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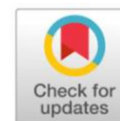
Original Research

Antioxidant protective effect of Nelumbo nucifera extract against spermatogenic cells in male mice due to 2-methoxyethanol exposure



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Abstract: One of the causes of infertility in men is influenced by the compound *2-Methoxyethanol* (2-ME) that can increase *Reactive Oxygen Species* (ROS) which can distrub spermatogenesis. This study aimed to analyze the effect of antioxidant of *Nelumbo nucifera* extract on the histology of mice testicular spermatogenic cells. Male mice are divided into 6 groups. The negative control group (KN) was given distillated for 28 days *ad libitum*. The positive control group (KP) was injected subcutaneous (s.c) with 2-ME dose 200 mg/kg bw as much as 0.05 ml/ day for 7 days, the drug group (KO) was injected s.c with 2-ME (200 mg/kg bw) as much as 0.05 ml/ day for 7 days and then injected s.c with *Clomiphene citrate* with a dose 50 mg/kg bw as much as 0.2 ml/ day for 21 days. The treatment group (P1, P2, P3) was injected s.c with 2-ME (200 mg/kg bw) as much as 0.05 ml/ day for 7 days and injected s.c with *N. nucifera* extract at low dose 50 mg/kg bw (P1), medium dose 150 mg/kg bw (P2), and high dose 450 mg/kg bw (P3) as much as 0.2 ml/ day for 21 days. At the end of the study all mice were sacrificed and testicular collection was carried out. Testicular tissue was processed using Hematoxylin-Eosin staining and observed spermatogenic cells (spermatogonia, spermatocytes and spermatids). Data analysis using ANOVA test and advanced test Post hoct test. The results showed that there was a significant difference ($p < 0.05$) in number of spermatogenic cells of mice testicles between the negative group (KN), positive group (KP) and treatment group (P1, P2, P3). The optimal dose of *N. nucifera* extract that is most able to repair testicular tissue damage is a high dose.

Keywords: Antioxidant; Spermatogenic cells; histopatology, *Nelumbo nucifera*; 2-*Metoxyethanol*

INTRODUCTION

The prevalence of infertility in Indonesia is increasing every year, based on data there were 1,712 men and 2,055 women who experienced infertility[1]. Infertility is not only experienced by women but, in this case the male factor is responsible for 36% while 64% is in women[2]. Infertility in men is influenced by several factors, one of which is declining sperm quality, both in terms of sperm count, sperm morphology, and sperm motility[3]. 2-*methoxyethanol* (2-ME) is one example of a glycol ether compound also known as *ethylene glycol monomethyl ether* (EGME). This compound is often used as an organic solvent to produce various industrial materials, such as resins, wood paints, wall paints, varnishes, nail polishes, and

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cellulose acetate, however, 2-ME is highly toxic to tissues that proliferate rapidly and have a high metabolic rate, such as the testes and thymus [4,5].

The process of spermatogenesis can be disrupted if exposed to excessive exposure to free radicals by damaging cell membranes. This compound can cause damage to *Deoxyribonucleic Acid* (DNA) spermatozoa and apoptosis of spermatozoa cells[6]. Sperm quality can affect a man's fertility potential. Oxidative stress is capable of decreasing the physiological capacity of fertilization, including capacitation, acrosome reactions, hyperactivation and sperm-oocyte binding, which will result in male infertility. Male fertility potential can be improved through the prevention and management of oxidative stress. Consumption of antioxidants in large quantities is a major factor to prevent oxidative stress[7]. Antioxidants are compounds that can prevent oxidative stress, one of the natural antioxidants is flavonoids. Flavonoids, which act as antioxidants, have the ability to inhibit ROS formation by inhibiting redox reactions that produce fresh oxidants[8].

Ethanol extract from *Nelumbo nucifera* revealed that this plant contains important phytochemical components phenols, flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, cardiac glycosides, coumarins and quinones[9]. Flavonoids are one of the powerful antioxidants that function as free radical chain breaking antioxidants[10]. Flavonoids will capture free radicals by releasing hydrogen atoms from their hydroxyl groups and breaking the free radical chain reaction[11]. The flavonoids compound can affect the reproductive process including spermatogenesis because it acts as free radical scavenging that causes infertility. Antifertility drugs have a mechanism of action through cytotoxic or cytostatic effects[12]. Spermatogenesis in mice takes 35.5 days to complete in one cycle, or 4 times longer than the seminiferous epithelial cycle. Proliferation, growth, maturation, and transformation or spermiogenesis are different phases of spermatogenesis[13]. Spermatogenesis is the process of producing sperm from spermatogonium, through complex and orderly development. Spermatogenesis occurs within the seminiferous tubules of the testes, through a series of processes such as proliferation, differentiation and transformation[14].

