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The Effects of *Imperata cylindrica* Root Extract on Spermatozoa Membrane Integrity Using Hypo-Osmotic Swelling Test

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ABSTRACT

High levels of reactive oxygen species (ROS) may trigger oxidative stress that harms the structural integrity of the sperm cell membrane. Concerning this case, ethanol extract from the alang-alang (*Imperata cylindrica)* root is considered capable of maintaining sperm quality. However, tests of spermatozoa membrane integrity in vitro have not been conducted. This research purposed to determine the effects of different concentrations of *I. cylindrica* root extracts on spermatozoa membrane integrity, analyzed by the hypo-osmotic swelling test. The *I. cylindrica root* extracts (concentrations of 1000 ng/mL, 2000 ng/mL, 3000 ng/mL, and 4000 ng/mL) were obtained through maceration extraction for 3 × 24 hours with 96% ethanol solvent. GC-MS analysis was then conducted to identify compounds in the extract. The sample used for the membrane integrity test was in vitro human spermatozoa. Priorly, it was washed, mixed with BWW medium, and incubated at 37°C for 60 minutes with *I. cylindrica* root extracts and the control group. Furthermore, semen was added to the HOS solution. The yields exhibited that the highest compounds in the ethanol extracts of *I. cylindrica* root were hexadecanoic acid, palmitic acid, octadec-9-enoic acid, and stearic acid. Further, the spermatozoa membrane integrity increased significantly with the higher concentrations of the *I. cylindrica* extracts. Moreover, the extracts could maintain the membrane integrity more than the control group, where the most effective result was achieved at 1000 ng/mL *I. cylindrica* extracts, since its minimum concentration can induce a biological response.

Keywords: Alang-alang (*Imperata cylindrica*); in vitro; hypo-osmotic swelling test; membrane integrity; spermatozoa

INTRODUCTION

Infertility refers to the inability of a couple to conceive even though they have had unprotected, frequent sexual intercourse after 1 year (Leslie et al., 2024). Data from the Central Statistics Agency in 2020 showed that the prevalence of infertility cases in Indonesia was 20% and continues to increase yearly (Amraeni et al., 2023). About 50% of all infertility cases worldwide are induced by factors from males. Based on previous research, the factors include pre-testicular and post-testicular disorders, immunological reactions, and environments that can cause poor sperm quality and decrease sperm number by 50% (Puspitaningrum & Nugraheni, 2022).

Attempts have been conducted to help infertility cases through assisted reproductive technology, one of which is In Vitro Fertilization (IVF). In IVF, liquid or frozen semen samples obtained from sperm donors are used for preservation. The preservation process with freezing and thawing can produce high ROSs. The high amounts of these species can break down sperm, reducing sperm quality, such as sperm movement and durability, and increasing sperm deoxyribonucleic acid (DNA) fragmentation. Furthermore, ROS can also harm and trigger cell apoptosis. Further, sperm quality impacts IVF effectiveness, where low quality can depress the success rate of IVF, meaning a declined pregnancy chance. Therefore, interventions are required to reduce sperm damage during cryopreservation and enhance sperm quality after freezing (Kumar et al., 2019).

One effort to maintain sperm quality is by introducing antioxidants. Antioxidants play a crucial role in inhibiting and neutralizing ROSs. They act as the body's defense that overcomes

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oxidative stress due to excessive amounts of ROS and can treat male fertility disorders (Azad et al., 2019; Permatasari et al., 2023). There are two types of antioxidants: endogenous and exogenous. Endogenous antioxidants, produced by the body, play a role in detoxifying and neutralizing free radicals. However, they undergo oxidation during the process, resulting in a reduction in their overall levels. Thus, relying solely on endogenous antioxidants is inadequate, making it essential to supplement with exogenous antioxidants from outside the body (Assidi, 2022).

