JTL 11 (2) DESEMBER 2023
Page: 89-100

Contents list available at Jurnal Teknologi Laboratorium

JURNAL TEKNOLOGI LABORATORIUM

Journal Homepage: www.teknolabjournal.com
ISSN 2580-0191(Online) I ISSN 2338 – 5634(Print)

Original Research

OPEN ACCESS

Bioactivity of Nelumbo nucifera extract on sperm recovery due to 2-methoxyethanol exposure: In vivo and in silico study

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Abstract: One of the causes of male infertility is influenced by the compound 2-Methoxyethanol (2-ME) that can increase Reactive Oxygen Species (ROS), which affect sperm quality. This study aims to analyze the effects of various doses of *Nelumbo nucifera* extract on sperm quality in mice. Methods: Male ddy mice were divided into six equal groups. The negative control group was given aquades for 28 days, the positive control group was injected with 0.05 ml of 2-ME for 7 days, compare control group was injected with 0,05 ml 2-ME and *clomiphene citrate* 50 mg for 21 days. The treatment groups were injected with 0.05 ml of 2-ME for 7 days followed by injection different doses of *N. nucifera* extract, namely 50 mg/kg body weight (BW), 150 mg/kg BW, and 450 mg/kg BW for 21 days. At the end of the experiment, the mice were sacrificed, and sperm suspensions were collected from the epididymis to measure morphology, concentration, and motility. In silico testing was performed by preparing ligands and the GSK3b protein receptor using PyMOL, and then tested to determine the binding energy using PyRx. Results: The administration of *N. Nucifera* extract can significantly improve sperm morphology, concentration, and motility ($p<0.05$). The dose of 450 mg/kg BW has a pronounced protective effect. Quercetin is the compound of *N. nucifera* extract with the highest inhibition of non-receptor protein kinase and the most significant antioxidant effect. *N. nucifera* extract can improve the decline in sperm quality caused by exposure to 2-Methoxyethanol.

Keywords: *Nelumbo nucifera*, sperm recovery, 2-methoxyethanol, in-vivo, in-silico.

INTRODUCTION

Infertility is a health issue that is increasingly prevalent in Indonesia. One of the causes of male infertility is exposure to chemicals that pollute the environment, with detrimental effects on sperm quality.[1,2] 2-Methoxyethanol (2-ME), used in plastic production and as a solvent for water-based organic materials,[3,4] is implicated in this context. 2-ME can enter the human body through various routes, including skin absorption, inhalation, and the consumption of contaminated food containing this compound. Once inside the body, 2-ME undergoes metabolism to a more toxic compound, namely methoxyacetic acid (MAA).[5,6,7] MAA then spreads throughout the body and accumulates in the testes, an organ highly sensitive to this substance. MAA is known to cause an increase in Reactive Oxygen Species (ROS) and lipid peroxidation, leading to decreased sperm motility and morphology.[8,9]

The toxic effects of 2-ME also result in increased expression of the cortactin gene, particularly evident in spermatocytes. Cortactin is a protein involved in the

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DOI: 10.29238/teknolabjournal.v12i2.450
Received 17 September 2023; Received in revised form 24 December 2023; Accepted 29 December 2023

regulation of cell cytoskeleton and cell movement. Elevated cortactin levels cause damage to cell function, contributing to the degeneration and necrosis of spermatocytes. Cortactin activity is induced by a non-receptor kinase enzyme, which is linked to the regulation of endocytosis processes and sperm formation.[10,7] Antioxidants may serve as inhibitors of non-receptor kinase. To counteract the negative effects of oxidation induced by 2-ME, substances with strong antioxidant properties are required.

