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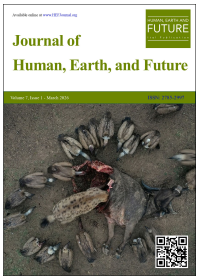

Male Fertility Suppression by *Piper nigrum*: Insights from In Vivo and In Silico Analysis

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Abstract

Piper nigrum L. (black pepper) is a widely used spice known for its diverse pharmacological effects, including potential activity on male reproductive function. This study aimed to explore the anti-fertility mechanisms of ethanolic extract of *P. nigrum* through a combination of in vivo and in silico molecular docking evaluation. Phytocompounds identified via GC-MS were docked against key reproductive targets, including the androgen receptor, follicle-stimulating hormone receptor (FSHR), follicle-stimulating hormone (FSH), testosterone, proprotein convertase subtilisin/kexin type 4 (PCSK4), mothers against decapentaplegic signaling (SMAD3) proteins, and cAMP-responsive element modulator (CREM). Compounds such as hinokinin (-9.9 kcal/mol) and piperine (-8.2 kcal/mol) exhibited strong binding affinities, particularly with the androgen receptor, SMAD3, and CREM, suggesting disruption of hormonal signaling and spermatogenic regulation. In vivo analysis in male rats confirmed these predictions, revealing dose-dependent alterations in sperm quality parameters. Lower to moderate doses (KP1 and KP2) significantly reduced sperm motility and viability and increased abnormality rates, indicating compromised fertility potential. However, the highest dose Group IV (KP3) showed a partial recovery trend, with the greatest increase in sperm concentration, motility levels comparable to the control group, and improved viability, though abnormalities remained elevated. These findings suggest a hormetic effect, where high doses may trigger adaptive protective mechanisms after initial impairment at lower doses. Overall, the results demonstrate that *P. nigrum* contains bioactive compounds capable of modulating male reproductive health through both molecular and physiological pathways. The combined approach highlights its potential as a reversible male contraceptive, warranting further research on dose optimization, treatment duration, and long-term safety. These findings also underscore the relevance of regional phytochemical variability in determining biological activity.

Keywords: Black Pepper; Reversible Male Contraceptive; Molecular Docking; SMAD3; CREM; PCSK4.

1. Introduction

The global population is expected to exceed 8 billion in 2022, with a peak of roughly 10.4 billion in the 2080s [1]. Rapid expansion in developing countries, particularly Indonesia, places additional strain on natural resources, healthcare

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systems, and socioeconomic infrastructure [2]. Indonesia, the world's fourth most populous country, is experiencing a demographic bonus era in which a large working-age population has the potential to boost economic growth but requires careful planning to avoid unemployment and resource overstrain [3]. Family planning efforts worldwide have primarily focused on female contraceptives, with limited options for men. Male contraceptives remain restricted to condoms, vasectomy, or coitus interruptus, each with significant limitations such as irreversibility or high failure rates [4]. This imbalance places an undue burden on women and leaves a critical gap in reproductive healthcare innovation. Research into safe, reversible, and culturally acceptable male contraceptive methods is essential to achieving shared responsibility in family planning [5]. The global pursuit of safe, effective, and reversible male contraceptive methods remains a significant challenge in reproductive health. Despite advancements in female contraceptives, the options for males are still largely limited to condoms and vasectomy, both of which have limitations in terms of effectiveness, reversibility, or user compliance. Hence, there is an increasing demand for the development of novel, plant-derived male contraceptives that are both reversible and free from serious side effects.

The utilization of phytochemicals that disrupt male fertility by influencing sperm function or hormone control is an encouraging area of research in this field. Local herbs such as *Carica papaya*, *Momordica charantia*, *Piper betle*, *Hibiscus rosasinensis*, *Curcuma domestica*, *Areca catechu*, and *Andrographis paniculata* have all shown antifertility potential in preclinical studies [6, 7]. Black pepper, or *Piper nigrum* L., is a medicinal plant that has lately come to light due to its possible antifertility effects; it is well-known for its medical and culinary uses. *P. nigrum*, which is a member of the Piperaceae family, is an important medicinal plant. It is considered to be the "King of spices" among the many different kinds of spices, and it is one of the spices that is used the most frequently. Numerous tropical regions, such as Brazil, Indonesia, Malaysia, Thailand, Madagascar, West Africa, and India, are responsible for the cultivation of black pepper [8]. It has been used as a remedy for gastrointestinal, respiratory, and inflammatory disorders, and its bioactive compound piperine exhibits broad pharmacological activity, including antimicrobial and antioxidant properties [9]. In addition to these uses, black pepper's potential as a natural contraceptive has recently drawn scientific interest [10-12]. Emerging evidence indicates that piperine, a major alkaloid in black pepper, may interfere with spermatogenesis and inhibit fertility in animal models [13]. Ethanolic extracts of black pepper have been reported to alter hormonal pathways and exhibit spermicidal properties, providing a basis for further investigation into its anti-fertilization potential [14]. Previous research results indicate that the administration of ethanol extract from black pepper fruit reduces the diameter of seminiferous tubules, spermatozoa concentration, the number of primary spermatocytes, and spermatozoa motility [10, 15].

In traditional medicine systems, *P. nigrum* has long been recognized for its therapeutic versatility. It is a key component of Tri-Ka-Tuk, a traditional Thai herbal formulation that also includes *Zingiber officinale* and *Piper retrofractum*, and is commonly used to support digestion, enhance metabolic function, and restore internal balance [16]. Beyond its digestive applications, modern pharmacological studies have shown that *P. nigrum* possesses neuroprotective properties, including the ability to regulate oxidative stress and modulate α-synuclein aggregation—two factors implicated in the progression of Parkinson's disease [17]. Furthermore, phytochemical analyses reveal that *P. nigrum* is rich in flavonoids and phenolic compounds, with total phenolic content reported at 85.3 mg QE/g, contributing to its broad antioxidant potential and supporting its relevance as a bioactive medicinal plant [18].

However, despite a growing body of evidence supporting the antifertility potential of various medicinal plants, including *P. nigrum*, studies examining the underlying molecular mechanisms, long-term safety, and reversibility of these effects remain scarce. Phytochemical studies have identified a variety of active compounds in *P. nigrum*, including essential nutrients such as carbohydrates, proteins, and minerals (e.g., calcium, magnesium, potassium, and iron), along with secondary metabolites like vitamin C, tannins, flavonoids, and carotenoids [19]. These constituents contribute to the plant's broad pharmacological activity. Notably, *P. nigrum* has been shown to affect reproductive, endocrine, and antioxidant pathways, underscoring its potential role in male fertility regulation [20]. Most available data are preliminary, often limited to basic histological or spermatogenic observations, without comprehensive analysis of the specific biochemical pathways involved. Furthermore, the potential hormonal imbalances, systemic toxicity, and reproductive recovery following treatment discontinuation are not well understood. These gaps in knowledge present a critical barrier to the development of plant-based male contraceptive agents that are both effective and safe for long-term use.

This study is grounded in a phytopharmacological framework that integrates principles of reproductive toxicology, hormonal biochemistry, and computational biology. Rather than focusing on a single compound, the research investigates the combined effects of *P. nigrum's* diverse bioactive constituents—such as alkaloids, flavonoids, and essential oils—on male reproductive function. Previous experimental studies have shown that ethanolic extracts of *P. nigrum* can improve or alter sperm morphology and concentration in hormone-disrupted animal models, indicating significant interaction with spermatogenesis and testicular function [11]. Other studies and pharmacological analyses of *P. nigrum* highlight its ability to influence reproductive hormones and oxidative stress pathways, both of which are central to male fertility regulation [21, 22]. These findings are further supported by molecular docking analyses conducted in this study, which explored the binding affinity of key metabolites, such as piperine and related compounds, to reproductive hormone receptors and enzymes involved in spermatogenesis. The docking results provide mechanistic

insights into how these phytoconstituents may modulate androgen signaling or disrupt sperm development at the molecular level. Taken together, this theoretical approach aims to characterize *P. nigrum's* antifertility effects as reversible and targeted, positioning it as a promising candidate for natural male contraception without inducing systemic toxicity.