In particular, the root of alang-alang (*I. cylindrica*) is a grass group plant spread throughout the world's tropics and subtropics. The root is often used as a sore throat medicine and antipyretic, and it has a diuretic effect. Among Indonesia's many medicinal herbs, *I. cylindrica* stands out for its rich antioxidant properties. Research exposed that the ethanol extract of *I. cylindrica* possessed a 56.03% ability to scavenge DPPH radicals, with a low IC50 of 0.098 mg/mL. The smaller IC50 value denotes stronger antioxidant activity (Suhendra et al., 2019). Besides, the metabolite compounds in *I. cylindrica* root have been reported to suppress the formation of ROS in the body.

Lubis et al. (2018) reported that the ethanol extract of *I. cylindrica* root could maintain normal sperm motility and morphology in old mice against degenerative effects and oxidative stress (Lubis et al., 2018). However, Widyastuti et al. showed the opposite, that the administration of *I. cylindrica* root extract caused a decrease in the weight of reproductive organs, reducing sperm quality (Widyastuti et al., 2017).

Given the variations in rat study results and the lack of human-based research, particularly on spermatozoa, additional studies on *I. cylindrica* root extract are essential. Besides, the continuous increase in infertility cases in Indonesia further emphasizes the pressing need to conduct this research promptly. Finally, findings from this study are expected to support the development of *I. cylindrica* extract as a promising herbal alternative to enhance male fertility and increase IVF outcomes.

MATERIALS AND METHODS

This research was approved by the Health Research Ethics Committee of the Faculty of Medicine, Palangka Raya University (No. 39/UN24.9/LL/2024). It used an experimental design with a post-test-only control group design.

Materials

The study utilized *I. cylindrica* root and human sperm samples classified as normozoospermic criteria collected following a 3-day abstinence period. Reagents includes ethanol 96%, Hypoosmotic Swelling Test (HOS) solution composed of sodium citrate (0.735 g) and fructose (1.351 g) (both from Sigma-Aldrich, St.Louis, USA) dissolved in 100 mL of distilled water, BWW (Bigger, Whitten & Whittingham) medium, and 50% percoll (Sigma-Aldrich, St.Louis, USA). The instruments employed were a centrifuge tool (Hettich, Germany), an analytical balance (Radwag, Poland), an incubator (Memmert, Germany), a Laminar Air Flow (ThermoScientific, America), a rotatory evaporator (Hahn Vapor, Hahn Shin Scientific Co., Korea), and a microscope (Olympus, Japan).

Methods

Preparation of 96% Ethanol Extract of *I. cylindrica* Root

I. cylindrica were obtained from Sei Gohong, Palangka Raya, Central Kalimantan. Subsequently, a determination test was conducted at the National Research and Innovation Agency (BRIN), InaCC Characterization Laboratory, with certificate No. B-495/II.6.2/IR.01.02/2/2024.

Approximately 1 kg of *I. cylindrica* roots were washed and cut to a size of 1.5 cm. Next, the *I. cylindrica* roots were dried by exposure to direct sunlight. Then, they were blended and filtered into simplicia. It was extracted through the maceration method utilizing 96% ethanol as the solvent. Maceration was conducted for 3 days, where every 24 hours, the simplicia was filtered and replaced with a new solvent and then stirred. After that, the macerate was filtered using filter paper to obtain the residue and 96% ethanol extract of *I. cylindrica* root. The extract was then processed using a rotary evaporator at 60°C to get a thick extract (Nuryadin et al., 2018; Sirait et al., 2024).

Analysis of Compounds by GC-MS

Gas Chromatography (GC) type Agilent 6890N with capillary column HP-5ms (30 m x 0.25 mm x 0.25 μm) and detector *Mass Spectrometry* (MS) type Agilent 5973 (GC-MS) analysis was conducted at the Biochemistry Laboratory, Faculty of Medicine, Lambung Mangkurat University (No. 38/UN8.1.17.2.2/PP/2024).