Nelumbo nucifera, an abundant aquatic plant, has not been optimally utilized as an antioxidant source.[11] Recent research shows that ethanol extracts of *N. nucifera* reveals that the plant contains essential phytochemical components such as phenols, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, glycosides, coumarins, and quinones[12]. This study demonstrates that lotus extract can enhance sperm viability and reduce lipid peroxidation levels by up to 80%. The petals of *N. nucifera* extract have activity as antioxidant against mancozeb, it's an toxic agent on male reproductive expecially on spermatogenesis, the mayor chemical compound in *N. nucifera* petals is tochopherol and flavonoid.[13]

On this research aims to explore the potential of *N. nucifera* petal extract in protecting and repairing damaged sperm due to 2-ME exposure, as well as how ligand activities of lotus phytochemical compounds interact with the glycogen synthase kinase-3. Glycogen synthase kinase-3 (GSK-3β) is a serine/threonine kinase that regulating various signaling pathways, GSK-3β GSK-3β plays a role in depleting beta-catenin, which subsequently enters the cell nucleus to regulate the expression of specific genes. GSK3b increase in the expression of the cortactin gene, which is particularly prominent in spermatocytes as receptor or GSK-3β as a kinase inhibitor playing a key role in the regulation of various signaling pathways.[14,15]

This study is intended to identify the optimal dosage of *N. nucifera* petal extract with potential compounds and to identify potential compounds that may serve as candidates for anti-infertility drugs. In this context, the research holds significant relevance in the field of phytopharmaceuticals and can contribute to our understanding of the potential use of lotus petals as a natural alternative medicine to address male infertility issues.

MATERIAL AND METHOD

In Silico Testing

Phytochemical compounds present in lotus petal extract were subjected to Druglikeness evaluation using Lipinski's rule through the Way-2-drug web server. Sample preparation involved collecting target protein through the Protein Data Bank and ligand samples through the Pubchem website. The next step included the sterilization of unnecessary molecules, including water molecules on the target protein. For ligands, minimization and docking were performed using PyRyx software. The docking results were visualized using PyMoL software to understand the interactions between ligands and proteins.

Ethical Approval

All procedures in this research, including the use of mice as animal models, were approved by the Ethics Committee, Department of Research and Community Service, Brawijaya University, East Java, Indonesia, with the number No: 370-KEP-UB-2023.

Plant Material

Nelumbo nucifera was collected from Jotosanur Reservoir, Lamongan, East Java in June 2023. The material was identified and authenticated in the Biology Laboratory, Department of Biology, Universitas Muhammadiyah Lamongan.

Preparation of N. Nucifera Ethanol Extract and Suspension

Lotus flower petals were cut into small pieces and dried in an oven at 40°C for 60 minutes. The dried petals (500 grams) were ground electrically and macerated with 96% ethanol for 3 days at room temperature. The extract was then filtered and

concentrated with a rotary evaporator and heated in a water bath at 70°C. To make the suspension, the extract was homogeneously mixed with distilled water. Concentrations used were 0.5%, 1%, and 2%.[16]

Preparation of 2-Methoxyethanol Suspension

For a five-day stock, seven milligrams of lead acetate were weighed and dissolved in warm distilled water. After dissolution, all remaining distilled water was added to obtain a volume of 15 ml in the Na-CMC solution.

Materials and Equipment

The tools used included an analytical balance, beakers, stirring rods, pipettes, petri dishes, 1cc syringes, Eppendorf tubes, glass slides, cover glasses, slide boxes, digital scales, probes, light microscopes, micropipettes, mouse cages with food and water, Pasteur pipettes, Eppendorf tubes, a Neubauer counting chamber, a rotary vacuum evaporator, and a water bath. The materials used in this study included Lotus Flower Petal Extract, ddy strain mice, filter paper, ethanol 96%, ethanol 70%, distilled water, Nigrosin, NaCl 0.9%, 2-Methoxyethanol, Neutral Buffered Formalin (NBF 10%), Dpph, Chloroform, Methanol, hematoxylin-eosin tissue stain.

Animals

Thirty adult male ddy mice aged 6-7 weeks, 25-30 grams in weight, were obtained from the Center for Veterinary and Pharmacy, Surabaya, East Java. They were kept under standard laboratory conditions (temperature 28-30°C, light/dark cycle 12 hours/12 hours) and provided with food and water ad libitum.