2. Material and Methods

2.1. Study Design

This study employed a combined in vivo and in silico approach to evaluate the anti-fertility potential of *P. nigrum* in male rats (*Rattus norvegicus*). The objective was to assess both the physiological effects of the ethanolic extract on sperm parameters and the molecular interactions between the plant's active compounds and key reproductive targets. The integration of these two approaches allows for a more comprehensive understanding of how *P. nigrum* might influence male fertility, linking observable biological effects with possible molecular mechanisms of action. The entire experimental workflow, including extract preparation, compound analysis, docking, and animal testing, is summarized in Figure 1.

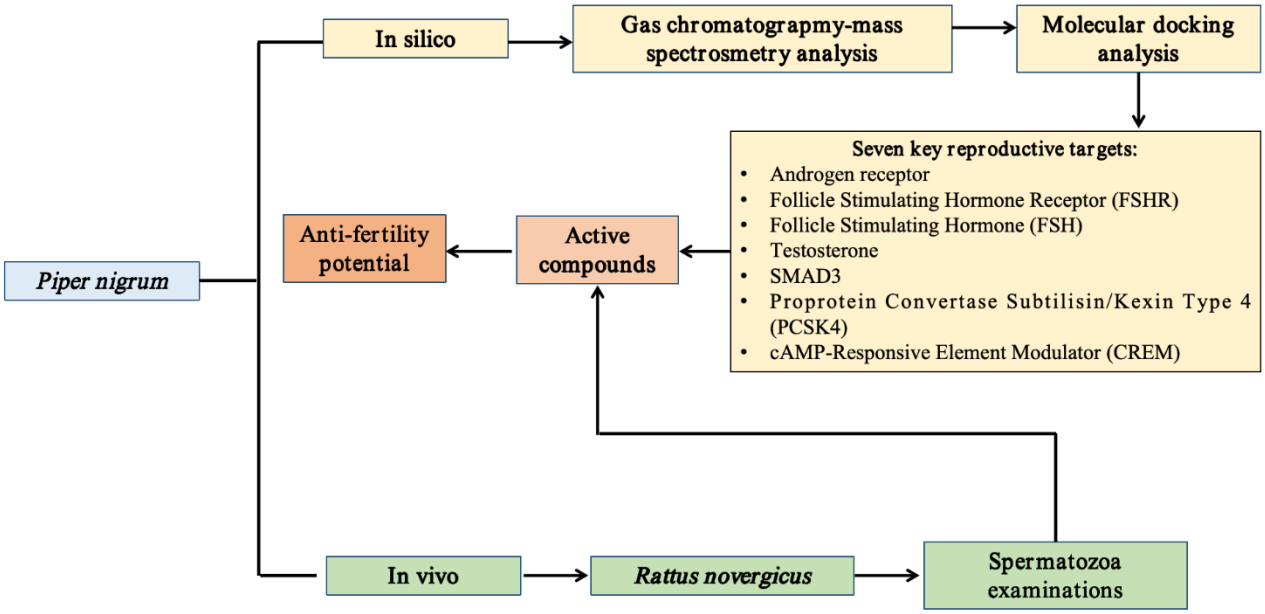


Figure 1. Methodological Flowchart of the Study

2.2. Sampling and Plant Preparations

Black pepper is cultivated in Bener Meriah Regency, Aceh Province, Indonesia [23]. The harvested black pepper fruits are rinsed with clean water to eliminate dust and debris adhering to them. The fruit is subsequently air-dried at ambient temperature until desiccated. The desiccated black pepper berries are pulverized to produce fine powder [24, 25]. The extraction process is conducted using maceration with a 70% ethanol solvent. The finely powder was macerated with a 70% ethanol solvent at a 1:10 ratio (100 grams of powder in 1 liter of 70% ethanol) for 7 days, with intermittent stirring of the mixture. Following the initial five days, the powder was immersed in 75% ethanol and intermittently agitated. The mixture is subsequently filtered through filter paper, and the residue is immersed in 25 volumes of 70% ethanol for 48 hours with intermittent agitation. Subsequently, filtration was performed utilizing filter paper till the extract and residue were acquired. The ethanol macerates 1 and 2 are amalgamated. The 70% ethanol macerate is evaporated with a rotary evaporator at 50°C until a viscous ethanol extract is obtained [26, 27].

2.3. Gas Chromatography-Mass Spectroscopy Analysis

A gas chromatography-mass spectrometry (GC-MS) analysis was carried out at the Chemical Instrument Laboratory of Universitas Syiah Kuala. The analysis was conducted in using a non-polar TraceGOLD TG-5MS column, which was manufactured by Thermo Fisher Scientific Inc. in the United States. The column measured 30 meters in length, 0.25 millimeters in diameter, and 0.25 micrometers in diameter. Helium was used as the carrier gas, and the flow rate through the column was 0.5 milliliters per minute. A single microliter of ethanol extract was introduced into the apparatus used for gas chromatography so that the mass spectrometry detector could do an analysis on it. The results of this compound separation were then analysed with the use of the mass spectral database maintained by the National Institute of Standards and Technology (NIST-MS) [28].

2.4. Molecular Docking Analysis

The selected target proteins including the androgen receptor, follicle-stimulating hormone receptor (FSHR), follicle-stimulating hormone (FSH), testosterone, proprotein convertase subtilisin/kexin type 4 (PCSK4), mothers against decapentaplegic signaling (SMAD3) proteins, and cAMP-responsive element modulator (CREM). The three-dimensional structures are available for download from the Protein Data Bank (PDB) at www.rcsb.org and AlphaFold [29, 30]. The downloaded proteins were dehydrated, optimized, and designated to the active sites. Target proteins were subsequently processed with BIOVIA Discovery Studio software and saved in .pdb format [31]. The active component in the target protein is the active chemical derived from the ethanol extract of black pepper. Dimethandrolone undecanoate and testosterone undecanoate served as the control ligand. Ligands can be obtained from the PubChem database [32]. Molecular docking of receptors utilizing Autodock Vina [33]. The ligand molecule will bind to the active site of the receptor, thereby inhibiting its function, and ultimately, the ligand may serve as a pharmaceutical agent [34]. The examination of docking data involved examining the nature of molecular bond interactions between proteins and ligands.

2.5. In Vivo Analysis

The subjects of the experiment consisted of 28 male Wistar rats of the *Rattus norvegicus* species. The rats were randomly allocated into four groups, each including six rats, with an extra rat per group to account for any attrition. Group I (K0) functions as the control group, receiving no therapy, and is provided with food in pellet form and unrestricted access to drinking water. Group II (KP1) is the treatment group that receives black pepper fruit extract at a dosage of 1 mg/kg body weight every four days for a duration of 48 days. Group III (KP2) was administered the extract at a dosage of 2 mg/kg body weight for 48 days, whereas Group IV (KP3) received the extract at a dosage of 4 mg/kg body weight for the identical duration. Each rat, in both the control and treatment groups, was weighed to determine the extract dosage according to its body weight. The extract was administered orally via a gastric tube in accordance with the prescribed dosage. The black pepper extract was delivered at 10:00 AM WIB. The extract was diluted in distilled water and combined with heated carboxymethyl cellulose (CMC) to create a homogenous solution. The extract solution is made anew for each administration based on the dosage specified for each treatment group.

2.6. Mouse Surgery, Spermatozoa Collection, and Spermatozoa Examination

Motility Test

On the 49th day, rats from the control and treatment groups were sacrificed for spermatozoa quality examination and testicular organ collection to examine the number of spermatogenic cells through Hematoxylin and Eosin (HE) staining. Samples of spermatozoa from male white rats were taken from the cauda epididymis using the slicing and gentle pressing method to obtain the spermatozoa. The assessment of spermatozoa motility is conducted by placing the sample on a glass slide, then adding a drop of physiological NaCl solution. Observation was conducted using a microscope with 40x magnification. The number of motile spermatozoa was counted based on their type of movement, which was categorized into rapid progressive (A), slow progressive (B), circular movement (C), and vibrating movement (D) [35]. Percentage of motility can be seen in Equation 1:

$$Percentage\ of\ motility\ spermatozoa\ (\%) = (A+B)/(A+B+C+D) \times 100 \quad (1)$$

Viability Test

The viability of spermatozoa is assessed by placing a drop of the sample on a glass slide, then adding a drop of eosin-nigrosin stain. The preparation is evenly spread, fixed using a spirit lamp, and observed under a 40x magnification microscope. Dead cells will appear red because they absorb the dye, while living spermatozoa do not absorb the dye, making them appear white. The number of spermatozoa is counted, compared to the total visible cells, and the result is expressed as a percentage in Equation 2 [35]:

$$Percentage\ of\ live\ spermatozoa\ (\%) = (Number\ of\ live\ spermatozoa)/(Total\ of\ live\ and\ dead\ spermatozoa) \times 100 \quad (2)$$