The GC-MS technique utilizes gas chromatography alongside mass spectrometry to identify and isolate organic substances. The separation principle relies on differences in

compound volatility and their interactions with the stationary phase (capillary column). Bioactive compounds were analyzed using helium as the carrier gas at a flow rate of 1 mL/min and a column temperature of 60°C. The 60°C system was held for 5 minutes and then increased by 4°C/min to 220°C for 20 minutes. The system uses split injection mode with electron impact. The number of peaks on the chromatogram indicates the number of compounds in the analyzed sample extract. The types of compounds in the *I. cylindrica* root extract were identified by interpreting spectral data from each peak using a library and database reference method.

Spermatozoa Preparation and Concentration Determination

The research samples were human sperm with normozoospermia criteria, determined using the consecutive sampling formula from Stanley Lemeshow. Accordingly, 15 male donors fulfilled specific inclusion criteria: age between 20–30 years, abstinence period of at least 3 days, semen volume over 1.5 mL, sperm concentration above 50 million per milliliter as per WHO standards, and sample collection within 6 hours of ejaculation (Permatasari, Halisa, et al., 2023). The semen sample, placed in a sterile container, was left at room temperature for 30 minutes to allow liquefaction. Then, 3 mL of 50% percoll gradient was used to wash the spermatozoa, followed by centrifugation at 1900 rpm for 30 minutes. The supernatant was removed, and the resulting pellet was rinsed with 3 mL of BWW. After that, the second centrifugation was done at 1900 rpm for 15 minutes, and the purified sperm pellet was suspended in 1 mL BWW and homogenized (Permatasari, Halisa et al., 2023). Subsequently, the spermatozoa concentration was determined by combining 5 µL of purified sperm with 95 µL of sperm-diluting solution in an Eppendorf tube, followed by homogenization. 10 µL of samples were pipetted and carefully loaded into the Neubauer counting chamber. Subsequently, the sperm concentration was counted three times under a microscope at 400x magnification using the standard semen analysis method recommended by WHO, by counting sperm in 5 medium squares located in the central grid of the Neubauer chamber (Hajizah et al., 2014). To prepare a stock solution with a concentration of 100.000 ng/mL, 0.1 mg of *I. cylindric*a extract was weighed and dissolved in 1 mL of BWW solution. Then, the working solutions with concentrations of 1000, 2000, 3000, and 4000 ng/mL were prepared by diluting the stock solution accordingly.

Based on the previous study, the sperm samples were divided into five groups: one control group (treated with BWW and four treatment groups exposed to ethanol extracts of *I. cylindrica* root at concentrations of 1000, 2000, 3000, and 4000 ng/mL (Permatasari, Halisa, et al., 2023). All groups were incubated for 1 hour at 37°C. Before and after processing, sperm were not stored at room temperature only (Syarpin et al., 2023; Permatasari et al., 2023)

Examination of Spermatozoa Membrane Integrity

The integrity of the sperm plasma membrane was evaluated using the hypo-osmotic swelling test method by combining 1 mL of HOS solution with 100 µL of the semen ample. The mixture was then incubated at 37°C for 30 minutes. After incubation, 10 µL of the sample was transferred onto a slide, covered with a coverslip, and observed under a microscope at 400× magnification (Baskaran et al., 2021; Tanga et al., 2021). The percentage of intact sperm membranes is characterized by a swollen tail of a minimum of 100 spermatozoa (Hajizah et al., 2014). All data were processed using SPSS software, analyzed with one-way ANOVA followed by a post hoc (LSD) test with a significance level set at $p<0.05$.

RESULTS

Extraction Results of *I. cylindrica*

The extraction results of *I. cylindrica* root are shown in Table I.

Table I. Extraction results of *I. cylindrica* root

Initial Weight	Dry Weight	Simplicia Powder	Thick Extract
1 kg	500 g	306 g	10.7 g

GC-MS Analysis Results

The yields of the GC-MS analysis of the ethanol extracts of *I. cylindrica* root are presented in Table II.