Experimental Design

After one week of acclimatization, the animals were randomly divided into six equally sized groups (n=10) as follows: negative control group received distilled water for 28 days (KN), positive control group received subcutaneous injection of 0.05 ml 2-ME at a dose of 200 mg/kg BW for 7 days (KP), comparative control group received subcutaneous injection of 0,05 ml 2-ME at a dose of 200 mg/kg Bw for 7 days and continously injection with commercial medicine (*Clomiphene citrate*) 50 mg for 21 days (K0). Treatment groups received subcutaneous injection of 0.05 ml 2-ME at a dose of 200 mg/kg BW for 7 days. Subsequently, subcutaneous injection of 0.2 ml with different doses of *N. nucifera* petal extract was given to each treatment group. The first treatment group received 50 mg/kg BW (P1), the second group received 150 mg/kg BW (P2), and the third group received 450 mg/kg BW (P3) for 21 days. After the procedure, all mice were sacrificed using chloroform. The cauda epididymis was then collected, and sperm suspension was made for sperm analysis.

Sperm Analysis

In this study, spermatozoa suspension was used to measure motility, morphology, and sperm count. For sperm motility analysis, 10µL was placed in a hemocytometer chamber and analyzed under a light microscope. Ten sperm were observed for the duration of their movement and measured using a stopwatch. For sperm count evaluation, in a sperm solution, 10µL of sperm suspension was transferred to each hemocytometer counting chamber and left for 5 minutes. Then, sperm heads were counted with a light microscope at 40x magnification and expressed as million/ml of suspension.[17] Sperm morphology was also determined using the eosin-nigrosin staining method. For this purpose, spermatozoa suspension was dropped on a glass slide to make a smear preparation and air-dried. The smear preparation was fixed with methanol, stained with 1% eosin and nigrosin solution, and allowed to dry. The preparation was rinsed with distilled water and dried.[18] The preparation was observed under a light microscope at 400x magnification to determine the morphology of 100 mouse sperm. The final step was to calculate the percentage of normal and abnormal sperm.

RESULTS AND DISCUSSION

The Lipinski test is conducted in the early stages of in silico testing in the field of vaccine and drug design.[19] The Lipinski test comprises a set of empirical rules used in drug research and pharmacology to identify the potential of new drug molecules, aiming to predict whether a molecule is likely to be a successful drug. Molecules with a molecular weight of less than 500 Daltons are more likely to penetrate cell membranes and reach biological targets within the body. The Log P (octanol-water partition coefficient) measures how well a molecule dissolves in fat compared to water. Molecules with a Log P of less than 5 tend to have better bioavailability. The number of hydrogen bond donors (H-Donor) should be less than 5, as molecules with too many hydrogen atoms functioning as donors tend to have less chemically stable properties. Similarly, the number of hydrogen bond acceptors (H-Acceptor) should be less than 10, as molecules with too many atoms that can function as hydrogen acceptors tend to have less chemically stable properties.[20]

Table 1. Lippinski Result Test

Ligands	Molecular Weight (<500)	Log P (<5)	Hydrogens Binding Donor (<5)	Acceptors (<10)	Explanation
Astragalin	448.0	-0,43	7	11	Not Eligible
Diosmetin	**300.0**	**2,42**	**3**	**6**	**Eligible**
Delphinidin	303.0	2,42	6	7	Not Eligible
quercetin	**302.0**	**2.01**	**5**	**7**	**Eligible**
Trifolin	448.0	-0.43	7	11	Not Eligible
Quercitrin	448.0	0.29	7	11	Not Eligible
Isoquercitrin	464.0	-0.73	8	12	Not Eligible
Myricetin	318.0	1.71	6	8	Not Eligible
Syringetin	**346.0**	**2.32**	**4**	**8**	**Eligible**
Cyanidin	**287.0**	**2,71**	**5**	**6**	**Eligible**
Peonidin	**301.0**	**3.02**	**4**	**6**	**Eligible**
petunidin	**317.0**	**2.72**	**5**	**7**	**Eligible**
asimilobine	**267.0**	**2,81**	**2**	**3**	**Eligible**
N-Nornuciferine	**281.0**	**3,11**	**1**	**3**	**Eligible**
Nuciferine N-Oxide	**311.0**	**3,09**	**0**	**3**	**Eligible**
O-Nornuciferine	**281.0**	**3,15**	**1**	**3**	**Eligible**
Norjuziphine	**285.0**	**2,53**	**3**	**4**	**Eligible**
Florinbundine	**281.0**	**3,15**	**1**	**3**	**Eligible**
Pronuciferine	**311.0**	**2,51**	**0**	**4**	**Eligible**
Clomiphene	405.5	5,95	0	2	Not Eligible

