

Photochemical and bioactivity examination of fractionated saluang belum root extract (*Lavanga sarmentosa*) on in-vitro human sperm motility

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Original Article

ABSTRACT

ARTICLE INFO

Keywords:

Phytochemical test, methanol fraction, chloroform fraction, *Lavanga sarmentosa*, Sperm Motility

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DOI: 10.20885/JKKI.Vol14.Iss2.art3

History:

Received: November 8, 2022

Accepted: April 11, 2023

Online: August 31, 2023

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Background: Saluang belum (*Lavanga sarmentosa*) is one of Kalimantan's typical plants, which is as efficacious as a traditional medicine to increase sexual activity and male fertility. Based on previous studies, the content of flavonoid and steroid in 70% ethanol extract of *L. sarmentosa* were able to affect sperm quality of mice. Studies related to *L. sarmentosa* are still limited for phytochemical test and their bioactivity on human spermatozoa motility in vitro.

Objective: This study is to perform phytochemical tests of compound content in fractionation with eluents of high and low polarity, namely methanol and chloroform, and then to test their bioactivity on the motility of human spermatozoa in vitro.

Methods: *L. sarmentosa* was extracted with 96% ethanol and fractionated by using a vacuum chromatography column with chloroform and methanol as the eluent. Then obtained samples were analysed by a quantitative phytochemical test. The samples used in-vitro human spermatozoa were divided into eleven groups: control group, group administered with *L. sarmentosa* extract eluent chloroform of 10, 50, 100, 500, and 1000 ng/mL, and same concentration with extract eluent methanol. Furthermore, the sperm motility was analysed by using a *Computer Assisted Sperm Analyser* (CASA).

Results: The methanol and chloroform fraction of *L. sarmentosa* root extract contained metabolites, namely terpenoids, flavonoids, steroids, alkaloids, saponins, and tannins. The sperm motility increased significantly at the treatment group of the methanol and chloroform fractions compared to the control group. There was a significant difference between the sperm motility incubated with methanol and that with chloroform fraction at concentration 500 and 1000 ng/mL.

Conclusion: The results of sperm motility were higher in the methanol fraction than those in the chloroform fraction.

Latar Belakang: Saluang belum (*Lavanga sarmentosa*) merupakan salah satu tanaman khas Kalimantan yang sering digunakan sebagai herbal untuk meningkatkan gairah seksual dan kesuburan pria. Ekstrak etanol 70% *L. sarmentosa* memiliki pengaruh terhadap kualitas sperma mencit. Penelitian mengenai uji fitokimia dan bioaktivitasnya terhadap motilitas spermatozoa manusia secara in-vitro masih terbatas.

Tujuan: melakukan uji fitokimia kandungan senyawa pada fraksinasi dengan eluen yang kepolarannya tinggi dan rendah yaitu methanol dan kloroform serta uji bioaktivitasnya terhadap motilitas spermatozoa manusia secara in vitro.

Metode: *L. sarmentosa* diekstraksi menggunakan etanol 96% dan dilakukan fraksinasi menggunakan kolom vakum kromatografi menggunakan pelarut kloroform dan etanol kemudian dianalisis uji fitokimia kuantitatif. Sampel menggunakan spermatozoa in-vitro yang dibagi 11 kelompok yaitu kontrol, pemberian fraksi ekstrak *L. sarmentosa* eluen kloroform dengan konsentrasi akhir 10, 50, 100, 500, dan 1000 ng/mL, dan konsentrasi

yang sama untuk fraksi eluen metanol. Motilitas sperma dianalisis dengan Computer Assisted Sperm Analyser (CASA).

Hasil: Fraksi metanol dan kloroform ekstrak akar *L. sarmentosa* mengandung senyawa metabolit yaitu terpenoid, flavonoid, steroid, alkaloid, saponin, dan tannin. Motilitas sperma mengalami peningkatan signifikan pada kelompok perlakuan yang menggunakan fraksi metanol dan kloroform dibandingkan kontrol. Terdapat perbedaan secara signifikan pada nilai motilitas sperma yang diinkubasi fraksi metanol dan kloroform pada konsentrasi 500 dan 1000 ng/mL.