Concentration of Spermatozoa

The spermatozoa concentration is determined by extracting a sample to the 0.5 mark on the pipette, followed by the addition of a 3% NaCl solution until the volume reaches the 101 marks. The entire mixture is subsequently homogenized for 2-3 minutes, and a small quantity of the semen solution is eliminated to guarantee uniformity. A Neubauer counting chamber was prepared and covered with a cover slip to enumerate the spermatozoa. The semen solution is thereafter placed into the Neubauer counting chamber and examined under a microscope (Olympus CX21, Japan) at 40x magnification. The quantification of spermatozoa was conducted by enumerating the spermatozoa observable in five big squares within the Neubauer counting chamber [36]. The concentration of spermatozoa (million/mL) is determined using the Equation 3:

$$Concentration\ spermatozoa = (Number\ of\ spermatozoa\ x\ dilution)/(Volume\ of\ the\ erythrocyte\ counting\ chamber) \quad (3)$$

Abnormality Test

Observation was performed by placing spermatozoa and eosin-nigrosin on a glass slide, fixing it with a spirit lamp, and then examining it under a microscope at 40x magnification. Morphological inspection reveals anomalies categorized into main abnormalities (e.g., disproportionate head size, bifurcated heads or tails, and irregular head forms) and secondary abnormalities (such as cranial injury, tails fractured at the neck or middle, and contorted tails) [35]. The observed spermatozoa totalled at least 200 cells, and the calculation was executed using the subsequent Equation:

$$Percentage\ of\ normal\ sperm\ (\%) = (Number\ of\ normal\ sperm)/(Normal\ sperm + Countable\ abnormal\ sperm) \times 100 \quad (4)$$

2.7. Examination of the Number Spermatogenic Cells with HE Staining

Testis Organ Procurement

The removal of the testicular organ was performed after cervical dislocation in the rats. Next, surgery was performed, and the testis organs were washed with a 0.9% NaCl physiological solution, then placed in a 10% BNF fixation solution for 24 hours, and subsequently, the testis organs were transferred to a 70% ethanol solution as a stopping point.

Histological Examination

The process of making histological preparations begins with the fixation of testicular tissue in a 10% neutral buffered formalin (NBF) solution. The tissue is cut into 5x5 mm pieces, soaked in 70% alcohol for 6-7 hours, then undergoes gradual dehydration with alcohol (80%, 90%, 95%, and absolute) and clarification using xylol. Next, the tissue was infiltrated with liquid paraffin at 60°C three times, then embedded into tissue blocks. The blocks were cut with a microtome to a thickness of 3-5 µm and placed on poly L-lysine coated glass slides. The preparation was stained using the hematoxylin-eosin (HE) method as applied.

Hematoxylin-Eosin (HE) Staining

The staining process begins with deparaffinization, which involves the removal of paraffin using xylene solution three times, each for two minutes. This step is followed by rehydrating the tissue using graded alcohol solutions (absolute, 96%, 90%, and 80%) for two minutes per concentration. After that, the sample was rinsed with running water for five minutes. Hematoxylin staining was performed for one minute, with the results checked under a microscope to ensure the quality of nuclear staining. The sample is rinsed again with running water before being stained with eosin for five minutes, which is also monitored under the microscope to ensure optimal cytoplasmic staining. The next process is dehydration with graded alcohol solutions, followed by clearing using xylene, and ending with mounting using Entellan® adhesive. The stained tissue samples were observed using an Olympus CX41 microscope, with digital images taken using a DP12 camera. The resulting images were then processed and analyzed using the JP Image device.

Observation of Staining Results

The staining results were observed by counting the quantity of spermatogonia, spermatocytes, and spermatids in five seminiferous tubules of the testes from each control group and treatment group. This procedure was performed in order to determine these results. Counting was carried out on five distinct fields of vision while the magnification was set to 400 times. The data that were collected were then evaluated to make a comparison between the control group and the treatment group in terms of the quantity of spermatogenic cells.

2.8. Data Analysis

Data analysis using SPSS 30 software was conducted in stages through normality tests and data homogeneity tests, followed by analysis of differences in sperm quality and the number of spermatogenic cells in the seminiferous tubule tissue of the control and treatment groups using one-way ANOVA.

3. Results and Discussion

The GC-MS analysis of ethanolic extract of *P. nigrum* revealed the presence of 19 distinct compounds with varied retention times and peak areas, indicating a rich phytochemical profile (Table 1). The major compound identified was chavicine (64.01%), a geometric isomer of piperine known for its bioactivity. This finding is consistent with previous reports highlighting the dominance of chavicine and piperine in *P. nigrum* extracts [37]. Black pepper contains four isomeric forms of piperine, namely *trans-trans isomer* (piperine), *cis-trans isomer* (isopiperine), *cis-cis isomer* (chavicine), and *trans- cis isomer* (isochavicine) [38]. Known for its bioactivity, it may play a central role in the extract's pharmacological properties [39]. Other significant constituents include piperanine (6.90%), 2,4,10-hexadecatrienamide (5.20%), piperolein B (5.06%), and caryophyllene (4.69%), which are known to contribute to the plant's pharmacological actions, including antioxidant, anti-inflammatory, and fertility-modulating properties. Notably, caryophyllene, a sesquiterpene, has been reported as a prominent antimicrobial and cytoprotective agent found in *P. nigrum* essential oil

[40]. The presence of alkaloids such as piperidine derivatives and piperettine, along with fatty acid esters like hexadecanoic acid, methyl ester, suggests the extract's potential to affect cell membrane dynamics and receptor signaling, possibly contributing to the observed anti-fertility activity in vivo. Interestingly, although compounds like β-sitosterol (0.64%) were present in low concentrations, their biological significance is notable, particularly in steroidal pathways and hormonal modulation [9]. The detection of compounds such as cadinol, β-bisabolene, and α-selinene further supports the complexity and synergistic nature of the extract's bioactivity. The diversity of amides, terpenoids, and fatty acid derivatives reflects a robust pharmacological profile, supporting the results of the docking studies and in vivo assays conducted in this research. Variability in phytochemical composition, as influenced by geographical origin and extraction method, has been well-documented and likely plays a role in the bioactivity observed in Bener Meriah, Aceh-Indonesia.

Table 1. GC-MS analysis results of ethanolic extract from Bener Meriah

No.	Retention time (min)	Compounds name	Similarity index	Area (%)
1	20.35	α-cubebene	95	0.51
2	21.50	Caryophyllene	100	4.69
3	23.17	β-Selinene	98	0.57
4	23.38	α-Selinene	98	0.69
5	23.62	β-bisabolene	97	0.53
6	26.89	Cadinol	100	0.79
7	32.58	Hexadecanoic acid, methyl ester	98	0.66
8	32.91	2,4-Decadienamide, N-isobutyl-, (E,E)-	94	0.56
9	35.87	*trans*-13-Octadecenoic acid, methyl ester	99	1.33
10	44.18	Piperolein A	92	1.20
11	4463	Piperanine	94	6.90
12	45.43	Piperettine	85	0.51
13	46.49	2,4,10-Hexadecatrienamide, N-(2-methylpropyl)-, (2E,4E,10E)-	81	5.20
14	48.24	Piperyline	95	1.41
15	48.63	Chavicine	97	64.01
16	49.27	Pipersintenamide	84	4.10
17	50.68	Hinokinin	79	0.62
18	51.47	Piperolein B	99	5.06
19	53.93	β-sitosterol	97	0.64

The molecular docking analysis evaluated the binding affinities of compounds identified through GC-MS from the ethanolic extract of black pepper collected from Bener Meriah (Figure 2). Molecular docking analysis were performed against multiple receptor targets to identify interaction strength and trends. In this study, lower (more negative) binding affinity values indicate stronger ligand-receptor interactions [31]. The phytocompounds from ethanolic extract of *P. nigrum* from Bener Meriah exhibited binding affinities ranging from -3.1 to -9.9 kcal/mol, suggesting a wide spectrum of interaction strengths across the tested receptors. Notably, several compounds demonstrated high affinity, particularly toward the androgen receptor and CREM, which are key regulators of hormonal and reproductive pathways. The strongest interactions were observed for hinokinin (-9.9 kcal/mol) and pipersintenamide (-9.5 kcal/mol), as indicated by the darkest blue regions on the heatmap. These results emphasize the therapeutic potential of the Bener Meriah extract, especially in modulating androgen-related signaling pathways.