Lipinski rule of five is important for determining compounds such as drug candidate molecules. In this study, it was found that there are 13 bioactive compounds that match Lipinski rules of five, namely: chlorogenic Diosmetin, Quercetin, Syringetin, Cyanidin, Peonidin, Petunidin, asimilobine, N-Nornuciferine, Nuciferine N-Oxide, O-Nornuciferine, Norjuziphine, Florinbundine, and Pronuciferine. The PASS Online analysis results show that all drug candidate

compounds have good activity (Table 1). In Table 1, during the Lippinski test, clomiphene does not meet the criteria. However, clomiphene serves as the reference drug in this case. In pharmaceuticals, a compound developed into a drug undergoes a rigorous evaluation process to maintain a balance between effectiveness and potential risks before production. However, it can be concluded that some phytochemical compounds of the flavonoid group in lotus petal do not meet the criteria as potential drug candidates.

Table 2. Glycogen synthase kinase-3β (GSK-3β) Inhibitor Docking Result

Ligands	Type	Protein Target	Pa	Pi	Binding Energy	Hydrogen Bindings
Astragalin	Flavonoid	GSK-3β	0,837	0,004	-7,6	6
Diosmetin	Flavonoid	GSK-3β	0,922	0,002	-7,5	3
delphinidin	Flavonoid	GSK-3β	-	-	-7,3	2
quercetin	Flavonoid	GSK-3β	0,809	0,005	-9,1	5
Trifolin	Flavonoid	GSK-3β	0,837	0,004	-7,7	2
Quercitrin	Flavonoid	GSK-3β	0,863	0,004	-8,3	6
Isoquercitrin	Flavonoid	GSK-3β	0,843	0,004	-7,6	6
Myricetin	Flavonoid	GSK-3β	0,958	0,001	-7,6	2
Syringetin	Flavonoid	GSK-3β	0,945	0,002	-7,2	3
Cyanidin	Flavonoid	GSK-3β	0,863	0,004	-7,3	8
peonidin	Flavonoid	GSK-3β	-	-	-7,3	2
petunidin	Flavonoid	GSK-3β	-	-	-7,1	4
asimilobine	Alkaloid	GSK-3β	0,418	0,058	-8,2	0
N-Nornuciferine	Alkaloid	GSK-3β	0,318	0,125	-8,0	1
Nuciferine N-Oxide	Alkaloid	GSK-3β	-	-	-7,7	1
O-Nornuciferine	Alkaloid	GSK-3β	0,352	0,095	-8,3	0
Norjuziphine	Alkaloid	GSK-3β	0,347	0,099	-7,0	2
Florinbundine	Alkaloid	GSK-3β	0,352	0,095	-8,3	1
Pronuciferine	Alkaloid	GSK-3β	0,077	0,043	-7,5	0
Clomiphene	kontrol	GSK-3β	0,643	0,029	-7,2	0

