kg)	Sperm per milliliter ($\times 10^6$ /mL)
Control	17.50 $\pm$ 1.34
EEHR 400	17.00 $\pm$ 0.97
EEHR 800	13.33 $\pm$ 0.49*
EEHR 1600	11.17 $\pm$ 0.70***

** $P < 0.01$, *** $p < 0.001$ vs control

Biochemical analysis

Treatment with EEHR at all doses did not show any significant changes in glucose, AST, ALT, ALP, bilirubin, urea, and creatinine levels compared to control (Table 5).

Organ weight

Treatment with EEHR at all doses did not show any significant changes in absolute organ weight of the testis and epididymal cauda compared to control ($p > 0.05$).

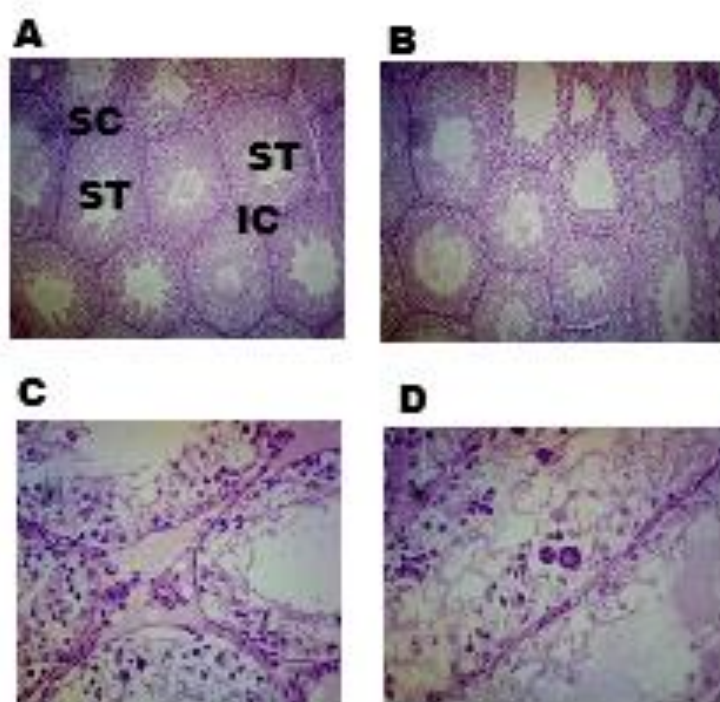
Table 5: Effect of ethanolic extract of *Hibiscus rosa-sinensis* leaves (EEHR) on biochemical parameters (mean $\pm$ SEM; n = 6)

Group (mg/kg)	Glucose (mg/dL)	AST (U/l)	ALT (U/l)	ALP (U/l)	Bilirubin (μ mol/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control	91.48 $\pm$ 4.65	91.17 $\pm$ 5.74	47.05 $\pm$ 3.09	113.38 $\pm$ 5.03	14.18 $\pm$ 0.36	41.85 $\pm$ 0.76	0.76 $\pm$ 0.04
EEHR 400	89.55 $\pm$ 4.95	91.78 $\pm$ 5.10	51.18 $\pm$ 5.60	111.67 $\pm$ 7.75	13.97 $\pm$ 0.71	47.72 $\pm$ 8.44	0.81 $\pm$ 0.04
EEHR 800	87.75 $\pm$ 2.78	86.97 $\pm$ 3.80	49.68 $\pm$ 1.69	110.62 $\pm$ 3.19	14.93 $\pm$ 0.55	47.08 $\pm$ 2.57	0.83 $\pm$ 0.03
EEHR1600	90.30 $\pm$ 3.70	90.27 $\pm$ 3.05	49.27 $\pm$ 2.20	113.12 $\pm$ 9.79	14.68 $\pm$ 1.43	51.75 $\pm$ 3.94	0.80 $\pm$ 0.04

Table 6: Effect of ethanolic extract of *Hibiscus rosa-sinensis* leaves (EEHR) on the weight of the epididymis caput and epididymis cauda (mean $\pm$ SEM; n = 6)