Kesimpulan: Hasil motilitas sperma lebih tinggi pada fraksi metanol dibandingkan dengan fraksi kloroform.

INTRODUCTION

Indonesia, especially Central Kalimantan, has various plants that can be developed into herbal medicine. Saluang Belum (*Lavanga sarmentosa*) is one of Kalimantan's typical plants, which is as commonly used as traditional medicine. The plant has long been believed to have practical benefits as a stamina-enhancing drug.¹ The plant, *L. sarmentosa*, is one of the endemic plants of Central Kalimantan, and the Dayak people use the roots of this plant to increase sexual activity, cure sexual dysfunction, and male fertility. It can be simply consumed as the ethnic Kalimantan community only boils and drinks the marinade from the stems or the roots of the plant daily.² The results of phytochemical screening that had been carried out in previous studies indicated that they contained phenolic compounds, flavonoids, and steroids.³ These secondary metabolites have the potential to become antioxidant compounds.

Another study related to the potential of *L. sarmentosa* root on the quality and viability of mice sperm by using 70% ethanol extract, which was divided into five dose groups and observed for 14 days, indicated the effect of motility and viability of mice sperm from 100 mg/KgBW 200 mg/KgBW and 400 mg/KgBW.¹ However, this evidence has not yet been tested for its effect on human spermatozoa, so further research in vitro is necessary. The flavonoid compounds in the root extract of *L. sarmentosa* are thought to act as an antioxidant in maintaining sperm quality.^{1,4} Fertilization can occur with good-quality sperm. Decreased sperm quality can cause infertility. The basis for the analysis of the spermatozoa quality is spermatozoa with a good number,

motility, and morphology.⁵ Sperm motility is the ability of sperm to swim in order to reach the oocyte and to fertilize. Sperm motility must be considered one of the most important parameters in evaluating the fertility potential of a semen specimen. Sperm motility is one of criteria for analysing spermatozoa quality, including the type of movement and the speed of sperm movement.⁶

Studies related to *L. sarmentosa* are still limited to phytochemical test and bioactivity of its root extract. Further research needs to be conducted, namely grouping the compounds by graded fractionation by using eluents with high and low polarity, such as methanol and chloroform. Then it is to identify the levels of compound content in the two fractions through phytochemical and bioactivity tests of the two fractions of *L. sarmentosa* root extract on in-vitro human sperm motility.

METHODS

Tools and Materials

The materials of this study were an amount of 5000 g *L. sarmentosa* root taken from Desa Bubut, Muara Teweh, Central Borneo, ethanol 96%, aqua dest, human sperm, TBS (tris Buffer) BWW (Bigger, Whitten & Whittingham) medium, percoll 50%, chloroform, and methanol. The tools were silica for KVC, silica for KKG, centrifuge, analytical balance, LAF (Laminar Air Flow), rotatory evaporator, water bath, and CASA microscope.

Extraction Methods and Fractionation

This study was approved by the Ethics Committee for Medical Research, Faculty of Medicine, Universitas Palangka Raya (No.103/UN24.9/LL/2022). An amount of 5000 grams of *L. sarmentosa* root was washed and dried for seven days to make simplicia by using a blender, and then sifted by using a 60-mesh strainer. A 2.5 kg 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 by using a vacuum chromatography column with chloroform and 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 and 5 x 250 mL of chloroform. Each fraction was accommodated and then

concentrated by using a rotary evaporator.

Phytochemical Test

Quantitative phytochemical analysis was applied to identify content in the *L. sarmentosa* root. The content analysed was flavonoids, terpenoids, saponins, alkaloids, and steroids.

