



The Effect of Methanol Fractionated of *Luvunga sarmentosa* on In Vitro Sperm Membrane Integrity

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DOI: 10.31964/mltj.v10i1.571

Abstract: Infertility cases in the world are increasing in 2020, with 186 million individuals affected worldwide. The most common cause of male infertility is due to decreased sperm quality. Sperm membrane integrity is known to play an essential role in the fertilization ability of spermatozoa. The methanol fraction of *Luvunga sarmentosa* root extract has the highest antioxidant activity compared to other fractions and can improve sperm motility in vitro. This study aims to determine the effect of the methanol fraction of *Luvunga sarmentosa* root extract on human sperm membrane integrity and the effective concentration of *Luvunga sarmentosa* methanol extract fraction to improve sperm membrane integrity in vitro. The samples used were washed human spermatozoa in vitro, then added BWW medium, and incubated at 37°C for 1 hour with methanol fraction of *Luvunga sarmentosa* root extract at concentrations of 100, 500, 1000, and 5000ng/ml as well as the control group (BWW only). Furthermore, 100uL of each sample was added to 1mL of Hypo-osmotic swelling (HOS) solution, then incubated at 37°C for 30 minutes to see the integrity of the spermatozoa membrane from the tail swelling. The results showed that sperm membrane integrity significantly increased at 100, 500, 1000, and 5000ng/mL concentrations compared to the control group. The methanol fraction of *Luvunga sarmentosa* root extract was most effective at a concentration of 100ng/mL. Methanol fraction of *Luvunga sarmentosa* root extract can improve human sperm membrane integrity.

Keywords: *Luvunga sarmentosa*; methanol fractionation; sperm membrane integrity.

INTRODUCTION

The WHO (World Health Organization) reported an increase in infertility cases in 2020, with 186 million individuals affected worldwide (Brenda C., 2022). The National Population and Family Planning Agency in 2020 showed that 2 million couples in Indonesia experience infertility problems every year (Puspitaningrum et al., 2022). Infertility cases, which account for 30-80%, are often attributed to idiopathic factors indicated by increased concentrations of reactive oxygen species (ROS), which, present in abnormal quantities, will cause oxidative stress, which affects sperm quality which decreases due to damage to sperm membrane integrity (Assidi, 2022).

Sperm membrane integrity plays a crucial role in the fertilization ability of spermatozoa, including sperm metabolism, capacitation, acrosome reaction, and binding of spermatozoa to the surface of the egg sperm membrane integrity contains

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polyunsaturated fatty acids (PUFAs) and has low levels of antioxidants, making it highly sensitive to free radicals. Oxidative stress will cause peroxidation of spermatozoa membrane lipid, leading to decreased sperm quality due to damage to sperm membrane integrity (Alahmar, 2019; Assidi, 2022).

Antioxidant administration can be an alternative treatment for infertility patients. It is known that antioxidants can play a role in reducing the amount of oxidative stress, which is a contributing factor to idiopathic infertility. Exogenous antioxidants have been shown to improve both the quality and quantity of sperm, including reducing malondialdehyde (MDA) levels, a parameter for sperm membrane damage oxidized by ROS. Examples of exogenous antioxidants that can be used include alkaloids, amino acid derivatives, and terpenoids. Research by Derbak et al. showed that alkaloid administration was shown to protect sperm from oxidative damage and improve sperm membrane integrity in ram semen (Derbak et al., 2021). Alkaloids and amino acid derivatives have prevented cell apoptosis (Li et al., 2023; Vardiyani et al., 2020). Wijayanti et al. reported that terpenoids found in the methanol fraction of Parijoto fruit (*Medinilla speciosa*) can increase spermatozoa motility in Sprague Dawley male rats (Wijayanti et al., 2021)

Research by Syarpin et al. identified that phytochemical components contained in the methanol fraction of *Luvunga sarmentosa* root extract have the highest antioxidant activity compared to other fractions. The highest percentage of compounds in the methanol fraction of *Luvunga sarmentosa* root extract was alkaloids, followed by amino acid derivatives, phenylpropanoids, and terpenoids (Syarpin et al., 2023). However, the bioactivity of the active compounds identified in the methanol fraction of *Luvunga sarmentosa* root extract in vitro sperm membrane integrity testing is still limited. Therefore, it is necessary to carry out research aimed at analyzing the effect of the methanol fraction of *Luvunga sarmentosa* root extract on the integrity of spermatozoa membranes using the Hypoosmotic Swelling Test (HOS). The HOS test will predict the integrity of the plasma membrane by looking at its ability to maintain the osmotic balance between spermatozoa cells and the environment.