This study aimed to explore the potential of *Nelumbo nucifera* extract in protecting and repairing sperm damage due to 2-ME exposure. This study was intended to identify optimal doses of *Nelumbo nucifera* extracts with potential compounds and identify potential compounds as anti-infertility drug candidates. In this context, this research has significant relevance in the field of phytopharmaceuticals and may contribute to the understanding of the potential use of *Nelumbo nucifera* as a natural alternative medicine to address infertility problems in men.

MATERIAL AND METHOD

Ingredients used include *Nelumbo nucifera* extract, male mice strain ddy, filter paper, ethanol 100%, ethanol 96%, ethanol 95%, ethanol 90%, ethanol 80%, ethanol 70% and ethanol 50%, distilled water, 2-*Methoxyethanol*, *Neutral Formalin Buffer* (NBF 10%), parafin (liquid), 2,2-difenil-1-pikrilhidrazi (DPPH), chloroform, normal buffer formaline (NBF) 10%, xylol, hematoxylin-eosin tissue dye. tools used include analytical balances, beers, stirrers, pipettes, petri dishes, 1cc syringes, Eppendorf tubes, object glass, glass covers, slide boxes, digital scales, probes, light microscopes, micropipettes, mice cages with food and water, Pasteur pipettes, Eppendorf tubes, Neubauer counting chambers, rotary vacuum evaporators, and water baths.

Ethical Approval

All procedures in this study, including the use of mice as animal models, have been approved by the Ethics Committee, Department of research and Community Services, Universitas Brawijaya, East Java, Indonesia, with number No: 370-KEP-UB-2023.

Plant material

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

Preparation of ethanol extract and dosage of *Nelumbo Nucifera*

Nelumbo nucifera are cut into small pieces and dried in an oven at 40°C for 60 Minutes. Dried (500 grams) are electrically ground and macerated with 96% ethanol for 3 days at room temperature. The extract is then filtered and concentrated with a rotary evaporator and heated in a water bath at a temperature of 70°C. The dosage used are 50, 150, and 450 mg/kg bw. Each mice receive 0,02 ml/day for twenty one days[15].

Preparation of 2-*Methoxyethanol* suspension

For a seven-day stock of 200 mg/kg bw per individual mice, thirty five milligrams of 2-*Methoxyethanol* are weighed and dissolved in 0,35 mL of Na-CMC solution. Each mice receive 0,05 ml/day[15].

Prepartion of *Clomiphene citrate* drug suspension

For a twenty one day stock of 50 mg/kg bw per individual mice, ten milligrams of chlomiphene citrate are weighed and dissolved in 4,2 mL of Na-CMC solution. Each mice receive 0,2 ml/day[15].

Animals

Thirty six adult male mice of ddy strain aged 6-7 weeks, weighing 25-30 grams, were obtained from the Center for Animal Health and pharmacy, Pusvetma, Surabaya, East Java. They were kept in standard laboratory conditions (temperature 28-30°C, 12-hour/12-hour light/dark cycle) and fed and watered *ad libitum*.

Experimental design

After one week of acclimatization, the mice were randomly divided into six equally large groups (n=6) as follows: the negative control group (KN) received distilled water for 28 days *ad libitum*, the positive control group (KP) received subcutaneous injections of 0.05 ml of 2-ME at a dose of 200 mg/kg bw for 7 days. The drug group (KO) received subcutaneous injections of 0.05 ml of 2-ME for 7 days and received subcutaneous injection of 0,2 ml of *Clomiphene citrate* at a dose 50 mg/kg bw for 21 days. The treatment group (P1, P2, and P3) received subcutaneous injections of 0.05 ml of 2-ME at a dose of 200 mg/kg bw for 7 days. Subsequently, subcutaneous injections of 0.2 ml at different doses of *N. nucifera*. The first treatment (P1) received a low dose (50 mg/kg bw), the second group (P2) received a medium dose (150 mg/kg bw), and the third group (P3) received a high dose (450 mg/kg bw) for 21 days. After the procedure, all mice were sacrificed using chloroform. The testis organ were weighed, and fixed into NBF 10% for histopatological analysis.