Lavanga sarmentosa Bioactivity Test on Sperm Motility

This study used human sperm samples with normozoospermic criteria obtained from male donors who agreed to its informed consent. The location of the sample collection and sperm analysis was in laboratory of department of biology medicine FKUI. The human sperm was obtained by masturbation after abstinence for at least 48 hours and collected in sterile containers. The inclusion criteria for the samples in this study were fertile and healthy men aged < 40 years. Then its exclusion criteria were men with suspicion, azoospermia, oligospermia, and asthenozoospermia. The number of the samples was calculated based on the one-proportion estimation formula with simple random sampling from Stanley Lemeshow et al., as in the following:⁷

$$n = \frac{Z^2_{1-\alpha/2} P(1-P)}{d^2} \dots\dots\dots(1)$$

Based on this formula, $Z_{1-\alpha/2}$ was a 1.960 (95% of confidence level); P was an unknown population (maximum 0.5), with D (degree of accuracy by 25%), so the number of samples was rounded up to 15 people.

The cement was collected in a sterile container and left at a room temperature for 15 minutes for the liquefaction process. The spermatozoa were washed with a 50% Percoll gradient. Then the tube was centrifuged at 1900 rpm for 30 minutes. The supernatant was discarded, and the pellet was washed with 3 ml of medium biggers, whitten, and whittingham (BWW). Next, the tube was centrifuged again at 1900 rpm for 15 minutes. The supernatant was discarded, and the pellet in the form of pure spermatozoa precipitate was then resuspended with 1 ml of BWW and then homogenized. After that, the spermatozoa concentration was measured by giving 95 μ L of sperm diluting fluid and 5 μ L of washed spermatozoa in a 1.5 ml tube and then homogenized. After that, 10 L of the sample was taken and placed in the Neubauer chamber.

Furthermore, the calculation of the concentration was carried out under a microscope with a magnification of 400 x by using the standard semen analysis method according to WHO.

The spermatozoa were divided into eleven groups, each containing \pm 10 million cells in 500 mL BWW. The eleven groups were negative control (without treatment), administration of the *L. sarmentosa* extract eluent chloroform with final concentrations of 10, 50, 100, 500, and 1000 ng/mL, and administration of the *L. sarmentosa* extract fraction eluent methanol with final concentrations of 10, 50, 100, 500, and 1000 ng/mL. The eleven groups were then incubated at 37°C for 2 hours. The sperm motility was examined by Computer Assisted Sperm Analyser (CASA) using 2010 WHO criteria. 3 μ L of the spermatozoa sample was dropped into the CASA slide chamber (Leja, Netherland) and then observed under a CASA microscope. The CASA examination was located in INA Repromed IMERI FKUI Laboratory. The samples were statistically analysed by using One-Way ANOVA test and continued by using LSD method. Then the difference in mean sperm motility of the two fractions was determined by using the independent T-test.

RESULTS

Phytochemical screening

The active compounds in the methanol and chloroform fraction (*L. sarmentosa*) are showed in Table 1.

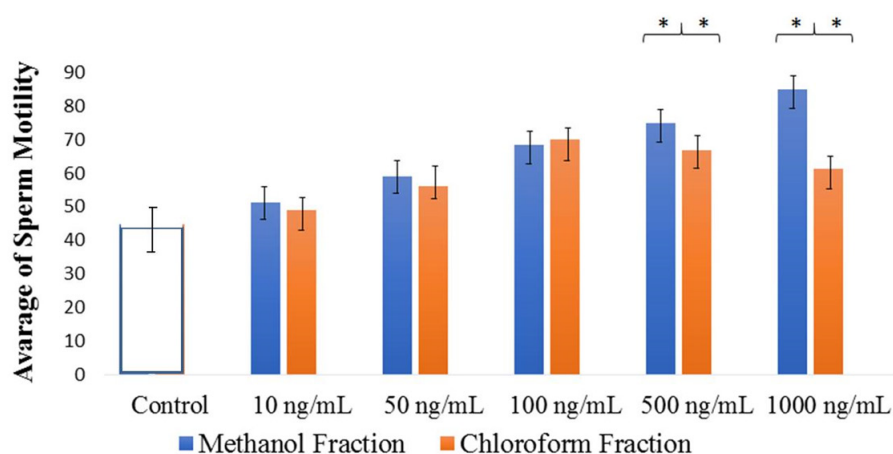
Bioactivity test of *L. sarmentosa* extract on sperm motility

