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Bioactivity Examination of *Uncaria gambir* (W.Hunter) Roxb on In Vitro Human Sperm Motility

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Abstract: Globally, 48 million couples experience infertility, where male infertility factors contribute to 50% of cases. Spermatozoa motility is a crucial parameter in assessing male fertility. Antioxidants act as the body's defence against excessive ROS and can be used as a treatment for male infertility. One of the local plants in Central Kalimantan that is potentially rich in antioxidants is Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb. However, there is limited research on the bioactivity of Bajakah Kalalawit on spermatozoa in vitro. This study aims to determine the effect of ethanol extract of *Uncaria gambir* (W. Hunter) Roxb on spermatozoa motility in vitro. Bajakah Kalalawit stems were extracted using a 3x24-hour maceration technique with 96% ethanol. After obtaining the concentrated extract, the secondary metabolite compound content was analyzed through a phytochemical screening. The sample used was in vitro human spermatozoa that were washed and added to Bigger Whitten Whittingham medium, then incubated at 37°C for 1 hour with a 96% ethanol extract of Bajakah Kalalawit at doses of 50ng/ml, 100ng/ml, 500ng/ml, and 1000ng/ml, as well as a control group (Bigger, Whitten & Whittingham medium only). The results showed that the compounds contained in the ethanol extract of *Uncaria gambir* (W.Hunter) Roxb were terpenoids, flavonoids, phenolics, steroids, saponins, alkaloids, and tannins. Spermatozoa motility significantly increased in the treatment groups starting from doses of 50ng/ml, 100ng/ml, 500ng/ml, and 1000ng/ml compared to the control group. Ethanol extract of *Uncaria gambir* (W.Hunter) Roxb could increase spermatozoa motility in vitro and succeeded in improving reproductive technology.
Keywords: Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb); spermatozoa; motility.

INTRODUCTION

Globally, 48 million couples experience infertility issues, with male infertility contributing to 50% of cases (Boitrelle et al., 2021). According to data from the Indonesian In Vitro Fertilization Association (Perfitri), in 2017, there were 1,712 men in Indonesia experienced infertility (Puspitaningrum & Nugraheni, 2022). There are various causes of male infertility, including environmental factors, lifestyle, ageing, varicocele, maldescended testes, hypogonadism, malignancies, immunological factors, systemic diseases, sperm autoantibodies, and idiopathic infertility (Minhas et al., 2021). Idiopathic infertility accounts for 30% of male infertility cases, with approximately 30-80% experiencing elevated levels of seminal reactive oxygen species (ROS)(Assidi, 2022).

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The oxidative reaction of ROS can induce oxidative damage to DNA by altering the composition of DNA constituents, leading to the emergence of various diseases. This is due to the oxidation of Deoxyguanosine (dG), a component of DNA, into 8-hydroxy-2'-deoxyguanosine (8-OHdG). Elevated levels of 8-OHdG serve as a marker for oxidative damage to spermatozoa DNA, where men experiencing infertility tend to exhibit higher levels of 8-OHdG. Furthermore, lipid peroxidation on the spermatozoa membrane reduces the flexibility of spermatozoa movement. DNA damage in the mitochondria, which decreases ATP production and lipid peroxidation on the spermatozoa membrane, results in impaired spermatozoa motility. Spermatozoa motility is a critical characteristic in assessing male fertility issues (Piomboni et al., 2012; Wagner et al., 2018).

Antioxidants play a pivotal role as the body's defence mechanism against and mitigators of oxidative stress caused by an excess of ROS, and they can serve as a therapeutic approach for male fertility disorders (Assidi, 2022). Endogenous antioxidants detoxifying free radicals also oxidise, resulting in a continuous decrease in their levels. Therefore, relying solely on endogenous antioxidants is insufficient, and external antioxidants, known as exogenous antioxidants, are needed to meet the body's requirements. Exogenous antioxidants can be obtained from nature, primarily from plants. Polyphenolic compounds, specifically flavonoids found in plants, can be employed as exogenous antioxidants for the body (Proklamasiningsih et al., 2019). In a study conducted by Silva et al., it was demonstrated that the flavonoid catechin can preserve the motility of frozen goat sperm (Silva et al., 2019).

Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb) is one of the varieties of bajakah found in Central Kalimantan. Research conducted by Hartanti on *Uncaria gambir* demonstrated potent antioxidant activity in both water and ethanol extracts (Hartanti et al., 2021). Rollando's study on Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb) revealed the presence of compounds such as flavonoids, terpenoids, tannins, phenolics, saponins, glycosides, and alkaloids (Rollando et al., 2022). Research conducted on white male rats administered Bajakah Kalalawit showed increased sexual activity, as measured by approach, climbing, and mating parameters. Additionally, hyperglycemic male rats given catechin isolates from *Uncaria gambir* exhibited elevated testosterone hormone levels and sperm count (Sari et al., 2018). however, research on Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb) ethanol extract on spermatozoa motility still needs to be improved. This research aims to determine the effect of ethanol extract of *Uncaria gambir* (W. Hunter) Roxb on spermatozoa motility in vitro.

MATERIALS AND METHODS

Tools and Materials

The materials of this study were an amount of 5000 g Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb) stem taken from Kelurahan Marang, Kecamatan Bukit Batu, Palangka Raya, Central Borneo, Indonesia in Figure 1 and were authenticated by Direktorat Pengelolaan Koleksi Ilmiah Badan Riset Dan Inovasi Nasional (BRIN) Cibinong (No.B-1155/II.6.2/IR.01.02.5/2023). Other materials were ethanol 96%, aqua dest, human sperm, BWW (Bigger, Whitten & Whittingham) medium (Sigma-Aldrich, St.Louis, USA), and percoll 50% (Sigma-Aldrich, St.Louis, USA). The tools were a centrifuge (Hettich, Germany), analytical balance (Radwag, Poland), rotatory evaporator (Hahn Vapor, Hahn Shin Scientific Co., Korea), water bath (Memmert, Germany), spectrophotometer UV-Vis (Shimadzu, Japan), incubator (Memmert, Germany) and microscope (Olympus, Japan).

Figure 1. Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb), the Stem is Reddish Brown and Realeases Water Within the Stem (Personal Documentation)

Extraction Methods

The Ethics Committee approved this study for Medical Research, Faculty of Medicine, Universitas Palangka Raya (No.38/UN24.9/LL/2023). 5000 grams of *Uncaria gambir* (W.Hunter) Roxb stem was washed and dried for seven days under direct sunlight to make simplicia using a blender and then sifted using a 60-mesh strainer. A 2000 g simplicia was macerated with 96% ethanol for 3 x 24 hours. The obtained macerate was concentrated by using a rotary evaporator. The concentrated extract obtained was 132.5 grams.

Phytochemical Test

To identify contents in *Uncaria gambir* (W.Hunter), Roxb stems quantitative phytochemical analysis. The analysis content is flavonoids, terpenoids, phenolics, saponins, alkaloids, and steroids. Alkaloids and saponins content were determined quantitatively using the gravimetric method. Flavonoids, terpenoids, phenolics and steroid content were determined quantitatively using the spectrophotometric method (Hutasuhut et al., 2022).

Bioactivity of *Uncaria gambir* (W.Hunter) Roxb on In-vitro Human Sperm Motility

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 and spermatozoa with a concentration of $\geq 50 \times 10^6$. The number of samples was calculated based on the one-proportion estimation formula from Stanley Lemeshow et al. with a consecutive sampling technique rounded up to 15 people (Quyen et al., 2020). 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 BWW. Then, the tube was centrifuged again at 3000 rpm for 25 minutes. The supernatant was discarded, and the pure spermatozoa precipitate pellet was resuspended with 1 ml of BWW 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 concentration calculation 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 containing ±10 million cells in 500 mL BWW. The samples were divided into five groups: negative control (without treatment), administration of the *Uncaria gambir* (W.Hunter) Roxb ethanol 96% extract with final concentrations of 50, 100, 500, and 1000 ng/mL. The samples are mixed homogeneously in an Eppendorf tube before incubation. The five groups were then incubated at 37℃ for 1 hour. Take ten µL of the sample and place it into the slide, and human sperm motility was examined by microscope with a magnification of 400 x. Percentage of sperm is: (Permatasari, et al., 2023; World Health Organization, 2021)

$$\%\text{Motility} = \frac{A+B}{200} \times 100\%$$

The assessment for calculating motility percentage is divided into:
A = Progressive Motility (straight and fast forward movement)
B = Non-progressive Motility (slow movement)
200 = 200 total sperm counted

According to the WHO (World Health Organization), normal sperm motility is defined as ≥ 50%, and if it is < 50%, it is referred to as asthenozoospermia (World Health Organization, 2021). Statistical analysis of the sample used the One-Way ANOVA test by SPSS, followed by the LSD method.