Follicle Stimulating Hormone or FSH, the principal regulator of spermatogenesis and male fertility, has been demonstrated to be linked with the Transforming Growth Factor Beta (TGF-β) pathway, via SMAD proteins, to modulate various facets of male reproductive function [41-43]. The SMAD proteins, especially SMAD3, are involved in modulating the production and reaction to FSH [41, 43]. The interaction of FSH with its receptor (FSHR) on Sertoli cells activates five biochemical pathways that influence spermatogenesis and phosphorylate the transcription factor cAMP-Responsive Element Modulator (CREM) at serine 117 [44]. The CREM molecule is crucial for activating genes that regulate meiosis and the development of spermatogenic cell morphology [45]. The molecule CREMr functions primarily in the testes as a regulator of the differentiation and maturation of spermatogenic cells during spermatogenesis. Proprotein convertase subtilisin/kexin type 4 (PCSK4) is a serine protease that is solely expressed in the testes [46]. This protein serves as a marker for ligand-binding receptors involved in fertilization. The PCSK4 enzyme facilitates the acrosome reaction via a proteolytic mechanism [47].

The elevated negative binding affinity signifies that the ligand-receptor complex is more stable and possesses reduced free energy, indicative of a robust connection. This stability is achieved through a combination of various types of

interactions, including conventional hydrogen bonds (Met:742 and Gln:711), which strengthen the specific binding of the molecule in the binding pocket, and carbon hydrogen bonds (Gln:738 and Met:745), which serve as additional stabilizers from the interaction of hinokinin with receptor androgen (Table 2). Pi-sigma and π-π stacked interactions strengthen the bond through direct contact between the ligand's aromatic groups and the receptor's aromatic residues, such as phenylalanine and tyrosine, while alkyl and π-alkyl interactions with hydrophobic residues (such as leucine and valine) add strength by keeping the ligand within the receptor's hydrophobic environment [48]. The interplay of these interactions reduces the free energy of the complex, enhances binding affinity, and enables the molecule to effectively block androgen receptor function, hence underscoring its potential as a male antifertility medication. The hinokinin demonstrates a binding affinity to FSHR that exceeds that of the positive control, suggesting significant promise as an FSHR inhibitor (Table 3). This molecule is ranked top because of its robust mix of contacts, primarily through π-π stacking and hydrophobic alkyl-π interactions.

In addition to furanone, piperine demonstrates a notably high binding affinity score, surpassing dimethandrolone undecanoate and testosterone undecanoate for proteins such as FSHR, FSH, and SMAD3. Dimethandrolone undecanoate (DMAU), which is also referred to as 7a,11b-Dimethyl-19-nortestosterone 17b-undecanoate, is a male hormonal contraceptive that is now in the experimental phase and contains a significant amount of promise. Dimethandrolone (DMA) is the active metabolite of DMAU, which is an oral experimental male hormonal contraception that operates as a prodrug and converts to its active phase in a short amount of time. Following the delivery of DMAU, the oral bioavailability of DMA is insufficient and inconsistent, which presents a substantial hurdle to the development of DMA as an oral drug [49]. Because of this, there is a substantial opportunity to examine potential candidates for anti-fertility medications.

Piperine is the primary bioactive alkaloid linked to numerous physiological effects of black pepper [50]. According to previous research, piperine has the ability to decrease the levels of antioxidant enzymes and sialic acid, which in turn leads to an increase in reactive oxygen species. This rise has the potential to cause harmful effects on the epididymal environment and the function of spermatozoa. Piperine reduces testosterone production in pubescent male rats by acting on the Extracellular Signal Regulated Kinase (ERK)1/2 and Protein Kinase B (PKB) pathways. However, piperine increases the development of Leydig cells in these rats [51].

The present study revealed that hinokinin exhibits superior binding affinity to androgen receptors (AR), FSHR, FSH, PCSK4, and CREM in comparison to testosterone undecanoate and dimethandrolone undecanoate. The compounds piperyline, and piperine exhibit the greatest binding affinity to SMAD3, whereas dimethandrolone undecanoate demonstrates a superior affinity score relative to the compounds present in the ethanol extract of black pepper fruit for the hormone testosterone.

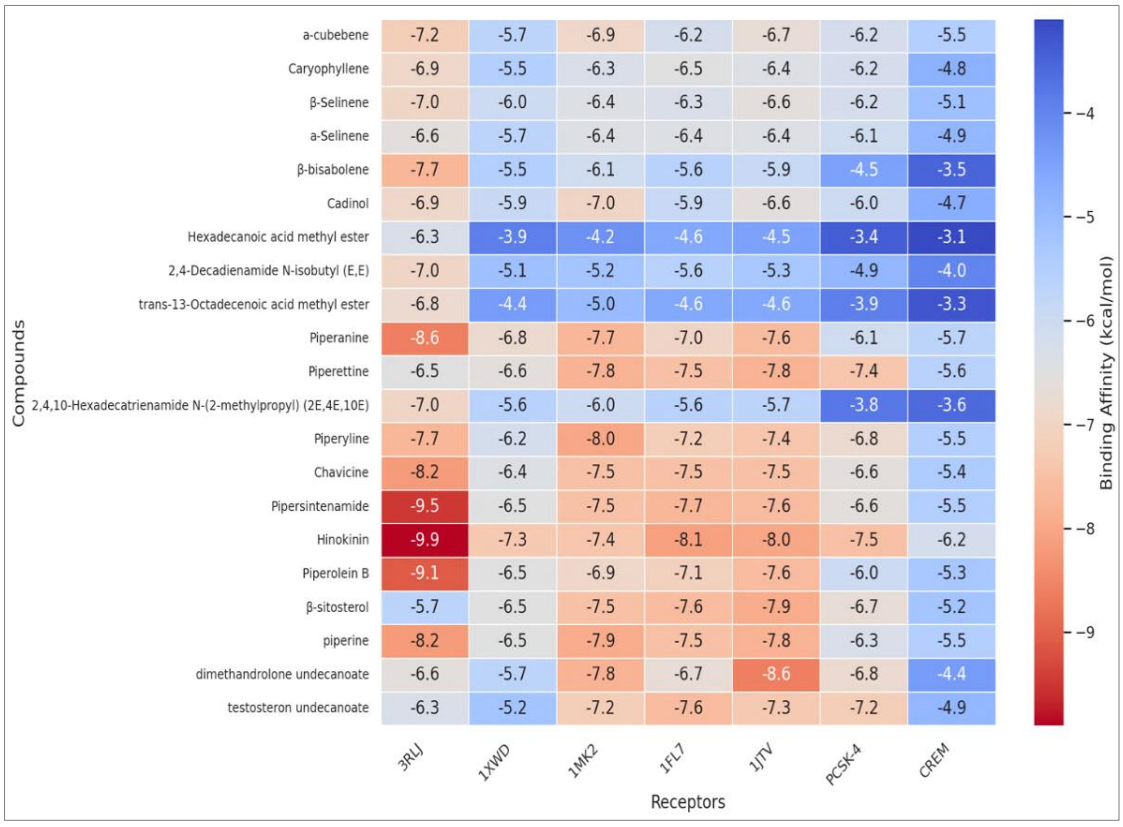
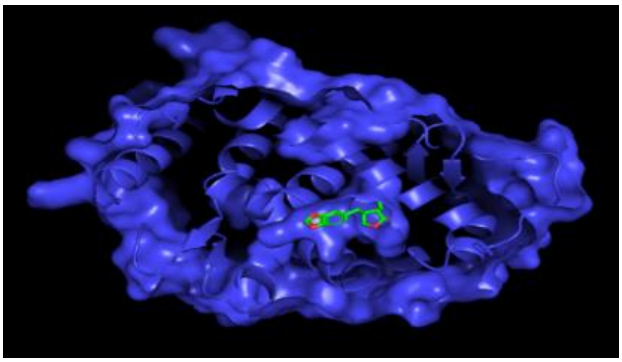
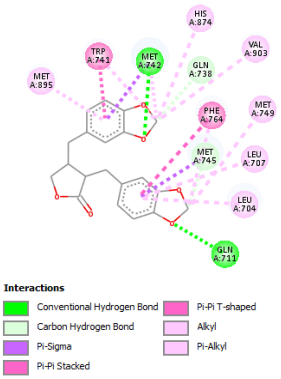
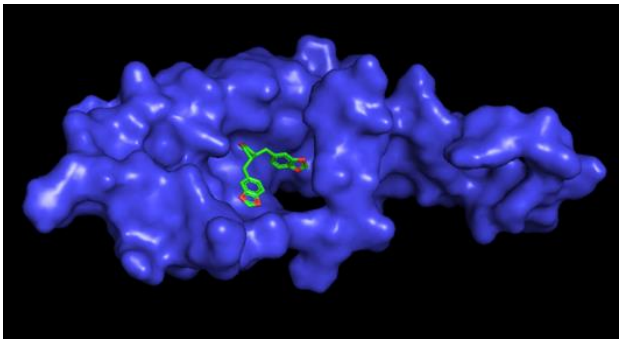
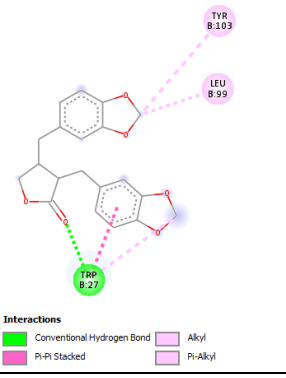
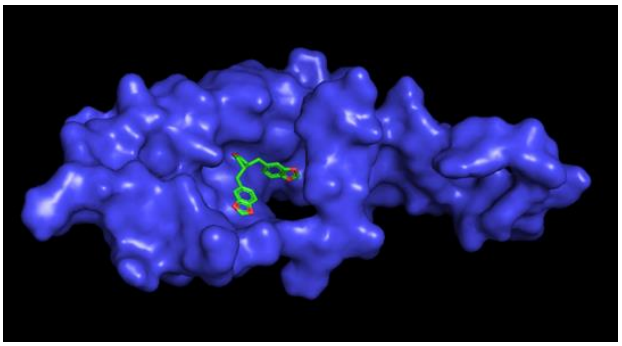
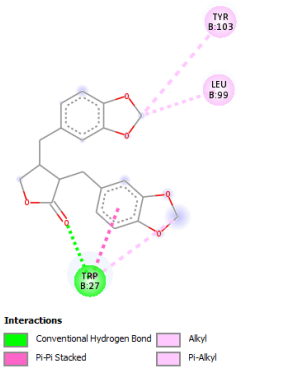