The results showed that the sperm motility value increased at the concentration of the methanol and chloroform fractions of the *L. sarmentosa* root extract compared to the control group. Figure 1 shows a difference of the average motility of sperm incubated with methanol and chloroform fractions using the T-test. There was a statistically significant difference of methanol and chloroform fractions in dose 500 and 1000 ng/mL ($p < 0.05$).

Overall, the results shown in Table 2 showed that sperm motility in the group induced by the chloroform and methanol fractions of *L. sarmentosa* extract was better than the control group based on CASA.

Table 1. Phytochemical screening of methanol and chloroform fractions from *L. sarmentosa* extract

Parameters	Methods	Contents of Fractions from <i>L. sarmentosa</i> extract	
		Methanol	Chloroform
Flavonoids (mg/mL QE)	Spectrophotometry	76.125 ± 0.177	34.250±0.081
Steroids (mg/mL)	Spectrophotometry	13.301±0.028	6.035±0.028
Terpenoids (mg/mL)	Spectrophotometry	94.300 ± 0.707	64.300±0.707
Tannins (mg/mL)	Spectrophotometry	0.421 ± 0.019	0.609±0.008
Saponins (%)	Gravimetric	14.650 ± 0.071	18.910 ± 0.013
Alkaloids (%)	Gravimetric	24.600±0.424	23.300 ± 0.283

Figure 1. The average of sperm motility for methanol and chloroform in the *L. sarmentosa* extractTable 2. The average of sperm motility in the control and treatment groups incubated with methanol and chloroform fractions of *L. sarmentosa* root extract

<i>Lavanga sarmentosa</i> Root Extract Fractions	Concentration	Number of samples (N)	Sperm Motility	
			Mean ± DS	SEM
Methanol Fractions	a. Control	15	44.832 ± 8.124 ^a	2.097
	b. 10 ng/mL	15	51.351 ± 8.632 ^b	2.229
	c. 50 ng/mL	15	59.217 ± 9.171 ^b	2.368
	d. 100 ng/mL	15	68.445 ± 8.661 ^b	2.236
	e. 500 ng/mL	15	75.140 ± 7.410 ^b	1.913
	f. 1000 ng/mL	15	85.064 ± 6.590 ^b	1.701
Chloroform Fractions	a. Control	15	44.832 ± 8.124 ^a	2.097
	b. 10 ng/mL	15	49.189 ± 8.356 ^a	2.157
	c. 50 ng/mL	15	56.355 ± 8.444 ^b	2.18
	d. 100 ng/mL	15	69.995 ± 8.870 ^b	2.29
	e. 500 ng/mL	15	66.972 ± 8.522 ^b	2.20
	f. 1000 ng/mL	15	61.251 ± 9.432 ^b	2.435

Notes: ^b(p<0.05); DS = Deviation Standard; SEM = Standard error of the mean.

DISCUSSION

Based on the phytochemical tests, secondary metabolite compounds in *L. sarmentosa* are

saponins, alkaloids, flavonoids, steroids, and terpenoids. This is supported by a previous study by Anggriani demonstrating that the chloroform

