Pharmaciana
Vol.x, No.x, Bulan 201x, Hal. xx-xx
ISSN: 2088 4559; e-ISSN: 2477 0256
DOI: 10.12928/pharmaciana.xxxx.xxxx

Evaluation of Sperm Quality in Male Rats Treated with *Sauropus androgynus* (L.) Merr. Leaf Fractions

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Submitted :..... Reviewed :..... Accepted:.....

ABSTRACT

Sauropus androgynus (L.) Merr. or katuk is one of the medicinal plants broadly used in Indonesia as it has active compounds that can, among others, stimulate reproductive hormones. This study was aimed to determine which active fraction of *S. androgynus* leaves that has the potential to improve the sperm quality of male rats based on three parameters, namely sperm count, viability, and motility. It employed fractionation using the liquid-liquid technique with *n*-hexane, ethyl acetate, and water solvents to obtain the fractions. A total of twenty mature male Sprague-Dawley rats were divided into four equal groups: the normal group (untreated group) received 0.5% Na-CMC suspense, and the three fraction groups were given the *n*-hexane, ethyl acetate, and water fraction p.o., respectively, at the dose of 11.85 $mg.kg^{-1}$ BW daily for seven days. Sperm count, viability, and motility were measured on Day 8 (after treatment) from the sperm samples collected at the cauda epididymis of the sacrificed test rats. The results showed that compared with the normal group, the *n*-hexane and ethyl acetate fractions significantly increased the three parameters ($p<0.05$). Therefore, the *S. androgynus* leaf fractions have the potential as a natural material that can increase the fertility of male rats.

Keywords: fertility, fractions, katuk, *Sauropus androgynus*, sperm

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INTRODUCTION

Infertility is defined as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (World Health Organisation, 2016). Male factor infertility is responsible for not less than 50% of the cases. In essence, identified contributors to sperm dysfunction and male factor infertility are mainly environmental, physiological, and genetic (World Health Organisation, 1992). Semen analysis remains the first step in the diagnosis of male infertility, which is generally seen as an alteration in sperm concentration, motility, morphology, or the combination thereof.

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Increased ROS and oxidative levels (oxidative stress) are among the proposed mechanisms leading to impaired spermatogenesis. Lacks of vitamin A, elements such as flavonoids, folate, and the overall reduction of antioxidants in the diet can be the reasons for infertility, especially oligospermia and asthenozoospermia in human (Ouladsahebmadarek *et al*., 2016). Using herbal medicine to increase fertility and improve its factors, such as hormonal imbalance, oligospermia, and low motility of sperm, has long been considered as a practical solution. Indonesia is a country rich in plants that can be made as traditional medicine, one of which is *Sauropus androgynous* (L.) Merr. or known as katuk. The ethanol extract of S. androgynous leaves contains steroids, tannins, alkaloids, flavonoids, and terpenoids (Rusdi *et al*., 2018). *S. androgynous* leaf extract can increase viability, motility, and concentration of spermatozoa in male mice exposed to cigarette smoke when administered at the dose of 6mg/20g BW (Khoironi, 2015). Similarly, at a dose of 11.85 $mg.Kg^{-1}$ BW, the *n*-hexane fraction can increase the sexual desire of male rats toward female rats, as observed from the number of mounts and precopulatory investigations, and the weight of testicular and vesicular of male rats (Rusdi *et al*., 2018).

Thus, an active fraction of 70% ethanol extract of *S. androgynous* leaves was analyzed for its effectiveness as medicinal ingredients in increasing sperm count, viability, and motility in male rats.

MATERIALS AND METHOD

Materials

Plant sample and authentication

The fresh *Sauropus androgynous* leaves were obtained from the Indonesian Medicinal and Aromatic Crops Research Institute (BALITTRO), Bogor, and were authenticated at the Botanical Research and Development Centre "Herbarium Bogoriense" LIPI, Bogor.

Experimental animals

Twenty-four sexually matured male Sprague Dawley rats aged 2-3 months and weighing around 150-250 g were obtained from Research Animal Breeder, Bekasi. These test rats were kept in a well-ventilated house, with a 12-12 photoperiod (12-hr light, 12-hr dark) and given pelleted rat feed and ad libitum access to water.