The molecular docking results aim to determine the stability of the interaction between the ligand and the target protein. The lowest binding affinity indicates the maximum level of stable interaction indicating that the ligand inhibitory activity against the target protein is larger, the negative values indicate an increasing energy.[21] All bioactive compounds have activity against target proteins (Table 2). However, the compounds to be analyzed next are those with the lowest binding affinity and that follow Lipinski rules of five. Quercetin from flavonoids group and Floribundine from alkaloids group are examples of such compounds. Based on the data in Table 2 on the bioactivity of ligands as kinase inhibitors, the flavonoid group shows a greater potential for activity compared to inactivity. The compound quercetin, with the highest binding energy of 9.1 kcal/mol and 5 hydrogen bonding sites, demonstrates the most significant inhibitory potential against non-receptor protein kinase. and in this context, Quercetin emerges as the compound with the highest potency, followed by other compounds from alkaloid group, Floribundine with the highest binding energy of -8,3 kcal/mol and 1 hydrogen bonding sites, demonstrates the most significant inhibitory potential against non-receptor protein kinase.

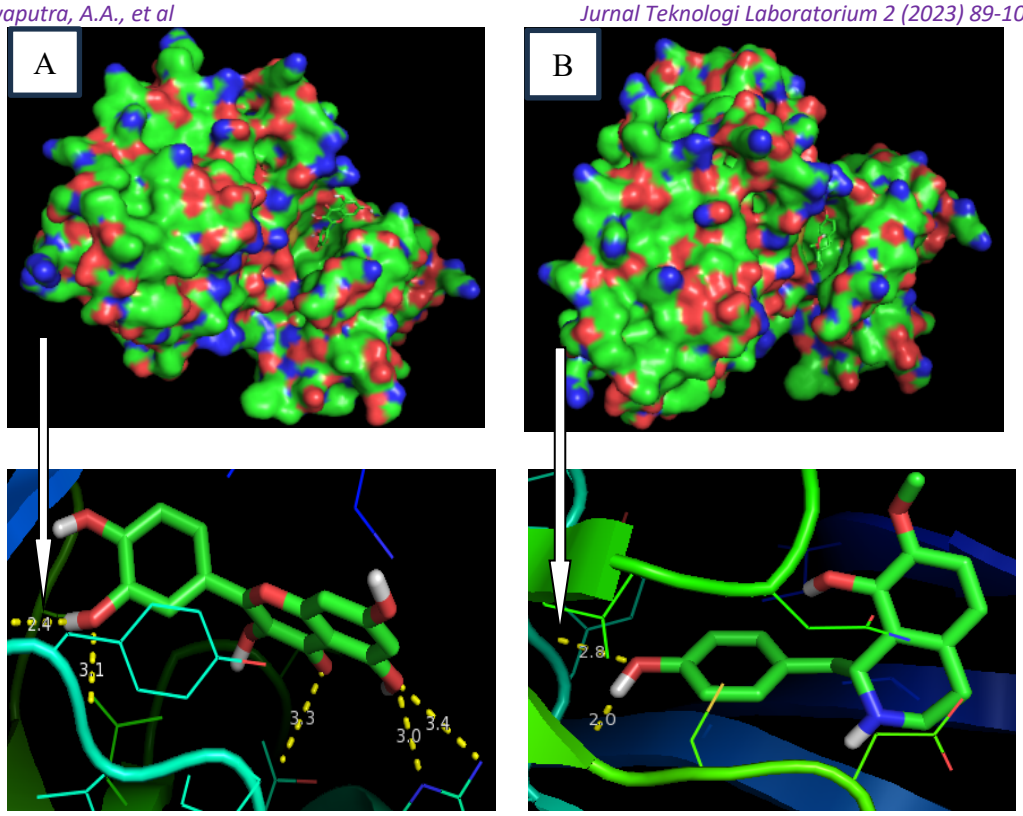

Figure 1. Interaction of ligands with the catalytic site of GSK-3β kinase. The structure of GSK-3β is depicted in granular form, with the catalytic site marked by hydrogen bonds represented by dashed yellow lines. (A. Quercetin, B.Floribundine)