MATERIALS AND METHODS

Tools and Materials

The materials of this study were a total of 2000 g of *Luvunga sarmentosa* root taken from Desa Bubut, Muara Teweh, Central Borneo, Indonesia. Other materials were ethanol 96%, methanol, Hypoosmotic Swelling Test (HOS) solution containing 0,735 g citrate sodium (Sigma-Aldrich, St.Louis, USA) and 1,351 g fructose (Sigma-Aldrich, St.Louis, USA) in 100 mL aqua dest, human sperm, BWW (Bigger, Whitten & Whittingham) medium, percoll 50% (Sigma-Aldrich, St.Louis, USA), and methanol. The tools were centrifuge (Hettich, Germany), analytical balance (Radwag, Poland), incubator (Memmert, Germany) Laminar Air Flow (ThermoScientific, America), rotatory evaporator (Hahn Vapor, Hahn Shin Scientific Co., Korea), and microscope (Olympus, Japan).

Extraction Methods and Fractionation

This study was approved by the Ethics Committee for Medical Research, Faculty of Medicine, Universitas Palangka Raya (No.39/UN24.9/LL/2023). An amount of 2000 grams of *Luvunga sarmentosa* root was washed and dried for seven days to make simplicia using a blender and then sifted using a 60-mesh strainer. A 1000 g simplicia was macerated with 96% ethanol for 3 x 24 hours. The obtained macerate was concentrated by using a rotary evaporator. The viscous extract was

fractionated using a vacuum chromatography column with methanol as the eluent and 50 g silica gel G60 was used as a stationary phase. Five grams of the thick extract was eluted with 5 x 250 mL of methanol. A rotary evaporator concentrates the methanol fraction (Syarpin et al., 2023).

Methanol Fractionation of *Luvunga sarmentosa* Effect on Human Sperm Membrane Integrity

The research used human sperm samples with normozoospermic criteria obtained from male donors who have agreed to informed consent. The location of sperm analysis is in the Biomedical Laboratory at the Medical Faculty of Universitas Palangka Raya. Human sperm is obtained by masturbation after abstinence for at least 48 hours and collected in sterile containers. The inclusion criteria for the sample in this study were fertile, healthy men aged <30 years. The number of samples was calculated based on the one-proportion estimation formula from Stanley Lemeshow et al. with a consecutive sampling technique rounded to 15 people. The cement was collected in a sterile container and left at room temperature for 30 minutes for liquefaction. Spermatozoa were washed with a 50% Percoll gradient. Then, the tube was centrifuged at 3000 rpm for 30 minutes. The supernatant was discarded, and the pellet was washed with 3 ml of medium BWB. Then, the tube was centrifuged again at 3000 rpm for 25 minutes. The supernatant was discarded, and the pellet in the form of pure spermatozoa precipitate was then resuspended with 1 ml of BWB and then homogenized. After that, the spermatozoa concentration was measured by giving 95 µL of sperm diluting fluid and 5µL of washed spermatozoa in a 1.5 ml tube and then homogenized. Take ten µL of the sample and place it in the Neubauer chamber. Furthermore, the calculation of the concentration was carried out under a microscope with a magnification of 400x using the standard semen analysis method according to WHO (Permatasari et al., 2023)

Spermatozoa were divided into five groups, each containing ±10 million cells in 500 mL BWB. The samples were divided into five groups: negative control (without treatment) and administration of the *Luvunga sarmentosa* extract eluent methanol with final concentrations of 100, 500, 1000, and 5000 ng/mL. The five groups were then incubated at 37°C for 1 hour. 100 µL of the sample from 5 groups mixed with 1 mL of HOS solution, then incubated for 30 minutes at 37°C (Yusrina et al., 2018). Place ten µL of the sample into a slide, and the sperm membrane integrity with HOS was examined by microscope with a magnification of 400 x. Percentage of integrity sperm membrane:

$$\frac{\text{Swollen tails}}{100 \text{ total sperm counted}} \times 100\%. \text{ (Yendraliza et al., 2019)}$$

Hypoosmotic Swelling Test (HOS) assesses the permeability characteristics of intact cell membranes, causing sperm to swell under hypo-osmotic conditions. The influx of water increases the volume of sperm cells, and the plasma membrane will swell. Normal sperm will show swelling under hypo-osmotic conditions. Statistical analysis of the samples using the One-Way ANOVA test continued using the LSD method.