Figure 2. Heat-map of binding affinity value from molecular docking analysis between phytochemical compounds. The receptors containing: Androgen receptor (PDB ID: 3RLJ); FSHR (PDB ID: 1XWD); SMAD3 (PDB ID: 1MK2); FSH (PDB ID: 1FL7); Testosteron (PDB ID: 1JTV), PCSK-4; and CREM.

The inclusion of control ligands, dimethandrolone undecanoate (synthetic androgen) and testosterone undecanoate (endogenous androgen), provides a clear benchmark for evaluating the docking performance of the natural compounds identified in the ethanol extracts of *P. nigrum*. These controls represent clinically established androgen receptor agonists, enabling a direct comparative assessment of binding efficiency and receptor selectivity. The comparative docking results reveal that several natural constituents, particularly hinokinin and piperine, demonstrate stronger or comparable affinities to both reference ligands across multiple receptors, including the androgen receptor (3RLJ), testosterone receptor (1JTV), and SMAD3 (1MK2). Specifically, hinokinin exhibited the strongest overall binding affinity (-9.9 kcal/mol) against the androgen receptor, substantially exceeding both dimethandrolone undecanoate (-6.6 kcal/mol) and testosterone undecanoate (-6.3 kcal/mol). This suggests that hinokinin may effectively interact with the ligand-binding domain through π-π stacking and hydrogen-bond interactions, potentially stabilizing receptor conformations in a manner analogous to steroidal ligands. Moreover, its concurrent high affinity for SMAD3 and FSH receptors indicates that it could exert multi-target modulatory effects, linking hormonal signaling to transcriptional regulation and anti-inflammatory or antiproliferative pathways. Similarly, piperine, the major alkaloid of *P. nigrum*, displayed consistently strong binding scores (-8.2 to -7.9 kcal/mol) across most receptors, closely matching those of the control androgens. Piperine's docking profile supports existing evidence of its bioenhancing and androgen-modulating properties, as it can influence steroidogenic enzyme activity and receptor sensitivity.

Table 2. The 3D and 2D visualization of hinokinin with seven receptors

3D Visualization	2D Visualization
	
Androgen Receptor	
	
FSH receptor	
	
FSH	

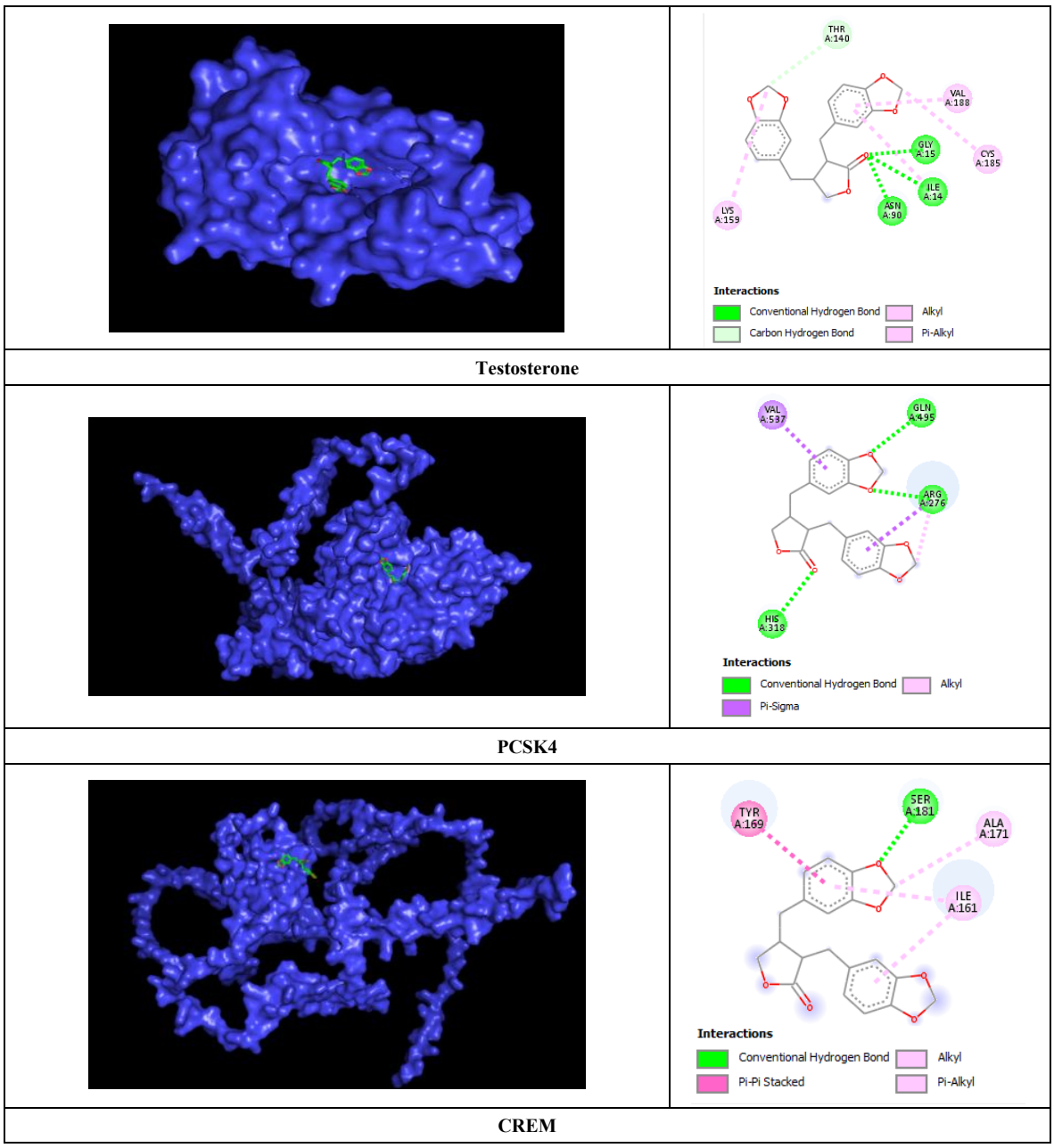


Table 3. Comparative analysis of binding free-energy value of selective ligands and control