Antioxidant test of *Nelumbo nucifera* extract

Conducted in vitro using the method of suppression of free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH). The test was conducted using UV-Vis spectrophotometry measured its absorption at a wavelength of 517 nm[16].

Antioxidant data analysis

The percentage of inhibition is the presentation that indicates the activity of the radical. Presentation of inhibition of DPPH-free radical from each concentration of solution the sample can be calculated by the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

Description: absorbance of control = absorbance on DPPH without sample, absorbance of treatment = absorbance on DPPH after adding the sample. The IC50 value of 50% ethanol extract was determined by determining the relationship between the concentration and the percentage of inhibition with the equation y= ax +b. the X axis is the sample concentration (ppm) and the y is the percentage of inhibition[17].

Histology preparation

Fixed testes in a 10% *neutral buffer formaline* (NBF) solution, then made histological preparations using paraffin method and hematoxylin eosin staining with a thickness of 3-5 µm[18].

Histological research parameters

The histological preparation of the testicular organ is examined under a microscope with a magnification of 400x. The seminiferous tubules look round in shape. The number of spermatogenic cells (spermatogonia, spermatocytes and spermatids) is a measured parameter. Cell count data collection was conducted by observing 5 field of view of fully spherical seminiferous tubules for each treatment and test. Then, a photo taking of the histological preparation takes place[19].

Analysis of histological data

The test results of the effect of *Nelumbo nucifera* extract on the number of spermatogenic cells in mice were analyzed using *One Way analysis of Variance* (ANOVA) and Post Hoct test with SPSS software, 95% confidence level (α =0.05%).

RESULTS AND DISCUSSION

Antioxidant Activity Test

Measurement of DPPH test results and obtained the value of percentage of inhibition of each concentration (Inhibition concentration/ IC50). IC50 value of ethanol extract of *Nelumbo nucifera* obtained 105.8 ppm. These values can be seen in (Table 1).

Table 1. Antioxidant Test Results of *Nelumbo nucifera* Extract

Concentration (ppm)	**Absorbance (Abs)**	**percentage of inhibition (%)**	**IC_{50} (ppm)**
20	0,156	0,87	
40	0,305	0,75	
60	0,376	0,69	105,8
80	0,447	0,64	
100	0,560	0,54	
Concentration (ppm)	Absorbance	percentage of inhibition (%)	IC_{50}

The calculation results with simple linear regression analysis can be presented in (Figure 1) with the IC50 value obtained from the equation y = 0.475x + 0.0838. The x value is the IC50 value and the y value is 50. The classification of antioxidants is divided into 5, namely <50 ppm (very strong), 50-100 ppm (strong), 100-150 ppm (medium), 150-200 ppm (weak) and >200 ppm is very weak[20]. The linear regression equation also shows that there is a significant relationship

between the solvent concentration and the percentage of inhibition indicated by the degree of tightness x. Based on the results of the study, the IC50 value was obtained from *Nelumbo nucifera* ethanol extract, which is 105.8 ppm, *Nelumbo Nucifera* extract has moderate antioxidant activity.

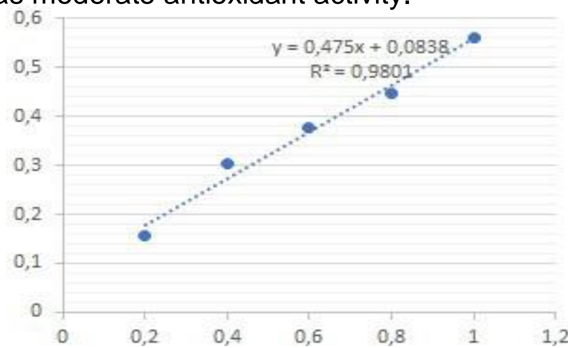

Figure 1. Regression values on the antioxidant activity graph

Mice Testicle Weight

The results of weighing the testes of mice that have been given *Nelumbo nucifera* extract after induced 2 ME there is a difference in weight in each group (Table 2). In the positive group (KP) has an average of the smallest testicular weight and in the negative group (KN) has an average value of the largest testicular weight as well as the number of spermatogenic cells.