RESULTS AND DISCUSSION

The mean percentage of spermatozoa membrane integrity was determined by observing the swelling of sperm tails from 100 spermatozoa, as shown in Table 1. Based on the data, the effective concentration starts from a concentration of 100 ng/ml. Overall, the results in Table 1 showed that sperm membrane integrity using

HOS in the group induced by methanol fraction of *Luvunga sarmentosa* root extract was better than the control. A microscopic image of a swelling spermatozoa tail can be seen in Figure 1.

Table 1. Average Results of Sperm Membrane Integrity in the Control and Treatment Groups Incubated with a Methanol Fraction of *Luvunga sarmentosa* Root Extract with a Concentration of 100-5000 ng/mL

<i>Luvunga sarmentosa</i> Root Extract Fraction	Concentration	Number of Samples (N)	Sperm Membrane Integrity Mean \pm DS
Methanol Fraction	a. Control	15	62,07 \pm 1,17 ^a
	b. 100 ng/mL	15	70,20 \pm 2,86 ^b
	c. 500 ng/mL	15	76,27 \pm 7,31 ^b
	d. 1000 ng/mL	15	82 \pm 3,16 ^b
	e. 5000 ng/mL	15	83,80 \pm 4,88 ^b

Notes: Superscript symbol (b) shows significant differences from the control (P<0.05) using LSD test \pm is a standard data deviation. DS = Deviation Standard

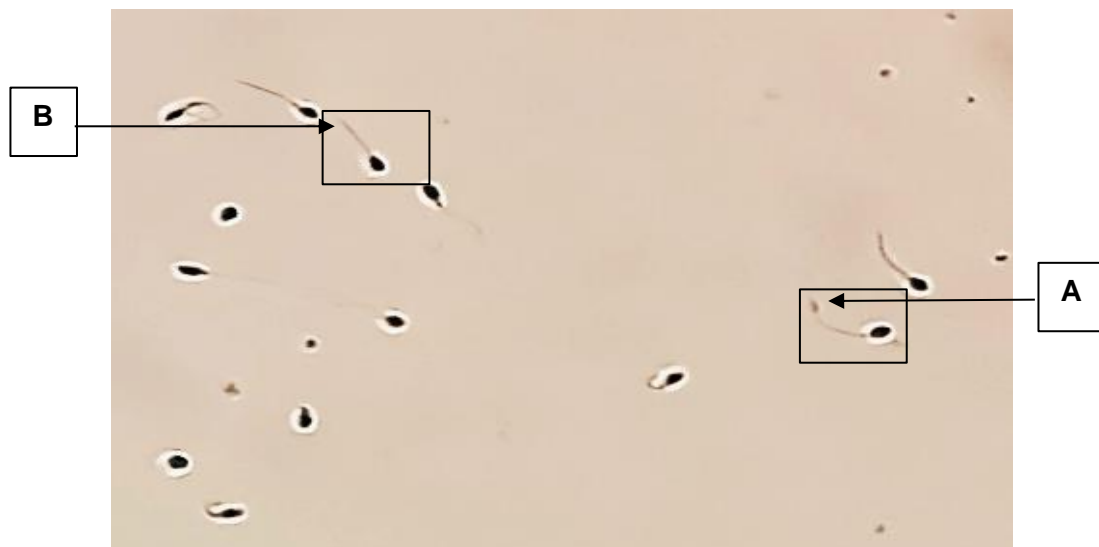


Figure 1. Microscopic Image of A: Swelling Spermatozoa Tail; B: Non-Swelling Spermatozoa Tail

The HOS test assesses the permeability properties of the intact cell membrane that causes sperm to swell under hypo-osmotic conditions. When sperm are exposed to hypo-osmotic conditions, water will enter the spermatozoa in an attempt to achieve osmotic balance. The influx of water increases the volume of sperm cells, and the plasma membrane will swell. The sperm tail is easily affected under hypo-osmotic conditions. The ability of the sperm tail to swell under hypo-osmotic conditions indicates that water transport is taking place normally, which means that membrane integrity and functional activity are normal. The HOS test will predict the integrity of the plasma membrane by looking at the ability of the plasma

membrane to maintain the osmotic balance between the spermatozoa cell and the environment (Check et al., 2023). The increase in spermatozoa membrane integrity, as seen from the swellings of the spermatozoa tail, is in line with the increase in the concentration due to the content of secondary metabolite compounds contained in the methanol fraction of *Luvunga sarmentosa* root extract (Table 1).