Compound	Type	3RLJ (Androgen Receptor)	1JTV (Testosterone)	1MK2 (SMAD3)	PCSK4	CREM
Hinokinin	Natural lignan	**-9.9**	-8.0	-7.4	-7.5	-6.2
Chavicine	Piperine isomer	-8.2	-7.5	-7.5	-6.6	-5.4
Piperine	Alkaloid	-8.2	-7.8	-7.9	-6.3	-5.5
Dimethandrolone Undecanoate (Control 1)	Synthetic androgen	-6.6	**-8.6**	-7.8	-6.8	-4.4
Testosterone Undecanoate (Control 2)	Endogenous androgen	-6.3	-7.3	-7.2	-7.2	-4.9

This comprehensive analysis underscores the significant impact of treatment on all assessed sperm quality parameters namely, concentration, motility, viability, and abnormality (Figure 3). Based on a comprehensive evaluation of all four sperm quality parameters namely concentration, motility, viability, and abnormality, KP3 stands out as the treatment group with the most favorable overall profile. Although it does not fully restore every parameter to the level observed in the control group, KP3 demonstrates the most balanced combination of beneficial effects. Notably, it yields the highest mean sperm concentration (150 million/mL), far exceeding that of the control and other treatment groups. As sperm concentration is a fundamental marker of male fertility potential, this finding is particularly significant. Moreover, KP3 is able to maintain motility (70.47%) at levels nearly identical to the control (70.99%), indicating that sperm remain functionally competent despite treatment exposure. Viability, while not entirely restored, shows a substantial recovery compared to the sharp declines observed in KP1 and KP2, suggesting an improvement in overall

cell health and membrane integrity. Importantly, KP3 also achieves a notable reduction in sperm abnormalities relative to KP1 and KP2, implying that the higher treatment dose may help mitigate the detrimental effects seen at lower doses.

These outcomes reflect a pattern consistent with a hormetic dose-response, a biphasic effect where low to moderate doses exert toxic or suppressive effects, while higher doses induce adaptive, protective biological responses [52]. This phenomenon is well recognized in both toxicology and reproductive biology, where the body's antioxidant systems or cellular repair mechanisms may be upregulated at threshold exposure levels, leading to improved resilience and function. In the context of male fertility, such adaptations can enhance critical sperm functions, including motility, morphology, and viability [53, 54]. Thus, among the treatments tested, KP3 appears to be the most effective, producing strong improvements in key fertility indicators while partially reversing the adverse effects seen in KP1 and KP2. However, it is important to acknowledge that the abnormality rate in KP3 remains higher than in the control, which may reflect residual stress or incomplete recovery. This highlights the need for further optimization of dosage, formulation, or treatment duration to maximize benefits while minimizing unintended cellular damage. Additional studies incorporating molecular markers such as DNA fragmentation and oxidative stress parameters would further clarify the underlying mechanisms and therapeutic potential of the treatment.

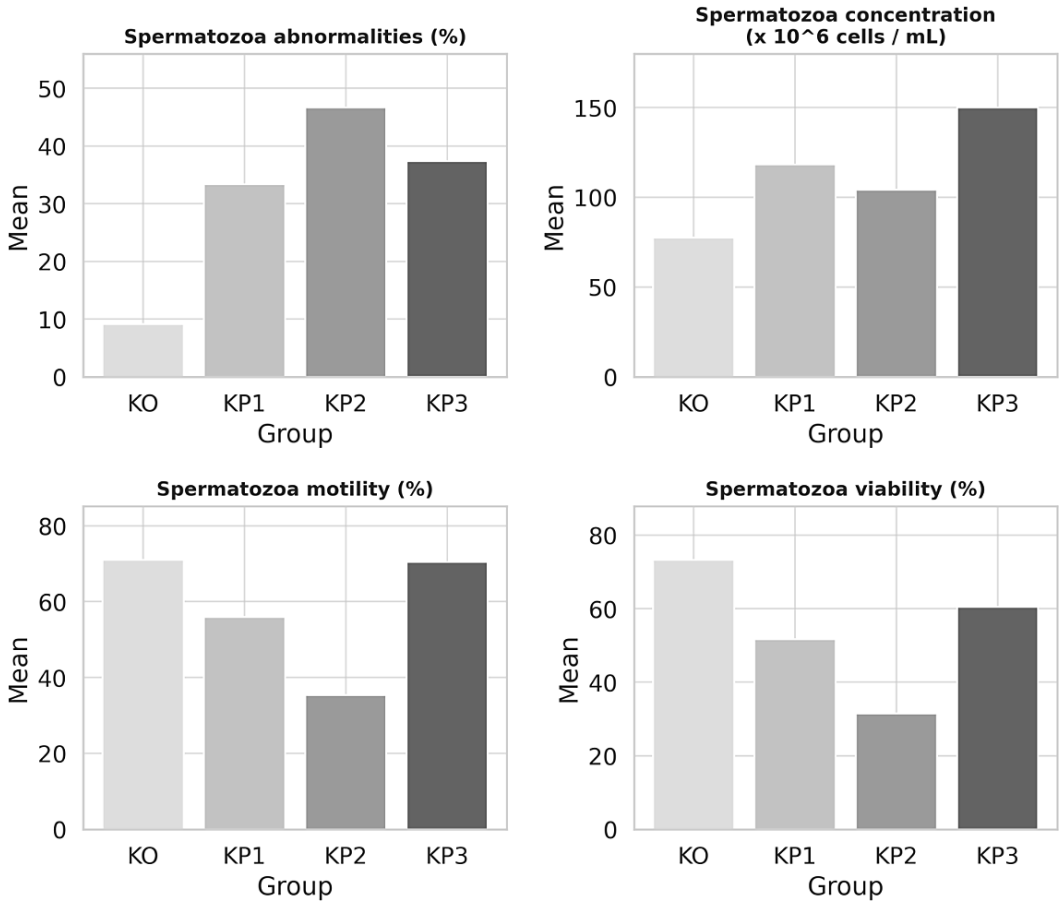


Figure 3. Comparison of sperm quality parameters across treatment groups.

The correlation analysis revealed notable relationships among key sperm quality parameters (Figure 4). Sperm abnormality demonstrated a moderate positive correlation with concentration ($r = 0.54$), suggesting that an increase in sperm output may be accompanied by a higher incidence of morphological defects. This could indicate a compensatory overproduction mechanism or potential stress-induced disruption in spermatogenesis, where the quantitative increase compromises quality. Additionally, abnormality showed a moderate negative correlation with viability ($r = -0.48$), implying that sperm with structural abnormalities tend to have compromised membrane integrity or are more susceptible to cell death. This is consistent with existing literature indicating that morphologically abnormal sperm are often less functional and have lower survival potential [55]. On the other hand, sperm motility was moderately positively correlated with viability ($r = 0.55$), reinforcing the interdependence between structural integrity and functional performance. Viable sperm are more likely to maintain mitochondrial activity and intact membranes, which are essential for progressive motility [56]. Motility showed a weak or negligible correlation with both concentration and abnormality, indicating that movement capacity may be influenced by factors beyond simple sperm count or morphology, such as mitochondrial function or oxidative stress. Meanwhile, sperm concentration exhibited only weak correlations with motility and viability, suggesting that the quantity of sperm produced does not necessarily reflect their functional or structural quality. This distinction underscores the importance of assessing a comprehensive panel of semen parameters in fertility evaluations, as high sperm counts may obscure underlying deficits in motility or viability.