Table 2. Testicle weight of mice that have been given *Nelumbo nucifera* extract after induction of 2-*Methoxyethanol*

Treatment	Total	Average of testicular weight (gram)
KN	5	0,358
KP	5	0,195
KO	5	0,310
P1	5	0,220
P2	5	0,260
P3	5	0,320

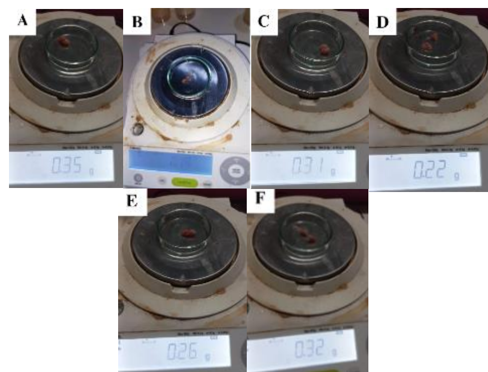

Figure 2. Weight of mice testes after induction of 2-*Methoxyethanol* and *Nelumbo nucifera* extract administration. Description: A: negative control (KN); B: Positive Group (KP); C: Drug Control: D: first treatment (P1: low dose *Nelumbo nucifera* of extract): E: second treatment (P2: medium dose *Nelumbo nucifera* of extract); F: third first treatment (P3: high dose *Nelumbo nucifera* of extract).

Histological Tests of The Testes of Mice

The results showed that the number of spermatogonia cells, spermatocytes, and spermatid cells in the positive control induced by 2-Methoxyethanol had lower values than the negative control (normal) and the group treated with *Nelumbo Nucifera* extract (Table 3). These results prove that 2-ME induction causes cell damage.

Table 3. Effect of *Nelumbo nucifera* extract after induction of 2-*Methoxyethanol* on the number of spermatogonia cells, spermatocytes, and spermatids

Treatment	Spermatogonia Cells	spermatocyte Cells	Spermatid Cells
KN	117,8 ± 3,99 ^{a}	120,8 ± 5,09 ^{a}	145,6 ± 4,77 ^{a}
KP	29,2 ± 3,99 ^{b}	25,4 ± 5,09 ^{b}	2,2 ± 4,77 ^{b}
KO	76,4 ± 3,99 ^{c}	116,4 ± 5,09 ^{a}	137,4 ± 4,77 ^{a}
P1	56,6 ± 3,99 ^{d}	62 ± 5,09 ^{c}	85 ± 4,77 ^{c}
P2	73 ± 3,99 ^{c}	85 ± 5,09 ^{d}	105,8 ± 4,77 ^{a}
P3	118,6 ± 3,99 ^{a}	115 ± 5,09 ^{a}	135,6 ± 4,77 ^{a}

Description: superscript differences in the same column indicate significant differences ($p<0.05$). The Data are presented in the mean of the ± SD

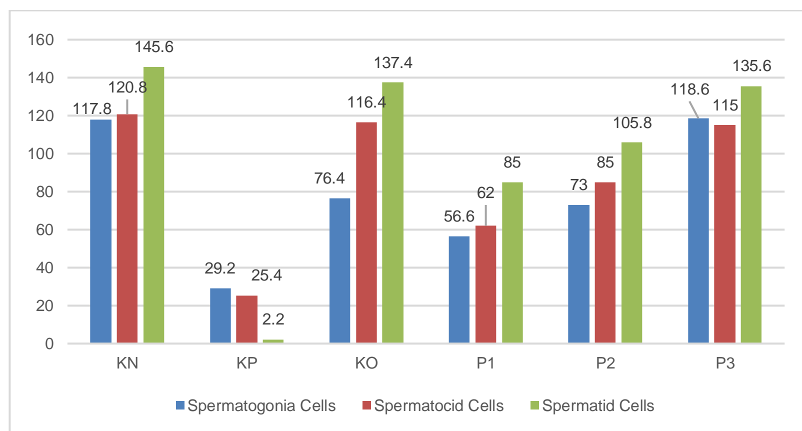

Figure 3. Histogram of spermatogenic cells number among control and treatment group. KN: negative control, KP: Positive Group, K0: Drug Control, (P1): low dose of *Nelumbo nucifera* extract (P2): medium dose of *Nelumbo nucifera* extract, (P3): high dose of *Nelumbo nucifera* extract.