Group (mg/kg)	Weight of testes (g)	Weight of epididymal caput/corpus (g)	Weight of epididymal cauda (g)
Control	3.10 $\pm$ 0.03	0.72 $\pm$ 0.02	0.43 $\pm$ 0.02
EEHR 400	2.93 $\pm$ 0.08	0.49 $\pm$ 0.06**	0.39 $\pm$ 0.02
EEHR 800	3.08 $\pm$ 0.05	0.48 $\pm$ 0.06**	0.41 $\pm$ 0.03
EEHR 1600	2.99 $\pm$ 0.06	0.49 $\pm$ 0.02**	0.39 $\pm$ 0.03

** $P < 0.01$, *** $p < 0.001$ vs control

**Figure 2:** Photomicrograph of a section of testis of animals administered EEHR. ST (seminiferous tubules), SC (sustentacular cells), IC (interstitial cells). (A) Sections of testis of EEHR 400 mg/kg administered animal showing the normal configuration. (B) Sections of testis of EEHR 400 mg/kg administered animal showing the normal configuration. (C) Sections of testis of EEHR 800 mg/kg administered animal showing vacuolated degeneration of Sertoli cells and spermatocytes. (D) Sections of testis of EEHR 1600 mg/kg administered animal showing vacuolated degeneration of Sertoli cells, loss of spermatogonia, spermatids and spermatocytes (Haematoxylin and Eosin staining, $\times 400$)

Furthermore, treatment with EEHR at 400, 800 and 1600 mg/kg resulted in significant reduction in epididymal caput/corpus compared to control (Table 6).

Histopathological examination

Treatment with EEHR at 400 mg/kg showed neither the thickness of the basement membrane

nor Intratubular germ cell neoplasia. The rat testes and spermatic cord were within normal limits. Treatment with EEHR (800 and 1600 mg/kg) showed vacuolated degeneration of Sertoli cells, loss of spermatogonia, spermatids and spermatocytes. Spermatogonia in EEHR 1600 mg/kg group were observed to have undergone apoptosis, and few tubules had fluid and edema. The interstitium of both EEHR at 400

mg/kg and 1600 mg/kg had dilated lymphatics and edema, while EEHR at 800 mg/kg was seen to have mild congestion and edema (Figure 2).

Docking validation

Redocking studies revealed binding energies of -11.19 and -11.99 kcal/mol, K_i values of 6.33 and 1.63 nM and reference RMSDs of 0.597 and 0.655 Å, for 1D2S and 1E3G proteins, respectively.

Molecular docking

The superimposed conformation of the docked internal ligand with DHT, concerning its crystallized conformation obtained from co-crystallized complex structure (PDB: 1D2S) and R18 concerning its crystallized conformation obtained from the co-crystallized complex structure (PDB: 1E3G) are presented in Figure 3.

1D2S protein

The detailed docking analysis data are shown in Table 7, the detailed interaction analysis is presented in Table 8 and the visual pose view is shown for the top three ligands in Figures 4 to 6. The results suggest that all ligands revealed

favourable binding energies and inhibition constants. Among the ligands, Quercitrin, (-)-epicatechin, and Quercetin displayed the most effective binding within the active site. These top three hits showed binding energies of -9.50, -8.03 and -7.91 kcal/mol and inhibition constants of 108.13, 1.31 and 1.60 μ M, respectively. These data are comparable to the co-crystallized ligand, which suggests how well the ligands fit into the sub-pockets of the protein's active site.

1E3G protein

The detailed docking analysis data are shown in Table 9 and the detailed interaction analysis is presented in Table 10, while the visual pose view is shown for the top three ligands in Figures 7 to 9. All the ligands revealed favourable binding energies and inhibition constants. Among the ligands, quercetin, rhamnetin, and quercitrin displayed most effective binding within the active site. These top three hits showed binding energies of -9.47 kcal/mol, -9.44 kcal/mol, and -8.25 kcal/mol and inhibition constants of 113.98 nM, 120.27 nM, and 894.60 nM, respectively. These data are comparable to the co-crystallized ligand, which suggests how well the ligands fit into the sub-pockets of the protein's active site.