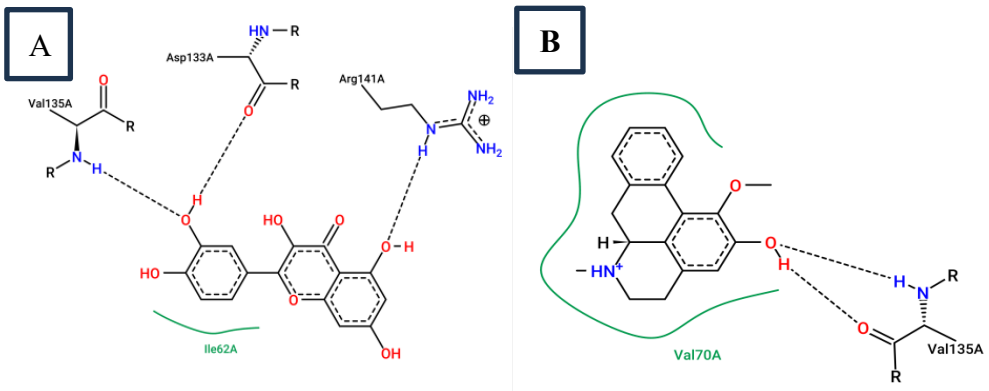

Figure 2. The binding visualization 2D of A) Quercetin and B) Floribundine targeting GSK-3β

Glycogen synthase kinase-3 (GSK-3β) is a serine/threonine kinase that regulating various signaling pathways, GSK-3β plays a role in depleting beta-catenin, which subsequently enters the cell nucleus to regulate the expression of specific genes. GSK-3β increase in the expression of the cortactin gene, which is particularly prominent in spermatocytes. The increased activity of cortactin is induced by non-receptor kinase enzymes (GSK-3β) Cortactin activated become a protein involved in the regulation of cell cytoskeleton and cell movement. The elevation of cortactin levels leads to cellular damage and dysfunction, contributing to the development of degeneration and necrosis in spermatocytes with the regulation of endocytosis processes and sperm formation.[14,10,15]

Some of these conserved sites are catalytic residues consisting of Aspartic acid (Asp), Arginina (Arg), and Valina (Val) this position can be used for molecular

docking in the development of multivariate anti-infertility. Based on the docking results (Figure 2), quercetin forms five hydrogen and hydrophobic bonds but only three bond connect with conservative residues (Asp 133A, Val 135A, Arg 141A). Meanwhile, floribundine has only one bond with catalytic residues Val135A. There are other conservative catalytic residues in this protein have the potential to bind to docked bioactive compound ligands, the presence of hydrogen bonds in catalytic residues aids in the stabilization of the ligands-target protein complexes, resulting in the desired interaction conformation play a role in the stability of drug molecules to trigger the inhibitory activity of the target protein.[22,23]

Table 3. In Vivo Test Result

Parameters	KN	KP	K0	P1	P2	P3
Motility (minutes/cell)	2,06±0,11	1,13±0,37	1,80±0,12*	1,03±0,11	3,10±0,15*	3,51±0,14*
Quantity (Million/ml)	22,1±2,38	10,8±0,97	12,8±3,39	12±1,44	20±1,09*	23±1,08*
Morphology normal (%)	99,1±1,95	77,5±2,29	72,6±3,31	71,4±3,20	81±2,42	83,2±2,37

Note: (*) indicates a significant difference compared to the control group ($p < 0.05$)