Observation of the Effects of Different Concentrations of Ethanol Extracts of *I. cylindrica* Root on Spermatozoa Membrane Integrity

Figure 1 shows the results of microscopic observations showing the swelling of spermatozoa in the tail, which indicates the integrity of the spermatozoa membrane. It illustrates that ethanol extract from *I. cylindrica* root improved the integrity of the spermatozoa membrane. Statistical analysis revealed a significant difference between the control group and treatment groups

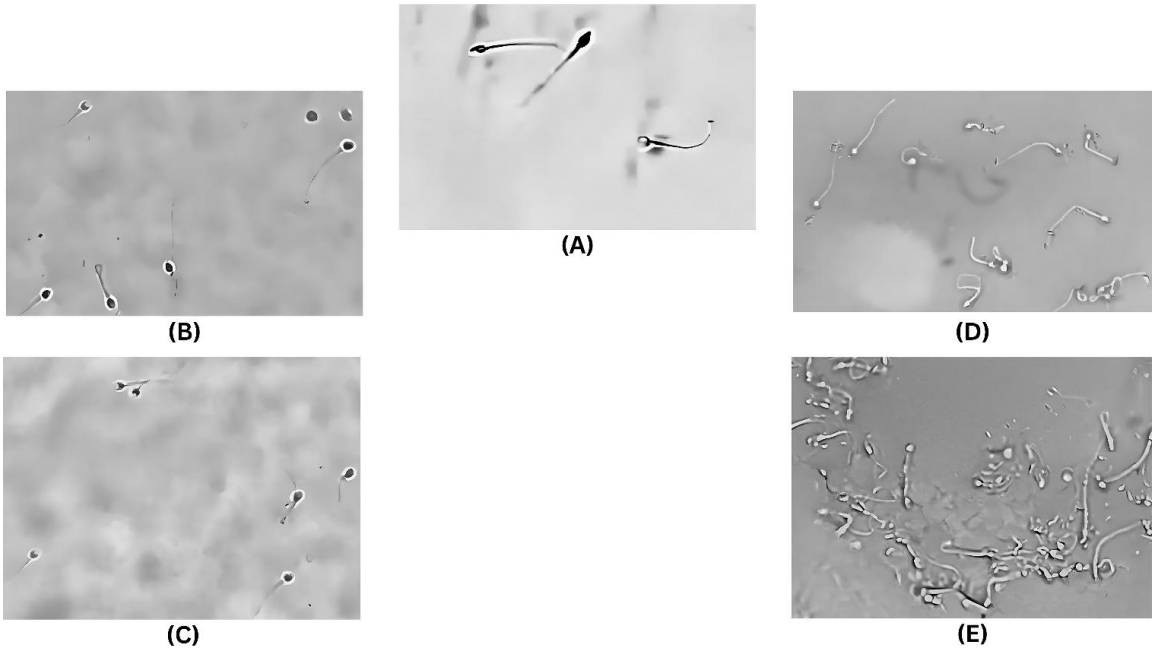

Figure 1. Microscopic observations at 400x magnification showing spermatozoa tail swelling treated with: (A) BWW only, (B) concentration 1000 ng/mL, (C) concentration 2000 ng/mL; (D) concentration 3000 ng/mL; (E) concentration 4000 ng/mL

Table II. GC-MS analysis results of ethanol extract of *I. cylindrica* root

No	Name	Compound Class	Retention Time	Peak area
1	Hexadecanoic acid (CAS) Palmitic acid	Saturated fatty acid	22.418	45497769
2	Octadec-9-Enoic Acid	Unsaturated fatty acid	23.708	29800436
3	Octadecanoic acid (CAS) Stearic acid	Saturated fatty acid	23.851	4244631
4	2(3H)-Furanone, dihydro-5-tetradecyl- (CAS) 4-Octadecanolide	Lactone	23.33	3220213
5	2-Undecenal (CAS) Undec-2-enal	Fatty aldehyde	15.05	2292205
6	1-Docosanol (CAS) Behenic alcohol	Saturated fatty acid alcohol	25.581	1455833
7	Octyl Heptanoate	Ester	25.113	1117929
8	Hexadecanoic acid, 1-[[(2-aminoethoxy) hydroxyphosphinyl]oct]methyl]-1, 2 ethanediyl ester (CAS) dipalmitoyl phospatidyl ethan	Saturated fatty acid	23.433	968420
9	9-Octadecenoic acid (Z)- (CAS) Oleic acid	Unsaturated fatty acid	22.955	465193

(1000 ng/mL, 2000 ng/mL, 3000 ng/mL, and 4000 ng/mL) with $p<0.05$, as shown in Tables III and IV.