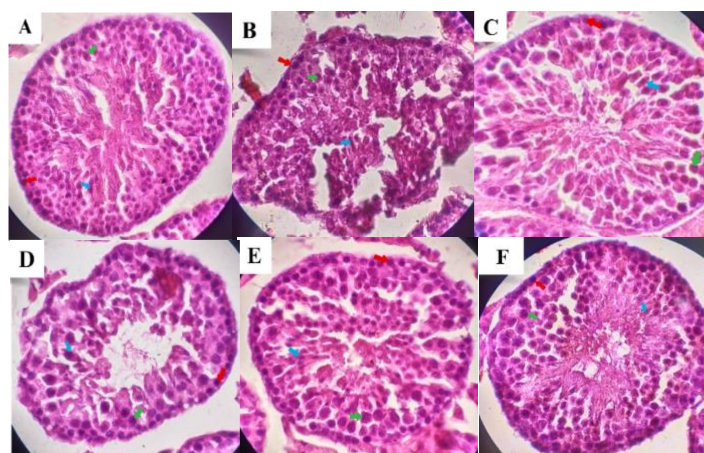

Figure 4. Cross-section of spermatogenic mice after treatment with extract *Nelumbo nucifera*. Description: A: negative control (KN); B: Positive Group (KP); C: Drug Control (KO): D: first treatment (P1: low dose *Nelumbo nucifera* of extract): E: second treatment (P2: medium dose *Nelumbo nucifera* of extract); F: third treatment (P3: high dose *Nelumbo nucifera* of extract). Red arrow ( )

showing spermatogonium cells; green arrow ( ) showing spermatocytes; and blue arrow ( ) showing spermatids; (Hematoxylin Eosin: 400x magnification).

Based on the Table 3 and Figure 3 and 4, there is a significant difference between the positive control group (KP) induced by 2-ME with the drug group (KO) and treatment group (P1, P2 & P3), namely in the observation of the number of spermatogonia cells, in the observation of spermatocytes cells there was no significant difference between the negative control group (KN) with the drug group (KO) and the treatment group (P3) and in the calculation of the number of spermatid cells there was also no significant difference between the control group negative (KN) with treatment group (P3). Thus, it can be concluded that the administration of *Nelumbo nucifera* extract at a dose of 450 mg / kg bw is significantly able to repair damage to spermatogenic cells damaged by 2-ME induction. The results of this study were able to indicate that the antioxidant content contained in *Nelumbo nucifera* extract was able to restore the quality of spermatozoa damaged by induction of 2-*methoxyethanol* (2-ME).

Reproductive and developmental toxicity in animal models has been linked to 2-*Methoxyethanol* (2-ME). This substance has demonstrated significant toxicity specifically in male reproductive systems. Exposure to 2-ME in experimental animals causes a decrease in testicular weight, a decrease in the number of spermatogonia, spermatocytes and spermatid cells[21]. 2-ME compounds are not found naturally in the environment because their presence in nature is the result of industrial and factory activities. 2-ME enters the body of animals and humans through inhalation, oral and topical, which will then be oxidized by *Alcohol dehydrogenase to methoxyaldehyde* (MALD); and MALD is rapidly oxidized by *aldehyde dehydrogenase* to 2-*methoxyacetic acid* (2-MAA) which is a stable and highly toxic metabolite. Exposure to MAA in male mice can cause disruption of the reproductive system, especially in the testes. The main disorders occur in the process of spermatogenesis, germinal epithelial degeneration, infertility, abnormal spermatozoa morphology, can also cause apoptosis in spermatocytes[22].

The testes are the main reproductive organs of men and are responsible for producing spermatozoa and hormones. Spermatogenic cells that have not developed into spermatozoa cells have the potential to experience disruption due to *Reactive Oxygen Species* ROS[23]. The presence of free radicals due to increased ROS can be caused by exposure to chemicals that are toxic and are strong oxidants, one of which is 2-*Methoxyethano*l (2- ME). These free radicals will be very dangerous for several organs, one of which is the reproductive organs. ROS can also cause disruption in ATP production and apoptosis in cells[24,25]. Emerging data suggest that reactive oxygen species (ROS) can fulfill this role in the GnRH receptor signaling through activation of MAP kinase signaling cascades, control of negative feedback, and participation in the secretory process[26].

Exposure to free radical compounds 2-ME affect the work of the central nervous system by the release of *Gonadrotopin Releasing Hormone* (GnRH) by the hypothalamus in stimulating the release of *Luteinizing Hormone* (LH) and *Follicle Stimulating Hormone* (FSH). These hormones function as regulators of spermatogenesis activity in the testes, such as LH which functions to stimulate leydig cells in producing testosterone, and FSH which functions to stimulate sertoli cells in the process of spermatogenic cell formation[27,28]. There is a decrease in levels of the hormone due to the content of compounds in 2-ME cause impaired spermatogenic cell formation and impact on the quality of sperm produced. This corresponds to the function of the testicles as genital organs to produce sperm. Severe testicular loss (atrophy) can result in infertility[29]. Giving 2-ME to mice BALB-C affects spermatogenesis, which is a decrease in the number of spermatogenic cells, especially in spermatocyte cells I.[30] A decrease in the number of spermatocyte cells I is thought to involve cell apoptosis. 2-*methoxyethanol* also affects the anatomy of the seminiferous tubules of the testes, which are characterized by epithelial degradation and cause atrophy in the testes.