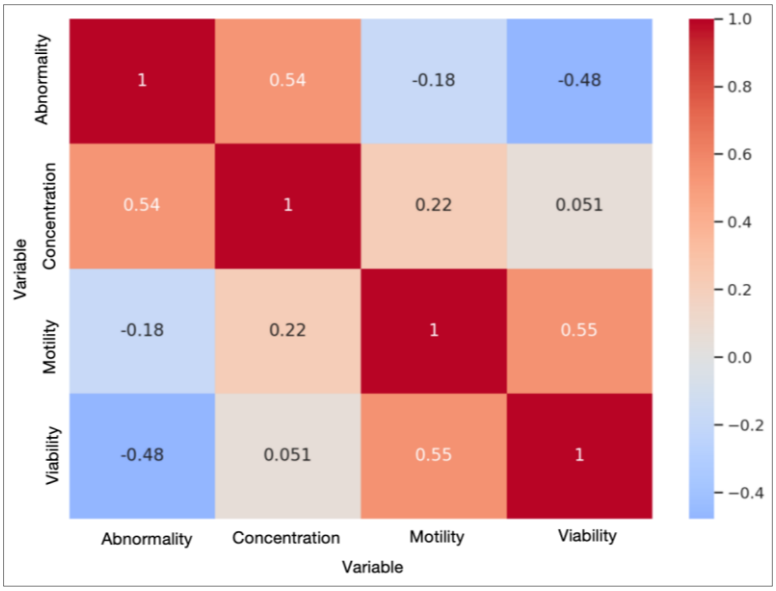


Figure 4. Correlation matrix between four variables

The violin plots in Figure 5 visualize the distribution and variability of biological variables across different experimental groups (KO, KP1, KP2, KP3). The violin plots show the density of data points across the range of each variable (spermatogonium, spermatocyte, round spermatid, elongated spermatid, Sertoli cell, Leydig cell). The width of the violins at different values indicates the concentration of data points, where a wider region reflects higher density, while a narrower region suggests less data concentration [57]. These plots provide a clear indication of the distribution of the data, highlighting the range and spread of values within each experimental group. In addition to the distribution, jittered points were overlaid on the violin plots to represent individual observations. These points help in visualizing the variation within each group. The addition of individual data points alongside summary plots like violin plots helps to avoid the problem of over-summarization and enhances the interpretability of complex datasets. By examining the spread of these jittered points, we can assess the variability within each experimental group. This result shows that the highest number of spermatogonium cells is in the KP1 group (50.17) and the lowest is in the KP2 group (46.20). The statistical test results indicate a significant difference (p=0.041), particularly between the control group and KP3 (p=0.026).

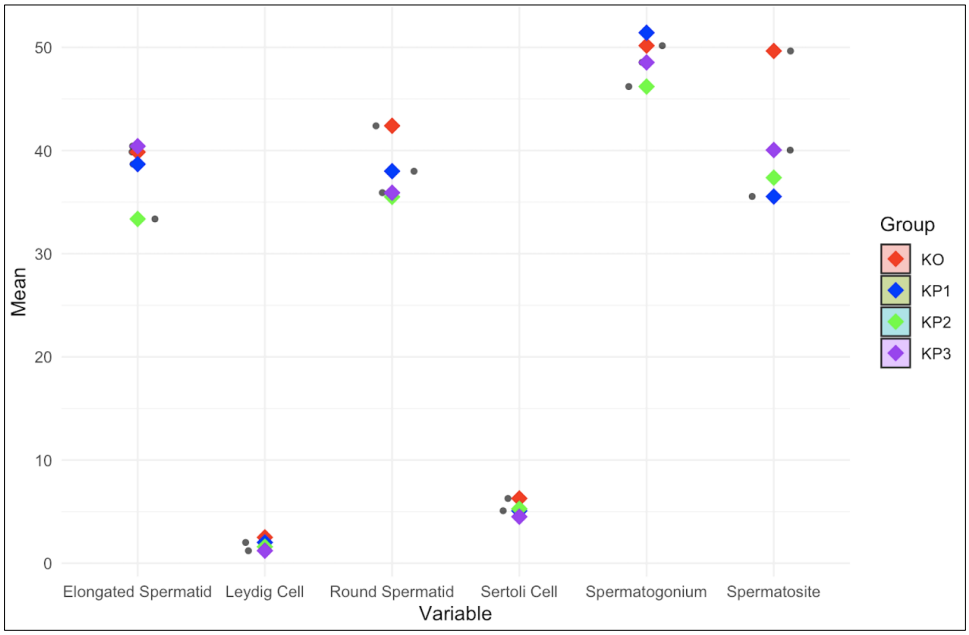


Figure 5. Violin plot with individual data point of spermatozoa

Figure 6 shows the histological differences in testicular tissue in each group. In the control group (K0), the seminiferous tubules appeared to have an intact and orderly structure. The lumen of the tubules is clean and clear, and the seminiferous tubular epithelium (ET) shows no signs of abrasion or pathological changes. The spermatogenic layer shows complete stages of spermatogenesis, starting from spermatogonia (SG) in the basal layer, spermatocytes (ST) in

the middle, to round spermatids (RS) and elongated spermatids (ES) adjacent to the lumen. Sertoli cells (SC) appear normal in supporting the development of spermatogenic cells, while Leydig cells (LC) in the interstitial space also do not show any significant morphological changes. In the KP1 treatment group (1 mg/kgBW), the tubular structure remains relatively intact, although there are slight changes in the tubular epithelium. The spermatogenic layer still shows complete stages, but the number of spermatogonia in the basal layer has started to decrease slightly. Sertoli cells and Leydig cells appear to be in normal condition without indications of fibrosis or significant vascular dilation. Overall, the effects of the extract on this group are still minimal and within the tissue tolerance limits. On the other hand, in the KP2 group (2 mg/kgBW), the structural changes are more clearly observed.

The epithelium of the seminiferous tubules began to show irregularities, with some areas experiencing abrasion or thinning. The number of spermatogonia shows a significant decrease, indicating a disruption in the early stages of spermatogenesis. In addition, the number of spermatocytes and spermatids also decreased, with some cells appearing not to have reached the optimal differentiation stage. Sertoli cells show signs of cellular stress, and around the tubules, there are early indications of fibrosis as well as slight dilation of the interstitial blood vessels. These changes indicate that the 2 mg/kgBW dose is beginning to have a significant impact on testicular function and sperm production. In the KP3 group (4 mg/kgBW), the histological changes appeared more extreme. The epithelium of the seminiferous tubules experienced quite clear abrasion, with some areas showing significant distortion. The number of spermatogonia has drastically decreased, with indications of atrophy in some areas. Spermatocytes and spermatids also decreased significantly, causing the spermatogenesis process to be severely disrupted. Sertoli cells appear to undergo morphological changes that can hinder their function in supporting the development of spermatogenic cells. Infiltration of polymorphonuclear (PMN) cells is beginning to be observed, indicating an inflammatory response in the testicular tissue. Fibrosis at the basal tubules becomes more pronounced, and vascular dilation is more dominant, indicating the presence of oxidative stress and inflammation due to the extract treatment. These changes indicate that at high doses, ethanol extract of black pepper can cause substantial structural disturbances, potentially significantly inhibiting sperm production.

The dosage selection in this study was informed by prior research on the antifertility effects and safety profile of *P. nigrum* extracts. Notably, Mishra & Singh (2009) administered *P. nigrum* fruit powder at 100 mg/kg/day for 90 days in mice, observing significant disruptions to spermatogenesis and testicular histoarchitecture. Importantly, partial recovery of reproductive function was reported following cessation of treatment, indicating the potential reversibility of its antifertility effects [15]. Similarly, Ekaputri et al. (2018) evaluated ethanolic extracts of *P. nigrum* in male Wistar rats at doses ranging from 3.33 to 13.32 mg/kg body weight over 55 days. Their findings included significant hormonal suppression and deterioration of sperm parameters without evidence of systemic or lethal toxicity [10]. Based on these results, the present study adopted a dosing strategy within this previously validated range, aiming to achieve measurable antifertility effects while minimizing toxicity and preserving the potential for reversibility.