RESULTS AND DISCUSSION

The results of quantitative phytochemical tests on Bajakah Kalalawit stems are shown in Table 1. Quantitative phytochemical tests on the 96% ethanol extract of *Uncaria gambir* (W.Hunter) Roxb compound of terpenoids, flavonoids, phenolics, steroids, saponins, alkaloids, and tannins.

Table 1**.** The Results of Quantitative Phytochemical Tests on Bajakah Kalalawit

No	Parameter	Compound levels (Mean ± SD)
1	Terpenoid (mg/ml)	392,800 ± 1,141
2	Flavonoid (mg/ml QE)	219,125 ± 0,530
3	Phenolic (mg/ml)	68,267 ± 0,519
4	Steroid (mg/ml)	49,238 ± 0,138
5	Saponin (%)	40,090 ± 0,665
6	Alkaloid (%)	29,575 ± 0,007
7	Tannin (mg/ml)	0,473 ± 0,008

The mean percentage of spermatozoa motility was determined by observing 200 spermatozoa, as presented in Table 1. Based on the data, the effective dose begins at the smallest dosage, 50 ng/ml. Overall, the results shown in Table 1 showed that sperm motility in the group induced by a 96% ethanol extract of *Uncaria gambir* (W.Hunter) Roxb stem was better than the control group.

Table 2**.** Average Results of Human Sperm Motility in the Control and Treatment Groups Incubated in Ethanol 96% Extract of *Uncaria gambir* (W.Hunter) Roxb with a Concentration of 50-1000 ng/mL

Concentration of *Uncaria gambir* (W.Hunter) Roxb Extract	Number of samples (N)	Sperm Motility Mean ± DS
a. Control	15	54,67 ± 5,43 [a]
b. 50 ng/mL	15	70,20 ± 3,08 [b]
c. 100 ng/mL	15	77,20 ± 4,16 [b]
d. 500 ng/mL	15	86,40 ± 2,55 [b]
e. 1000 ng/mL	15	92,90 ± 2,87 [b]

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

Based on the observed spermatozoa motility results, the administration of ethanol extract from Kalalawit bajakah (*Uncaria gambir* (W.Hunter) Roxb) demonstrated an increase in the motility of spermatozoa with straight and fast forward movement, as well as slow movement, in accordance with the criteria for spermatozoa motility calculation by the WHO, following incubation with various concentrations of Kalalawit bajakah extract (50, 100, 500, and 1000 ng/ml) compared to the control group (BWW medium) based on Table 2. The study conducted by Charit et al. reported that the percentage of spermatozoa motility that resulted in the highest success rate for intrauterine insemination was 78.69% (Charit & Sirait, 2020). In this study, the average percentage of spermatozoa motility incubated with Bajakah Kalalawit extract at a concentration of 500 ng/ml was 86.4%, and at a concentration of 1000 ng/ml, it was 92.9%. The addition of Bajakah Kalalawit extract allows for an improvement in in vitro fertilization (IVF) success. This effect is possibly caused by the presence of compounds such as flavonoids, terpenoids, phenolics, and steroids, which act as antioxidants capable of safeguarding spermatozoa from free radicals or ROS, thereby preventing damage to the spermatozoa membrane and enhancing spermatozoa motility (Permatasari et al., 2023).

Terpenoids exhibit antioxidant activity by scavenging reactive species such as superoxide and chelating metals (Fe2+ dan Cu2+) (Furi et al., 2020). Research conducted by Wijayanti et al. demonstrated that terpenoid compounds from the n-hexane fraction of parijoto acted as antioxidants, enhancing spermatozoa motility and protecting the spermatozoa membrane (Wijayanti et al., 2020).