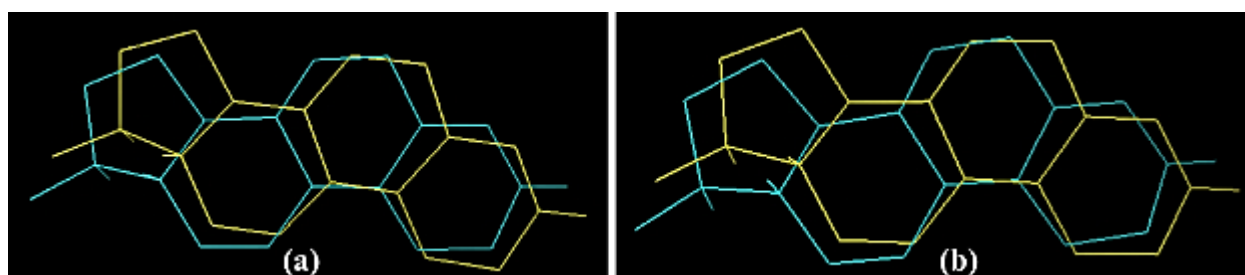


Figure 3: Superimposed conformation of the docked internal ligand with (a) DHT concerning its crystallized conformation obtained from the co-crystallized complex structure (PDB: 1D2S) and (b) R18 concerning its crystallized conformation obtained from the co-crystallized complex structure (PDB: 1E3G)

Table 7: Tabular representation of molecular docking analysis of various ligands with 1D2S protein

Ligand Name	Binding Energy (Kcal/mol)	Inhibition constant
Co-crystallized ligand	-11.19	6.33 nM
(+)-Catechin	-6.75	11.29 μ M
(-)-epicatechin	-8.03	1.31 μ M
Rhamnetin	-7.56	2.86 μ M
Quercetin 3,4'-diglucoside	-6.10	33.79 μ M
Quercetin 7,4'-diglucoside	-7.40	3.74 μ M
Quercetin 3,7'-diglucoside	-6.31	23.90 μ M
Hyperoside	-7.69	2.30 μ M
Isoquercitrin	-7.46	3.42 μ M
Kaempferol 3-O-glucoside	-7.74	2.13 μ M
Kaempferol 7-O-glucoside	-7.60	2.69 μ M
Quercitrin	-9.50	108.13 nM
Quercetin	-7.91	1.60 μ M

Table 8: Tabular representation of various interactions between the ligands and 1D2S protein

Ligand Name	H-Bonds with distance	Hydrophobic interactions
Co-crystallized ligand	Asn82 (2.73 Å)	Asp65, Leu80, Gly58, Trp66, Phe56, Ile141, Ser42, Ser41, Leu171, Thr40, Met107, Val105, Phe67
Quercitrin	Asn82 (2.80 Å), Asp65 (2.58 Å), Trp66 (2.44 Å), Ser128 (2.68 Å), Val105 (2.84 Å)	Gly129, Met139, Met107, Leu171, Ser42, Ser41, Thr40, Phe67, Leu80, His81
(-)-epicatechin	Asn82 (2.83 Å), Asp65 (2.84 Å), Gly58 (2.67 Å), Val105 (2.51 Å)	Trp66, Asp59, Met139, Phe67, Ser42, Phe56, Ser41, Thr40, Lys106, Met107
Quercetin	Asn82 (2.98 Å), Asp65 (2.66 Å), Thr60 (2.82 Å), Ser42 (3.00 Å), Val105 (2.81 Å)	Asp59, Gly58, Ile141, Leu171, Ser41, Val112, Phe67, Leu80, Trp66