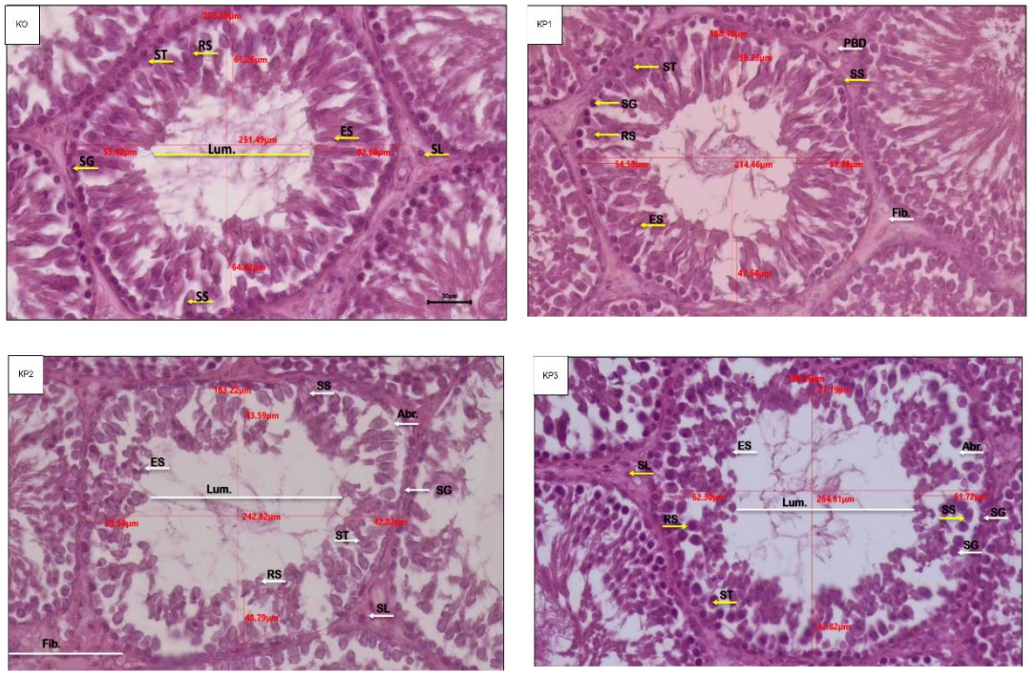


Figure 6. Histological appearance of the seminiferous tubules of rat testes after administration of ethanol extract of black pepper. K0=control, KP1=1 mg/kg bw extract dose, KP2=2 mg/kg bw extract dose, KP3=4 mg/kg bw extract dose. Descriptions: Seminiferous Tubule (ST), Spermatogonium (SG), Spermatocyte (SC), Round Spermatid (RS), and Elongated Spermatid (ES). Sertoli Cells (SS), Leydig Cells (SL), Lumen (Lum), Seminiferous Tubule Epithelium (ET), Fibrosis at the Basal Tubule (Fib), Vascular Dilation (PBD), Atrophic Spermatogonium (SG), Polymorphonuclear Cells (PMN), Abrasion of Seminiferous Tubule Epithelium (Abr). Magnification 400 times.

Comparative analysis between the control group and the treatment group shows a clear dose-response relationship. At a low dose (KP1), the changes that occur are still mild and do not significantly impact the structure or function of the seminiferous tubules. However, at moderate doses (KP2), changes begin to affect the balance of spermatogenic cells, whereas at high doses (KP3), the structural changes that occur are quite significant with indications of atrophy, inflammation, and apparent spermatogenesis dysfunction. The histological changes that occurred indicate that this extract can affect sperm production. The decrease in the number of spermatogonia and the disruption of spermatocyte differentiation to spermatids indicate an inhibitory effect on spermatogenesis. Furthermore, the inflammatory response and fibrosis at high doses can create a testicular environment that is less supportive of healthy sperm formation. Thus, ethanol extract black pepper has potential as a male contraceptive agent, although the specific mechanisms and long-term effects still require further research. These findings have important implications for reproductive research and clinical applications. Future studies should prioritize dose optimization, aiming to maximize therapeutic benefits while minimizing adverse effects. Additionally, the inclusion of more sensitive biomarkers such as DNA fragmentation indices, reactive oxygen species (ROS) levels, and mitochondrial activity would provide deeper mechanistic insights and help delineate the pathways through which these treatments exert their effects. A multi-parametric approach would enhance the predictive value of semen analysis in both experimental and clinical fertility assessments.

The findings of this study demonstrate that the ethanolic extract of black pepper (*P. nigrum*) contains several bioactive phytocompounds, notably hinokinin and piperine, which exhibit strong binding affinities toward key molecular targets involved in male reproductive regulation, including the androgen receptor, FSH and FSH receptor, PCSK4, SMAD3, and CREM (Figure 7). The integration of molecular docking and in vivo analyses suggests that these compounds may influence hormonal signaling and spermatogenic processes, leading to dose-dependent alterations in sperm quality observed in *Rattus norvegicus*. Collectively, these results indicate that black pepper phytochemicals possess a potential anti-fertility effect, likely mediated through disruption of androgenic and spermatogenic regulatory pathways. This integrated approach highlights the relevance of combining computational modeling with experimental validation to uncover the mechanistic basis of traditional medicinal plants and supports further investigation into the therapeutic and contraceptive potential of *P. nigrum* bioactive constituents.

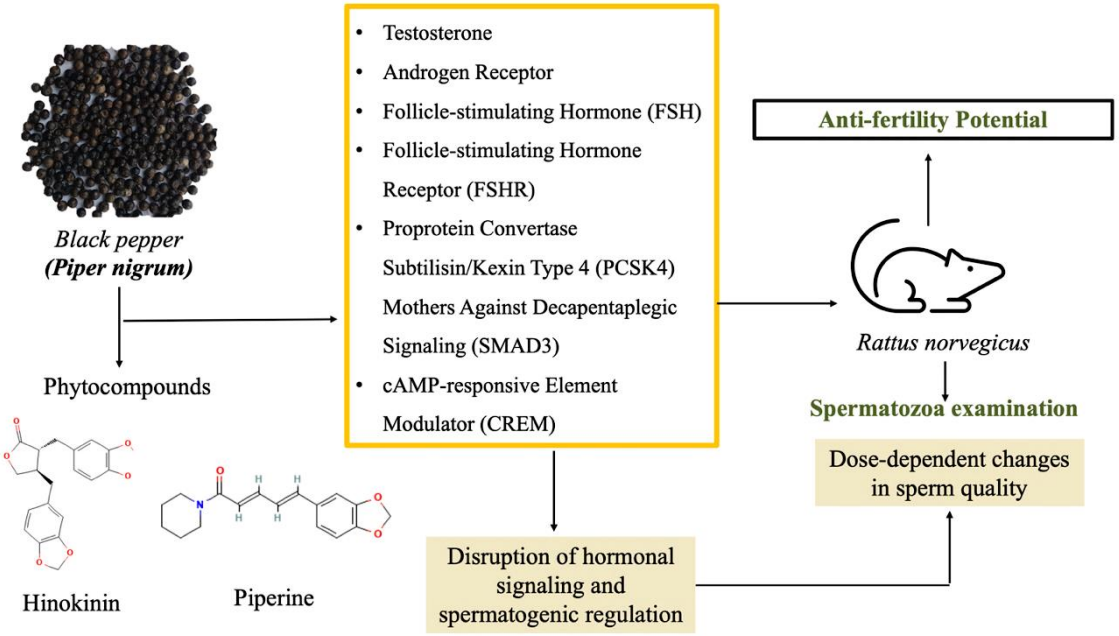


Figure 7. Schematic summary of the molecular docking and biological evaluation of ethanolic extract of *P. nigrum*

4. Conclusion

This study indicates that the ethanolic extract of *Piper nigrum* possesses promising anti-fertility potential in males. The extract significantly influenced key sperm quality parameters, including concentration, motility, viability, and morphological abnormality. Interestingly, although lower doses adversely affected sperm quality, the highest dose group showed partial improvement, suggesting a possible dose-dependent adaptive response. These physiological findings were further supported by molecular docking analyses, which revealed that major bioactive compounds such as piperine and hinokinin exhibit strong binding affinities toward critical reproductive proteins, including the androgen receptor and SMAD3, indicating potential modulation of hormonal and spermatogenic pathways. Collectively, these results suggest that *P. nigrum* may influence male fertility through both molecular and biological mechanisms, and that certain doses might produce reversible contraceptive effects. While these findings highlight the potential of *P. nigrum* as a natural male contraceptive agent, further studies are warranted to confirm its long-term safety, elucidate its mechanistic pathways, and determine the reversibility of fertility outcomes following treatment cessation.

5. Declarations

5.1. Author Contributions

Conceptualization., K.K. and M.A.; methodology, Y.F., K.K., T.Z.E., and M.A.; validation, Y.F., K.K., and M.A.; investigation Y.F. and, M.A.; writing—original draft preparation, Y.F., K.K., T.Z.E., and M.A.; writing—review and editing, Y.F. and K.K.; supervision, K.K., T.Z.E., and M.A. All authors have read and agreed to the published version of the manuscript.

5.2. Data Availability Statement

The data presented in this study are available in the article.

5.3. Funding and Acknowledgments

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5.4. Institutional Review Board Statement

This research has received approval from the Animal Experimentation Ethics Committee for Research at the Faculty of Veterinary Medicine, Syiah Kuala University, Ref. No. 299/KEPH/IV/2024.

5.5. Informed Consent Statement

Not applicable.

5.6. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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