Catechin and quercetin are antioxidant compounds belonging to the flavonoid group found in Bajakah Kalalawit (*Uncaria gambir* (W.Hunter) Roxb) (Hasanah, 2019). Research conducted by Cimini et al. demonstrated that incubating swine spermatozoa with catechin can enhance motility and the fertilization capability of spermatozoa in vitro (Cimini et al., 2023). Other studies have also shown increased human spermatozoa motility when incubated with quercetin at the appropriate dosage and incubation time (Karabulut et al., 2020). Both of these flavonoid compounds also function as phytoestrogens. Phytoestrogens can bind to estrogen receptors (ER), including ERα and ERβ, found on the surface of both capacitated and non-capacitated spermatozoa, with lower affinity than estrogen. The binding of phytoestrogens to ER can stimulate spermatozoa functions such as motility, acrosomal reaction, and capacitation (Skibińska et al., 2022).

Phenolic compounds, as antioxidants, possess hydroxyl groups bound to aromatic rings, making them susceptible to oxidation as they donate hydrogen atoms to free radicals (Dhurhania & Novianto, 2019). Research conducted by Kedechi et al. revealed that the sperm-washing process can reduce the viability and motility of spermatozoa. However, incubating spermatozoa with phenolic compounds can enhance sperm quality, reduce DNA oxidation, and improve motility compared to incubation without phenolic compounds. Phenolic compounds can protect spermatozoa from damage caused by ROS during the preparation process in assisted reproductive technology (ART) by acting as antioxidants that eliminate ROS and disrupt the free radical chain reaction (Kedechi et al., 2017). Gholami-Ahangaran et al. stated that ginger (*Zingiber officinale*), rich in phenolic compounds, exhibits vigorous antioxidant activity. Consequently, it can enhance sperm quality, particularly in motility, viability, morphology, and DNA integrity, by preventing free radicals and reducing oxidative stress on spermatozoa (Gholami-Ahangaran et al., 2021).

Steroid compounds play an antioxidant role by interrupting the chain reaction, thus reducing the formation of new free radicals and converting them into more stable products (Kartika et al., 2020). Furthermore, this is supported by research conducted by Wehrli et al., which demonstrated increased spermatozoa motility when incubated with a medium containing steroids compared to the control with BWW medium. Sperm-specific channels, known as CatSper (cation channel of sperm), located in the flagellar membrane, control intracellular Ca2+ levels and play a crucial role in spermatozoa function. Both endogenous and exogenous steroids can elicit Ca2+ influx and motility responses in human sperm (Wehrli et al., 2023). Steroids can stimulate an increase in intracellular Ca2+, leading to spermatozoa motility, hyperactivation, and acrosomal reactions. Steroids play a role in stimulating spermatozoa-related capacitation changes, such as Ca2+ influx that activates adenylyl cyclase (sAC). sAC converts ADP to cAMP, thereby stimulating protein kinase A (PKA). PKA then promotes increased global tyrosine phosphorylation in sperm proteins. This increase in tyrosine phosphorylation leads to capacitation and hyperactivation of spermatozoa motility (Pujianto et al., 2019). The presence of non-genomic progesterone receptors on human spermatozoa, along with steroid compounds, can activate sperm membrane receptors, leading to Ca2+ influx (Jeschke et al., 2021).

Saponin compounds exhibit antioxidant activity by quenching superoxide radicals through the formation of hyperoxide intermediates, thereby preventing biomolecular damage caused by free radicals. Tannin compounds are capable of donating hydrogen atoms from their OH groups to free radicals, converting the DPPH radical into DPPH-H (non-radical) (Hasan et al., 2022). Research conducted by Derbak et al. demonstrated that the alkaloid compounds in *Peganum harmala* extract increase spermatozoa motility, maintain membrane structure integrity, and preserve low lipid peroxidation in ram spermatozoa (Derbak et al., 2021).

Like most other studies, this research also has limitations as we had difficulty finding ejaculated semen with a sperm concentration that met the criteria. Another limitation is that it is not yet known whether incubation of spermatozoa with ethanol extract of *Uncaria gambir* (W.Hunter) Roxb), which increases spermatozoa motility in vitro, can also increase the success of sperm penetration into the ootid. This would be an interesting parameter to investigate.

CONCLUSION

The motility of human spermatozoa increases when incubated with 96% ethanol extract of *Uncaria gambir* (W.Hunter) Roxb compared to the BWW medium. Spermatozoa motility significantly increased in the treatment groups starting from doses of 50ng/ml, 100ng/ml, 500ng/ml, and 1000ng/ml compared to the control group. Ethanol extract of *Uncaria gambir* (W.Hunter) Roxb could increase spermatozoa motility in vitro and succeeded in improving reproductive technology.

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CONFLICT OF INTEREST

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

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