Based on the research conducted by Syarpin et al. regarding the phytochemical analysis of the methanol fraction of *Luvunga sarmentosa* root extract using the LCMS/MS method, it was determined that the methanol fraction of *Luvunga sarmentosa* root extract contains alkaloids, amino acid derivatives, phenylpropanoids, and terpenoids (Syarpin et al., 2023). Alkaloids are secondary metabolite compounds with the highest percentage present in this fraction. The mechanism of alkaloids as antioxidants involves donating hydrogen atoms to free radicals (Amelia & Rahmanisa, 2020). Excessive free radicals can lead to lipid peroxidation in sperm membranes. With antioxidants, lipid peroxidation can be prevented, and sperm membrane integrity can be maintained. The same research also mentions that low-rate antioxidants can protect sperm membranes from damage, thus enhancing fluidity, flexibility, and sperm membrane integrity, ultimately improving sperm quality (Derbak et al., 2021). The alkaloid compound found in the methanol fraction of *Luvunga sarmentosa* extract is isoquinoline. According to research by Li et al., isoquinoline works through the apoptosis inhibition pathway by reducing the activity of apoptotic factors such as Caspase-3 and Bax while increasing anti-apoptotic factors such as Bcl-2. (Li et al., 2023).

Other compounds found in the methanol fraction of *Luvunga sarmentosa* extract are amino acid derivatives. The amino acid derivative compounds found in the methanol fraction of *Luvunga sarmentosa* root extract are L-carnitine and L-proline. L-carnitine also acts as a scavenger of free radicals to increase the anti-oxidative ability in spermatozoa and reduce oxidative stress levels (Moretti et al., 2023). Research by Vardiyan et al. reported that L-Carnitine can inhibit apoptosis by increasing the anti-apoptotic protein Bcl-2 and reducing the pro-apoptotic protein Bax (Vardiyan et al., 2020). L-proline is an essential amino acid that can function as a plant's natural antioxidant and osmoprotectant. Sperm in the medium induced by L-proline can reduce caspase three and caspase nine by increasing the level of GSH / GSSG to protect mitochondria and prevent apoptosis (Zhang et al., 2022). Research by Feng et al. showed an increase in ATP levels by adding proline supplements to cryopreserved pig sperm (Feng et al., 2020).

Another compound found in the methanol fraction of *Luvunga sarmentosa* root extract is phenylpropanoid. Phenylpropanoid is a part of the phenolic group and acts as an antioxidant by scavenging free radicals, inhibiting lipid peroxidation, chelating metal ions, and binding to proteins (Neelam et al., 2020). According to Adami et al., it is known that sperm exposed to a medium containing phenolics can increase sperm motility and viability and prevent oxidation of DNA by reducing ROS levels (Adami et al., 2022).

Terpenoid compounds also act as antioxidants by inhibiting lipid peroxidation, chelating metals, and capturing reactive species. Research by Wijayanti et al. demonstrated that terpenoids in the n-hexane fraction of Parijoto fruit act as antioxidants and can increase sperm motility (Wijayanti et al., 2021).

The increase in the number of swelling sperm tails can already be seen from the first treatment group with a concentration of 100 ng/ml; based on pharmacology, effective concentration is the minimum concentration that produces a biological response (Grogan & Preuss, 2023). This study is consistent with previous research

that showed an increase in sperm motility induced by the methanol fraction of *Luvunga sarmentosa* root extract at low concentration, specifically, ten ng/ml (Permatasari et al., 2023) and another research reported that the antioxidants at low concentration could protect sperm membranes from damage, thus enhancing fluidity, flexibility, and sperm membrane integrity, ultimately improving sperm quality (Derbak et al., 2021). However, limitations in this study included a restricted search for patients willing to provide sperm samples and those meeting the inclusion criteria of this study. Another limitation is that the success rates of the fertilizing ovum through Assisted Reproductive Technology (ART) following the incubation of sperm methanol fraction of *Luvunga sarmentosa* root are currently unknown. Therefore, further research is needed to investigate and determine the success rate in this specific context.

CONCLUSION

The methanol fraction of *Luvunga sarmentosa* root extract could increase sperm membrane integrity. The most effective at a concentration of 100ng/mL. The success rates of sperm incubated with the methanol fraction of *Luvunga sarmentosa* root are not yet known in fertilizing ovum in Assisted Reproductive Technology (ART), so further research is needed.

CONFLICT OF INTEREST

All authors declared that there was no conflict of interest in this study.

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