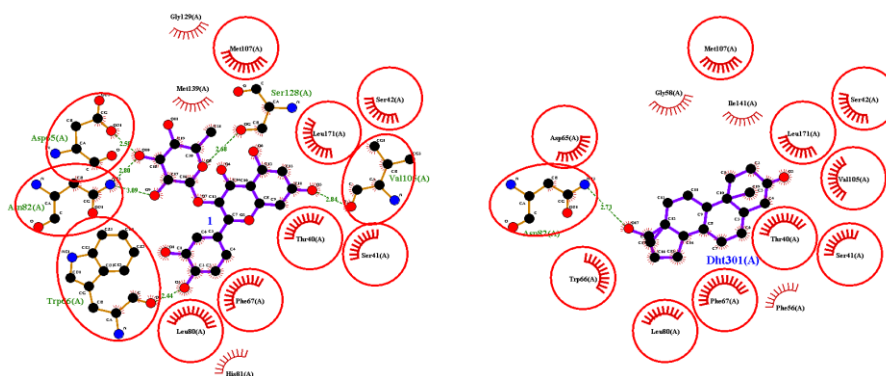
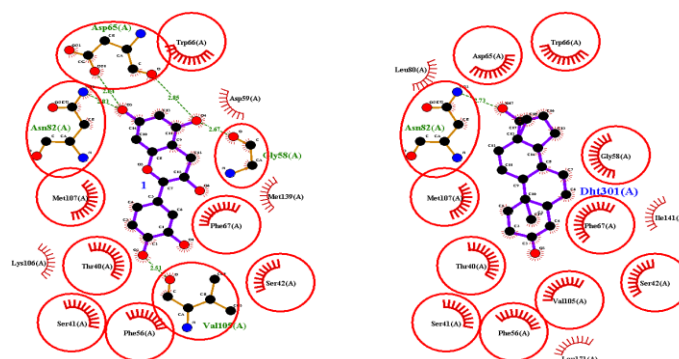
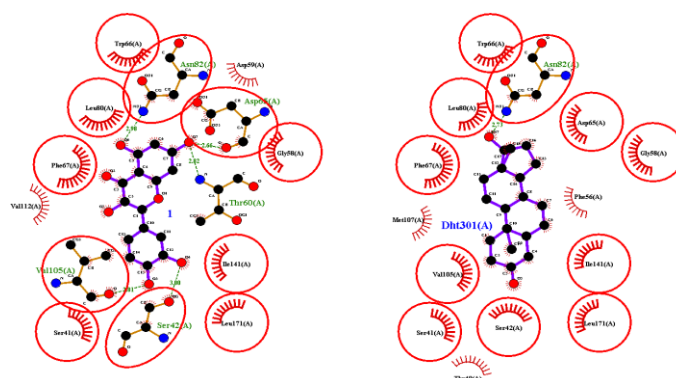
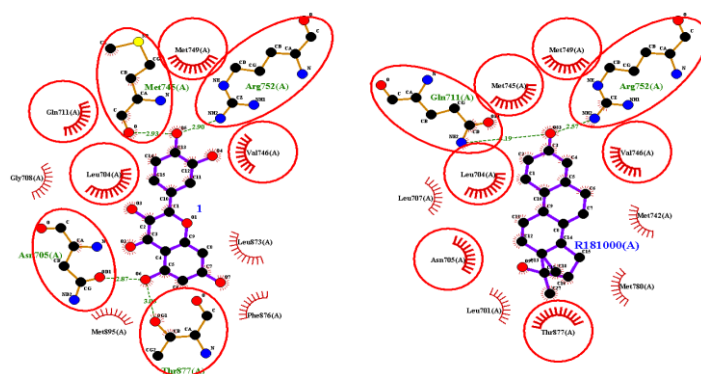
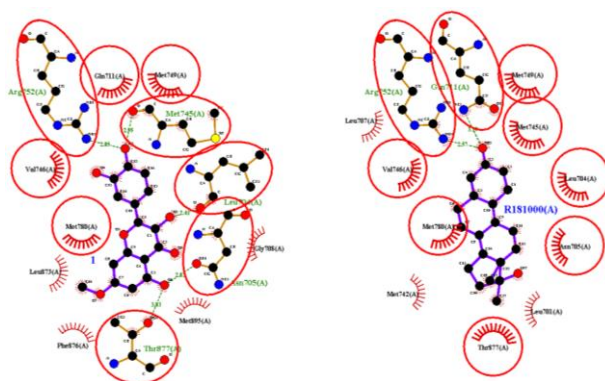
**Figure 4:** Overlay confirmation of Quercitrin with the co-crystallized ligand (DHT), highlighting the conserved residues (encircled red)**Figure 5:** Overlay confirmation of (-)-epicatechin with the co-crystallized ligand (DHT), highlighting the conserved residues (encircled red)**Figure 6:** Overlay confirmation of Quercetin with the co-crystallized ligand (DHT), highlighting the conserved residues (encircled red)