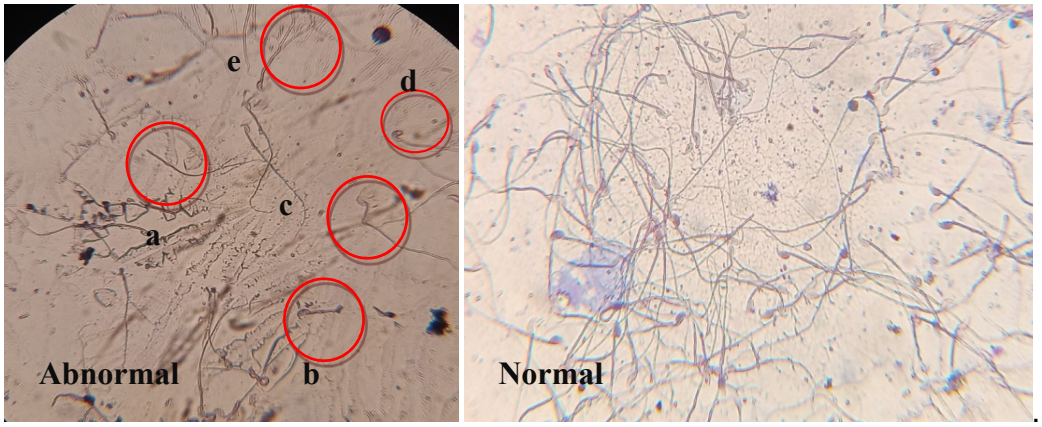

Figure 3. Normal and Abnormal Mature Mouse Spermatozoa (a. Headless, b. Tailless, c. Broken Neck, d. Folded Head Shape, e. Two-Tailed)

Spermatozoa Motility

The average mortality rate of sperm cells indicates that in the Positive Control group (KP), which was only injected with 2-ME, there is no significant difference compared to the Comparative Control group (K0). However, in groups P2 and P3, which received moderate and high doses of lotus petal extract, the sperm motility duration is above that of the out-of-room control group (KN). Meanwhile, in the P1 group, there has not been a significant increase in speed compared to the KN group.

Spermatozoa Quantity

Data from Table 3, obtained from the average of individuals in the treatment group, show that the cumulative sperm count in each chamber on the hemocytometer, when multiplied by 1,000,000, leads to the conclusion that, compared to P1 and P2 doses, the P3 group indicates a significant improvement in quantity, well above the KN group.

Spermatozoa Quality

Percentage calculations of sperm quality reveal that in groups P2 and P3, which were treated with lotus petal extract injections, the number of normal cells is higher compared to the Comparative Control group (K0), reaching only 72.6%. The percentage of normal cells increases with the rising extract dose. Along with the administration of ethanol

extract of Nelumbo nucifera, the percentage of observed normal morphology increasingly dominated. Table 3 shows that the percentage of sperm with normal morphology increased from 77.5% to 83.2%, approaching the percentage of the negative control. The decrease in abnormal cells indicates that lotus petal extract possesses antioxidant activity in influencing the development of degeneration and necrosis of spermatocytes caused by the effects of 2-Methoxyethanol (Figure 3).

2-ME causes damage to the spermatogenic process, as evidenced by the occurrence of azoospermia in the observation of sperm quality, the positive control group (KP) differs from the negative control group (KN), which was not given 2-ME. The effect of 2-ME on spermatogenesis can also be seen in the quantity and motility of spermatozoa in the positive control group (PC) and Negative (KN) in Table 3. The quantity, quality, and motility of spermatozoa in PC are always lower than NC, and this decrease indicates spermatogenesis disruption by 2-ME. Exposure to 2-ME in experimental animals causes a decrease in testis weight, a decrease in the quantity and motility of sperm, and an increase in the proportion of epididymal sperm with abnormal morphology. This indicates that these cells degenerate due to exposure to 2-ME.