extract method on *L. sarmentosa* identified some content of secondary metabolites, namely steroids, flavonoids, and saponins.⁸ These secondary metabolites have effects in spermatogenesis. In this study, high terpenoids in the methanol and chloroform fractions affected the sperm motility. This is supported by a previous study by Rusdi et al. explaining that terpenoids bound to saponins so that they affect sexual activity by forming hormone testosterone, which functions in spermatogenesis.⁹ In addition, triterpenoids have an effect in overcoming cytotoxicity and inflammation and can repair damaged cells by stimulating collagen more quickly.¹⁰ Other compounds contained in the extract fractions of *L. sarmentosa* are flavonoids, alkaloids and saponins which act as natural antioxidants to protect against oxidants or free radicals that affect sperm motility.^{8,11-13} Flavonoids function is to decrease the number of free radicals by donating hydrogen atoms to bind metal ions and also not to cause oxidative stress.^{11,14} Alkaloids act as primary antioxidants that can protect cells from toxic substances and can prevent genetic damage caused by H₂O₂ oxidants.¹⁵ Saponins can work to reduce superoxide through the formation of hydroperoxide intermediates, so they prevent damages caused by free radicals.^{8,16} Steroids also play a role in the continuity of spermatogenesis in the testes.¹⁷ The steroids contained in the methanol and chloroform fractions of *L. sarmentosa* root still do not have properties of anabolic steroids, or known as anabolic androgenic steroids (AAS), which work by binding to androgen receptors by entering the cell cytoplasm through the membrane in Leydig cells; therefore, with the help of luteinizing hormone (LH) they produce Testosterone, a hormone that plays a role in spermatogenesis.^{10,18}

The average motility of sperm incubated by methanol and chloroform fractions increased compared to the control group. This is because, according to a previous study by Syarpin et al. the major content of secondary metabolites in methanol fraction are compounds of amino acid derivatives and alkaloids, and in chloroform fraction are compounds of terpenoids, alkaloids, and phenyl propanoids. All the compounds act as an antioxidant to protect cells and affect the production of testosterone and spermatogenesis.¹⁹ Nazari et al. pointed out that antioxidant amino acid supplementation containing L-Carnitine

could increase sperm concentration and could improve sperm morphology in patients with oligoacetenoteratozoospermia.²⁰ In this current study, the sperm motility increased significantly in each concentration incubated with methanol fraction, with the most effective dose of 1000 ng/mL. The effect of administering the chloroform fraction of *L. sarmentosa* root extract increased the sperm motility with the most effective dose of 100 ng/mL. Meanwhile, the chloroform fraction decreased at large concentrations of 500 and 1000 ng/mL, but it was still higher than the control group. This is related to the relatively high content of saponins and tannins in the chloroform fraction compared to the methanol fraction. Some plants have a spermicidal effect which contains saponins and tannins. Saponins and tannins are cytotoxic or spermicidal on spermatozoa which affect cell membrane permeability and disrupted nutrient as a source of sperm energy so that spermatozoa will lack energy and decrease sperm motility.^{21,22} Toxicity of saponins also inhibits certain enzymes such as hyaluronidase and acrosine.²² However, because of the high antioxidant content such as terpenoids, flavonoids, and alkaloids, it is possible to suppress some of spermicide effect by incubating chloroform and methanol fractions.

Based on the results of this current study, flavonoids, steroids, terpenoids, and alkaloids in the methanol extract fraction were higher than in the chloroform extract fraction. The results of sperm motility were higher in the methanol fraction of *L. sarmentosa* extract than that in the chloroform fraction due to the high content of flavonoids, terpenoids, and alkaloids as antioxidants that could ward off free radicals to prevent damage to the sperm membrane. The energy used for sperm motility is supplied in the form of ATP by the mitochondria in the sperm tail. Therefore, if the mitochondria are disturbed or damaged, they will make less ATP. Mitochondrial cell membrane damage causes sperm metabolism to decrease. These antioxidant properties work naturally to help ward off free radicals or ROS in the testes so that the mitochondrial cell membrane is not disturbed, so that ATP supplies become abundant and cause increased sperm motility.^{23,24}

CONCLUSION

The methanol and chloroform fractions of *L. sarmentosa* root extract were contained

of terpenoids, flavonoids, steroids, alkaloids, saponins, and tannins. The methanol and chloroform fraction of *L. sarmentosa* root extract could increase sperm motility.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENT

The research was sponsored by LPPM Universitas Palangka Raya, especially Grant of 2022.

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