Table 9: Tabular representation of molecular docking analysis of various ligands with 1E3G protein

Ligand name	Binding energy (Kcal/mol)	Inhibition constant
Co-crystallized ligand	-11.99	1.63 nM
(+)-Catechin	-7.14	5.85 μ M
(-)-epicatechin	-8.83	339.48 μ M
Rhamnetin	-9.44	120.27 μ M
Quercetin 3,4'-diglucoside	+23.62	NA
Quercetin 7,4'-diglucoside	+31.87	NA
Quercetin 3,7-diglucoside	+23.26	NA
Hyperoside	-4.51	496.35 μ M
Isoquercitrin	-2.49	14.91 mM
Kaempferol 3-O-glucoside	-6.79	10.52 μ M
Kaempferol 7-O-glucoside	-4.01	1.16 mM
Quercitrin	-8.25	894.60 nM
Quercetin	-9.47	113.98 nM

Table 10: Tabular representation of various interactions between the ligands and 1D2S protein

Ligand name	H-Bonds with distance	Hydrophobic interactions
Co-crystallized ligand	Gln711 (3.19 Å), and Arg752 (2.57 Å)	Met745, Val746, Thr877, Met742, Met780, Leu704, Leu701, Asn705, Leu707, Met749
Quercetin	Met745 (2.93 Å), Arg752 (2.90 Å), Asn705 (2.87 Å), Thr877 (3.03 Å)	Met749, Val746, Leu873, Phe876, Met895, Gly708, Leu704, Gln711
Rhamnetin	Arg752 (2.85 Å), Met745 (2.95 Å), Leu704 (2.41 Å), Asn705 (2.83 Å), Thr877 (3.03 Å)	Gln711, Met749, Gly708, Met895, Phe876, Leu873, Met780, Val746, & Gln711
Quercitrin	Phe764 (2.75 Å), Gln711 (2.54 Å), Thr877 (3.00 Å), Asn705 (2.50 Å)	Val746, Leu707, Met745, Met742, Met895, Trp741, Ile899, Phe891, Leu704, Met787, Leu873, Met780, Met749, Phe876, & Arg752

**Figure 7:** Overlay confirmation of Quercetin with the co-crystallized ligand (R18), highlighting the conserved residues (encircled red)**Figure 8:** Overlay confirmation of Rhamnetin with the co-crystallized ligand (R18), highlighting the conserved residues (encircled red)

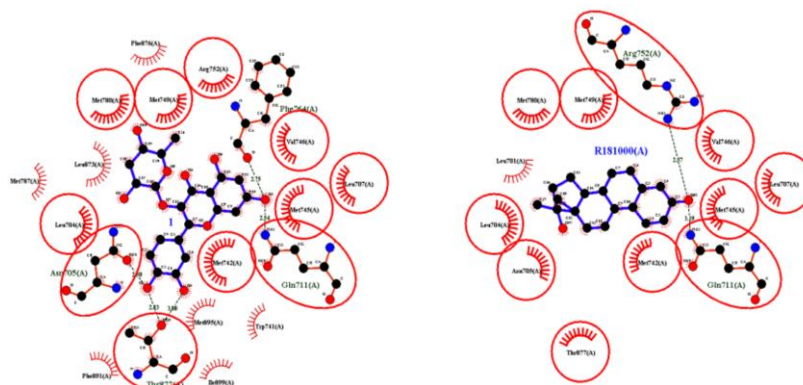


Figure 9: Overlay confirmation of Quercitrin with the co-crystallized ligand (R18), highlighting the conserved residues (encircled red)

DISCUSSION

This present study revealed that EEHR exhibited higher TPC and TFC contents, antioxidant activity and reduced sperm motility and count. Plant phenolics and flavonoids are a major group of compounds with free radical scavenging effects. Phenolics are considered a major food component in plants, and more than 8,000 phenolic compounds are present in plant sources. These compounds donate hydrogen atoms or chelate metals, which inhibit free radical chain mechanism and lipoxygenase [15]. These findings are in accordance with published reports on the antioxidant activity of flavonoids and phenolics [17,18].