DISCUSSION

Based on the results, the ethanol extracts of *I. cylindrica* root with all concentrations were found to better preserve the membrane integrity. This is evidenced by the increasing number of sperm tails swelling after incubation with all concentrations of ethanol extracts of *I. cylindrica* root, when compared to the control group. The proportion of sperm with swollen tails increased proportionally with the concentration of the extracts. The ethanol extract of *I. cylindrica* root appears to influence sperm membrane integrity in

Table III. Observation Result of *I. cylindrica* Root Extract on Spermatozoa Membrane Integrity In Vitro

Mean	62.53	71.07	76.80	84.47	88.93
SD	5.73	5.27	3.87	6.01	6.48
SEM	1.480	1.361	1.001	1.552	1.675

Table IV. The significant difference between the control group and the treatment group

Group	Control	1000 ng/mL	2000 ng/mL	3000 ng/mL	4000 ng/mL
Control		0.000*	0.000*	0.000*	0.000*
1000 ng/mL	0.000*		0.006*	0.000*	0.000*
2000 ng/mL	0.000*	0.006*		0.000*	0.000*
3000 ng/mL	0.000*	0.000*	0.000*		0.031*
4000 ng/mL	0.000*	0.000*	0.000*	0.031*	

* = The analysis results using post hoc LSD between groups showed significant differences with $p<0.05$.

a dose-dependent manner, as seen in the progressive swelling of sperm tails, which could change with varying extract concentrations. Spermatozoa membrane integrity began to improve at a concentration of 1000 ng/mL. This finding is consistent with the study of Permatasari et al., where an increase in swollen sperm tails was observed even at the lowest concentration. From a pharmacological view, the effective concentration is defined as the minimum concentration that produces a biological response (Grogan & Preuss, 2023). The plasma membrane is a complex structure composed of phospholipids, proteins, carbohydrates, and cholesterol. Phospholipids, which are derived from fatty acids (FAs), play a crucial role in sperm function. The composition of FAs influences membrane fluidity and flexibility, the acrosome reaction, motility, and sperm viability. FAs can be classified into Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA), and Polyunsaturated Fatty Acids (PUFA). Research by Martinez-Soto JC et al. suggests that SFA enhances plasma membrane integrity and sperm viability by increasing antioxidant activity in sperm, thereby contributing to improved semen quality (Martínez-Soto et al., 2013). SFA are classified into three primary types: palmitic acid, stearic acid, and myristic acid.

The GC-MS analysis of *I. cylindrica* root extract showed that palmitic acid was the compound with the highest concentration. Palmitic acid is the predominant class of SFA in spermatozoa, and it exhibits a strong correlation with the total sperm number, highlighting its role in the process of spermatogenesis (Yuan et al., 2023). According to Han et al. (2023) and Khophloiklang et al. (2024) reported that elevating palmitic acid levels can restore the spermatogenesis process impaired by asthenozoospermia in mice and protect sperm from cryodamage caused by freezing and thawing (Han et al., 2023; Khophloiklang et al., 2024). Zhu et al. (2020) found that adding palmitic acid to boar sperm during storage increased total spermatozoa motility. Exogenous fatty acids, such as palmitic acid, are crucial in sperm progressive motility and survivability. Additionally, the addition of palmitic acid to the extender can enhance the spermatozoa membrane integrity, helping to maintain the sperm quality (Zhu et al., 2020).