Metabolite MAA in the body has toxic and teratogenic properties, thus inhibiting the formation of DNA and RNA in primary spermatocytes, especially in pachytene spermatocytes. Pachytene spermatocytes are the most active cells in synthesizing RNA. Therefore, these cells are the most sensitive to MAA, resulting in them becoming the most degenerated cells. MAA can also increase membrane permeability, causing excessive Ca^{2+} influx (overload).[6,7,4] This increase allows calcium ions to bind to calmodulin protein (Chin, 2000), calmodulin will activate the signaling pathway by binding to the active side of protein kinase, too much protein kinase will increase protein phosphorylation which further impacts cell apoptosis. In addition, protein tyrosine kinase can also cause an increase in the expression of cortactin genes. Cortactin is a protein involved in the regulation of cell cytoskeleton and cell movement. Cortactin connects "ectoplasmic specialization" (ES) with the actin cytoskeleton and is considered important in regulating the release of sperm from the seminiferous epithelium and facilitating the movement of spermatids. In short, this actin-binding protein controls the dynamics of the actin cytoskeleton through nucleation, elongation, closure, binding, assembly, cleavage, and depolymerization, thus facilitating changes in cell shape and the location of spermatids in the epithelium during spermiogenesis.[24] However, an excess of cortactin can affect the ability of sperm to pass through the seminiferous epithelium properly and uncontrolled changes in the actin cytoskeleton that can interfere with the structural integrity of cells and their normal function and cause cell damage and dysfunction, contributing to the degeneration and necrosis of spermatocytes[25,10]. Overload Ca^{2+} can inhibit oxidative phosphorylation, so energy supply becomes reduced as it is used to pump out Ca^{2+} ions, while energy is crucial for sperm motility.[24,26]

The lotus petal (*N. nucifera*) extract contains bioactive substances from secondary metabolite compounds such as quercetin, quercitrin, isoquercetin, myricetin which have the potential as antioxidant preparations.[27,28] Antioxidants are effective in counteracting the effects of oxidative reactions and the toxicity of 2-methoxyethanol. Not only can they protect DNA from oxidative damage by inhibiting lipid peroxidation, but they can also capture free radicals by providing additional electrons to unstable molecules. Moreover, they play a crucial role in maintaining cell signaling regulation by modulating signaling pathways involved in inflammatory responses, and help regulate the redox balance in cells, thus not interfering with the regulation in spermatogenesis.[13,29] Based In silico analysis, lotus flavonoid compounds have also been proven to have the potential as ligands capable of inhibiting non-receptor protein kinase.[30] Non-receptor protein kinase (GSK-3β) is active due to the redox imbalance of Ca^{2+} ions in cells caused by the toxicity of 2-Methoxyethanol.[31,32] By inhibiting the activity of this protein, the

compound content of lotus can bind to the active side of non-receptor kinase and prevent over-expression of cortactin genes that can be the cause of degenerative spermatogenic cells and lead to infertility.[26,33] Therefore, compounds in lotus petals show potential as candidates for anti-infertility drugs. In general, it can be concluded from this study that the optimal dose for preventing spermatocyte degeneration is the administration of lotus petal (*N. nucifera*) extract at a dose of 450 mg/kg BW (Group P3). However, further research in the field of phytopharmaceuticals is needed to consider lotus petals as a potential drug.

CONCLUSION

This study reveals that lotus petal extract, particularly the flavonoid quercetin, shows potential as a non-receptor kinase inhibitor targeting GSK-3β, a key protein in spermatocyte signaling pathways. The administration of 450 mg/kg BW of lotus petal (*N. nucifera*) extract significantly improves sperm quality and mitigates the negative effects of 2-ME. This suggests the potential of lotus petal (*N. nucifera*) extract as a natural remedy for male infertility issues.

AUTHORS' CONTRIBUTIONS

Angella Ananda Syaputra: Project administration, Conceptualization, In-silico analyze. Badriatul Musyarofah: Data curation, Writing- Original draft preparation. Amelia Kartika Reza; Yunita Ainul Kasanah: Methodology, Visualization, Investigation; Helga Syasya Qatrunnada: Analyze data; Putri Ayu Ika Setiyowati: Resources, Supervision, Validation, and Reviewing.

ACKNOWLEDGEMENT

The author expresses gratitude to the Directorate General of the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for funding the research under the Student Creativity Program with number 2383/E2/DT.01.00/2023.

FUNDING INFORMATION

Directorate General of the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia under the Student Creativity Program with number 2383/E2/DT.01.00/2023.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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