Antioxidant activity was assessed using DPPH and hydrogen peroxide scavenging assays and the results revealed that EEHR extract exhibited scavenging properties at low concentrations against DPPH and hydrogen peroxide free radicals. This activity is attributed to the presence of phenolics and flavonoids in the extract. Furthermore, EEHR may act by donating a hydrogen atom, chelating metals, inhibiting the free radical chain mechanism, inhibiting lipoxygenase, and scavenging free radicals. These observations are in correlation with existing reports on the antioxidant activity of *Hibiscus rosa-sinensis* species [19]. The EEHR extract at higher doses (1600 mg/kg) decreased spermatogenic parameters, including sperm count and motility, and produced significant changes in absolute organ weight of the testis and epididymal cauda. These findings are in tandem with earlier reports on antifertility potential of *Hibiscus rosa-sinensis* flower extracts in various solvents [20, 21].

The histopathologic analysis of tissues treated with EEHR at 800 and 1600 mg/kg clearly revealed vacuolated degeneration of Sertoli cells,

dilated lymphatics and edema in the interstitium, loss of spermatogonia, spermatids and spermatocytes due to apoptosis in treated animal compared to control. These observed findings are in tandem with the histopathological findings reported in previous studies [20, 22]. It has been reported that atrophy of seminiferous tubules and degeneration of Sertoli cells lead to impaired male fertility [23]. This explains the antifertility activity of EEHR. Biochemical analysis showed that EEHR was not hepatotoxic, which is in tandem with earlier reports [24, 25]. Molecular docking studies showed that the binding between ligands such as quercetin, quercitrin and targeted proteins ID2S and IE3G was effective. Furthermore, ligand (-)-epicatechin displayed high binding with ID2S, whereas rhamnetin showed high binding towards IE3G Protein. The results were supported by high binding energies and high inhibition constants. These observations provide insights into possible mechanism of action of tested extracts for antifertility activity. These results also prove the intensity of ligands, fitting into sub-pockets of active site of targeted protein.

CONCLUSION

Hibiscus rosa-sinensis leaves extracts furnish an array of phytochemical compounds such as flavonoids and phenolic compounds, demonstrating significant antioxidant and antifertility effects with ligands that effectively bind to targeted proteins. Further isolation studies and molecular mechanistic studies need to be carried out to isolate specific compounds responsible for the contraceptive activity, specifically in males. These efforts would lay a platform for future studies that could end the search for male contraceptive agent.

DECLARATIONS

Acknowledgement/Funding

This study was funded by the Ministry of Higher Education (MOHE), Government of Malaysia, under Fundamental Research Grant Scheme (FRGS/1/2018/WAB13/AIMST/02/1). We would like to thank the Ministry of Higher Education (MOHE), Government of Malaysia, for providing financial assistance to conduct this study.

Ethical approval

Ethical approval was obtained from the AIMST University Human and Animals Ethics Committee (approval no. AUAEC/FOP/2021/18), and the study was conducted in accordance with the guidelines of Helsinki Declaration.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