Oleic acid compound is one of the MUFA class found in the ethanol extracts of *I. cylindrica* root, that can be employed as an indicator to asses spermatozoa membrane fluidity in mammals (A. Alizadeh et al., 2014). It is a class of omega-9 that has antioxidant potential. The antioxidant properties of oleic acid are evident in its ability to protect frozen sperm. As a fatty acid prone to oxidation, oleic acid can neutralize free radicals, thereby preventing damage to the spermatozoa's plasma membrane (Banihani, 2017). Oleic acid positively impacts membrane integrity and preserves the stability of sheep sperm during 48 hours of storage at low temperatures. It can reduce lipid peroxidation and boost antioxidant capacity, making its inclusion advantageous for enhancing semen quality (Mortazavi et al., 2020). Additionally, Zhu et al. reported a significant enhancement in the total motility of boar sperm (Zhu et al., 2020).

Stearic acid is another group of SFAs contained in the ethanol extracts of *I. cylindrica* root. It can enhance the activity of internal antioxidant enzymes (Mbulang et al., 2023). Stearic acid is the predominant component of the spermatozoa membrane. It has been suggested

to be positively correlated with sperm motility. As an antioxidant, it plays a protective role against oxidative stress through the phosphatidylinositol 3-kinase (PI3K) pathway, thereby helping to prevent cryodamage in sperm (Amirjannati et al., 2024; Khophloiklang et al., 2024). Yu et al. identified stearic acid as a biomarker for distinguishing high and low freeze ability in male donkey sperm, due to its association with membrane integrity and oxidative stress post-thaw (Yu et al., 2022).

Behenic alcohol is a class of fatty alcohol compounds that contribute to the sperm membrane stability, especially during capacitation. It also acts as an antioxidant, protecting membrane lipids from oxidative damage (Remedios et al., 2023; Shan et al., 2021). Meanwhile, Furanones are organic compounds with a five-membered lactone ring with a furan structure, known for their antioxidant properties. The structure allows them to scavenge free radicals and prevent oxidative degradation (Alizadeh et al., 2020).

The *I. cylindrica* root extract is considered to help maintain the spermatozoa membrane integrity. Excessive free radicals in sperm can damage the membrane structure. This membrane is rich in polyunsaturated fatty acids (PUFAs), which are highly susceptible to lipid peroxidation (Permatasari et al., 2024). Derbak et al. stated that low antioxidants could protect sperm membranes from damage, resulting in increased integrity, flexibility, and fluidity of spermatozoa membranes that maintain sperm quality (Derbak et al., 2021). The spermatozoa's viability, defined as the ability of spermatozoa to remain alive, is an important indicator of sperm health. It is closely correlated with sperm membrane integrity, which is essential for maintaining cellular function and overall fertilizing potential. Viability assessment is based on the permeability of the spermatozoa membrane, which serves as an indicator of membrane integrity (Gustina et al., 2022). This study had several limitations. It possessed a limited search for respondents who met the inclusion criteria. Besides, time management must be considered, particularly from the sample processing to the sperm observation stages, to ensure that the sperm is properly preserved. Furthermore, this study was limited to observing sperm membrane integrity, a key parameter in assessing male fertility. Therefore, further research is needed to investigate the success rate of the fertilizing ovum using Assisted Reproductive Technology (ART) following the incubation of sperm and *I. cylindrica* root extracts.

CONCLUSION

The yields revealed that the *I. cylindrica* root extracts were more effective in maintaining the integrity of the spermatozoa membrane than the control group, with an optimal concentration of 1000 ng/mL. The highest compounds found in the ethanol extracts of *I. cylindrica* root were hexadecanoic acid, palmitic acid, octadec-9-enoic acid, and stearic acid. The three compounds have antioxidant potential to maintain sperm membrane integrity. The results of this study suggest that the *I. Cylindrica* root extract is a promising herbal medicinal agent that can improve reproductive technology in the future. Hence, further research is needed to explore its full potential.

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