Various parts of the *Nelumbo nucifera* plant, such as leaves, roots, seeds, and flowers, contain several bioactive flavonoid molecules, including flavonols, flavones, flavan-3-ols, flavanones, and anthocyanins. Flavonols found in *Nelumbo nucifera* are *myricetin, quercetin, kaempferol,* and *isorhamnetin,* while the flavone molecules are *diosmetin, needletin, apigenin, luteolin*, and *chrysoeriol Nelumbo*[31]. *Quercetin* is also a polyhydroxy flavonoid that is widespread in plants, which has strong antioxidant and free radical exterminator abilities[32]. *Quercetin* is a flavonoid found in fruits and vegetables that has health benefits for example as, antimicrobial, anticancer, anti-inflammatory, antiviral and antioxidant[33].

The mechanism of action of flavonoid compounds can ward off free radicals, namely by reducing ROS. In the formation of ROS, oxygen will bind to free electrons that come out due to leaking electron chains[34]. This reaction between oxygen and free electrons is what produces ROS in mitochondria[35]. Antioxidants in flavonoids (*quercetin*) can donate hydrogen atoms so as to suppress the radical properties of free radicals. *Quercetin* will oxidize and bind to free radicals so that free radicals become more stable compounds[36]. In addition, *quercetin* can induce the antioxidant capacity of cells by activating the intracellular p38 MAPK pathway, increasing intracellular GSH levels and providing a source of hydrogen donation in counteracting free radical reactions[37].

The highest antioxidant activity test was found in White Lotus flower extract (*Nymphaea nouchali* L) with an IC50 value of 66.906 μg / mL, and the lowest was found in White Lotus leaf extract (*Nymphaea nouchali* L) with an IC50 value of 99.449 μg / mL[38]. *Quercetin* as a comparison standard has an IC50 value of 6.337 μg/mL with a "very strong" antioxidant activity category, while flower extract, flower stalk extract, leaf extract, and petiole extract have a "strong" antioxidant activity category. The maceration process of the extract uses a 70% ethanol solution. While in this study testing the antioxidant activity of *Nelumbo nucifera* extract has an IC50 value of 105.8 μg / mL, *Nelumbo nucifera* ethanol extract has moderate antioxidant activity. The maceration process uses a 96% ethanol solution. From the results of the study, there are different solutions used at the time of maceration and plant extracts used so that they produce different value results but both have moderate-strong antioxidant content that can delay or inhibit cell damage, especially through free radical antidote properties.

Therefore, compounds in the of *Nelumbo nucifera* show potential as candidates for anti-infertility drugs. In general, it can be concluded from this study that the optimal dose to prevent spermatocyte degeneration is the administration of *Nelumbo nucifera* extract at a high dose. However, more research in the field of phytopharmaceuticals is needed to consider *Nelumbo nucifera* as a potential remedy.

CONCLUSION

Antioxidant Potential of high-dose *Nelumbo nucifera* extract is able to repair spermatogenic cell damage after induced 2-ME. This shows the potential of *Nelumbo Nucifera* extract as a drug candidate against male infertility. But further research needs to be done on its potential in humans and to validate its use.

AUTHORS' CONTRIBUTIONS

Badriatul Musyarofah: Project administration, Conceptualization, in vivo examination, antioxidant measurement, analyze data; Angella Ananda Syaputra: Data curation, Writing- Original draft preparation. Amelia Kartika Reza; Yunita Ainul Kasanah: Methodology, Visualization, Investigation; Putri Ayu Ika Setiyowati: Resources, Supervision, Validation data, and Reviewing; Rofiatun Solekha: Supervision.

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FUNDING INFORMATION

Directorate General of the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia.

DATA AVAILABILITY STATEMENT

There were no studies involving humans and animals as test objects in this research.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are the author's own and do not necessarily reflect the views or policies of the author's institution. The data is original research by the author and has not been published elsewhere.

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