REFERENCES

- Grindlay K, Dako-Gyeke P, Ngo TD, Eva G, Gobah L, Reiger ST, Chandrasekeran S, Blanchard K. Contraceptive use and unintended pregnancy among young women and men in Accra, Ghana. *PLOS One* 2018; 13(8): e0201663.
- Solo J, Festin M. Provider Bias in Family Planning Services: A Review of Its Meaning and Manifestations. *Glob Health Sci Pract*. 2019; 7(3): 371-385. doi: 10.9745/GHSP-D-19-00130.
- World Health Organization. Traditional medicine. Questions and answers 2023. <https://www.who.int/news-room/questions-and-answers/item/traditional-medicine> (Accessed October 2025).
- Najimudeen M, Sachchithanantham K. An insight into low contraceptive prevalence in Malaysia and its probable consequences. *Int J Reprod Contracept Obstet Gynecol* 2014; 493-496.
- Nachimuthu H, Devi H, Karunanithi N. Knowledge, awareness, and perception on contraception among women of reproductive age attending Senawang Health Clinic. *MJMR* 2022; 06(02): 16-27.
- Shahidan WNS, Tuan Ismail TNN, Ponnuraj KT. Malaysian herbs in contraception: Public Perception. *JSKM* 2022; 20(1): 129-137.
- Gupta V, Bansal P, Garg A, Meena A. Pharmacopoeial standardization of *Hibiscus rosa sinensis* Linn. *Int J Pharm Clin Res* 2009; 1(3): 124-126.
- Gupta PC. Contraceptive potential of *Hibiscus rosa-sinensis* (Linn.) - An update. *Int J Pharm Res* 2012; 4(4): 1-3.
- Amtaghri S, Qabouche A, Slaoui M, Eddouks M. A comprehensive overview of *Hibiscus rosa-sinensis* L: its ethnobotanical uses, phytochemistry, therapeutic uses, pharmacological activities, and toxicology. *EMIDDT* 2024; 24(1): 86-115.
- Begashaw B, Mishra B, Tsegaw A, Shewamene Z. Methanol leaves extract of *Hibiscus micranthus* Linn exhibited antibacterial and wound healing activities. *BMC Complement Altern Med* 2017; 17(1): 337.
- Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *J Taibah Uni Sci* 2015; 9(4): 449-454.
- Garg D, Shaikh A, Muley A, Marar T. In-vitro antioxidant activity and phytochemical analysis in extracts of *Hibiscus rosa-sinensis* stem and leaves. *Free Radical Antioxidant* 2012; 2(3): 41-46.
- Parasuraman S, Huey LY, Jee YS. Effect of hesperidin in the prevention of aluminum chloride-induced testicular dysfunction in rats. *J Adv Pharm Tech Res* 2024; 15(3): 194-199.
- Patel SK, Singh S, Singh HK, Singh SK. Effect of standardized extract of *Bacopa monnieri* (CDRI-08) on testicular functions in adult male mice. *J Ethnopharmacol* 2017; 197: 101-109.
- Al-Snafi AE. Chemical constituents, pharmacological effects, and therapeutic importance of *Hibiscus rosa-sinensis*: A review. *IOSR J Pharm* 2018; 8(7): 101-119.
- Sharma Y, Sharma R. Pharmacognostical and phytochemical profiling of *hibiscus rosa-sinensis*: unveiling the healing potentials of a botanical treasure. *Int J Res Anal Rev* 2023; 10(3): 823-834.
- Dowara M, Gogoi I, Saikia S. Phytochemical screening and in vitro antibacterial activity of *Hibiscus rosa-sinensis* L. leaf extracts. *Asian J Biol Life Sci* 2024; 13(1): 35-39.
- Ghaffar F, El-Elaimy I. In vitro, antioxidant and scavenging activities of *Hibiscus rosa sinensis* crude extract. *J Appl Pharm Sci* 2012; 2(1): 51-58.

19. Rajesh M. Radical scavenging and antioxidant activity of *Hibiscus rosa sinensis* extract. *Afr J Pharm Pharmacol* 2011; 5(17).
20. Kholkute S, Mudgal V, Udupa K. Studies on the antifertility potentiality of *Hibiscus rosa sinensis*. Parts of medicinal value; selection of species and seasonal variations. *Planta Med* 1977; 31(01): 35–39.
21. Reddy CM, Murthy DR, Patil SB. Antispermato-genic and androgenic activities of various extracts of *Hibiscus rosa-sinensis* in albino mice. *Indian J Exp Biol* 1997; 35(11): 1170–1174.
22. Mishra N, Tandon V, Munjal A. Evaluation of medicinal properties of *Hibiscus rosa sinensis* in male Swiss Albino mice. *Int J Pharm Clin Res* 2009; 1(3): 112–114.
23. Behr R, Sackett SD, Bochkis IM, Le PP, Kaestner KH. Impaired male fertility and atrophy of seminiferous tubules caused by haploinsufficiency for *Foxa3*. *Dev Biol* 2007; 306(2): 636–645.
24. Wangikar P, Ahmed T, Vangala S. Toxicologic pathology of the reproductive system. In: *Reproductive and Developmental Toxicology*. Elsevier 2011: 1003–1026.
25. Tan CH. Is *Hibiscus rosa sinensis* Linn. a potential source of antifertility agents for males? *Int J Fertil* 1983; 28(4): 247–248.