Methods

Preparation of Fractions

The *S. androgynous* leaves were dried at room temperature and then pulverized. Following this was the extraction of 1.43 kg of the resultant dried powder by maceration using 70% ethanol for 24 hours. The filtrate was separated from the pulp by filtration. While the pulp was extracted again with 70% ethanol using the same procedure up to three times, the filtrate was evaporated using a vacuum rotary evaporator (EYELA) until thick extract was obtained. A known quantity (306.52 g) of the thick ethanol extract was fractionated with water and *n*-hexane solvents in a separating funnel and left to form two phases. Afterward, the two phases were separated. This procedure was also applied to

fractionation with ethyl acetate. Finally, the *n*-hexane, ethyl acetate, and water fractions were evaporated to produce fractions in their thick forms (Suprayogi *et al*., 2015).

Phytochemical Screening of Active Fractions using TLC method

Phytochemical screening was carried out by the TLC method to examine the presence of steroids and flavonoids in the active fraction that, in this procedure, was dissolved in ethanol. The TLC method used silica gel GF254 (Merck) as the stationary phase, while two different mobile phases were used to each detect the presence of steroid and flavonoid compounds. The mobile phase used to identify the presence of steroid compounds was chloroform:methanol (10:1), with vanillin-sulfuric acid as the detection reagent. Meanwhile, the mobile phase used to detect flavonoid compounds was *n*-hexane:ethyl acetate (1:1), with ammonia vapor as the detection reagent (Hanani, 2015).

Test Animal Preparation

The test animals were divided into four groups, each consisting of six rats. Before receiving the treatment, they were acclimatized for seven days, fed with pelleted rat feed, and allowed access to water ad libitum. Protocol no. 17-05-0438 was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia no. 631/UN2.F1/ETIK/2017.

Test Animal Treatment

All four groups received different treatments for seven days: Group I (normal control group) was given Na-CMC 0.5% each day, while Groups II, III, and IV were given the *n*-hexane, ethyl acetate, and water fractions, respectively at the dose of 11.85 $mg.Kg^{-1}$ BW per day. On Day 8, they were injected with ketamine at the dose of 33.35 $mg.Kg^{-1}$ BW i.m. and sacrificed by cervical dislocation. The epididymis was carefully separated from the testis, and the caudal epididymis was lacerated with scissors and collected for sperm count, motility, and viability analyses.

Sperm Count Analysis

The sperm count was evaluated by removing the caudal epididymis from the left and right testes. Then, it was cut into pieces, mixed with Ringer's solution, and left for 2 minutes. Ten µL of the semen suspension was collected and inserted into the hemocytometer, then immediately examined under the Olympus Microscope at ×40 magnification (Quadri & Yakubu, 2017).

Sperm Motility Test

Ten µL of the semen suspension in Ringer's solution was collected and inserted into the hemocytometer, then any moving and immovable spermatozoa were immediately examined under the Olympus Microscope at ×40 magnification. The percentage of motile sperm cells was determined by the progressive and nonprogressive movement of the sperm cells under the microscope. It was defined as the number of motile sperm cells divided by the total number of counted sperm cells (Quadri & Yakubu, 2017).

Sperm Viability Test

Ten µL of the semen suspension in Ringer's solution was collected, dropped into the object's glass, then added with one drop of 2% eosin. After homogenized, it was covered with a cover glass. The stained slide was immediately examined under the Olympus Microscope at ×40 magnification. The live sperm cells were unstained (transparent), while the dead ones absorbed the stain (red color). Sperm viability is expressed in percent (Quadri & Yakubu, 2017).

Data Analysis

The data were statistically analyzed using one-way ANOVA, followed by a Tukey's test to find significantly different means. A statistical significance was identified from $p<0.05$.

Results and Discussion

The extraction yield of powdered dried *Sauropus androgynous* leaves by maceration with 70% ethanol was 21.99% (w/w). The higher the concentration of ….., the more the bioactive flavonoid compounds detected with 70% ethanol, mainly due to its higher polarity than pure ethanol. Adding water to the pure ethanol up to 30% in the preparation of 70% ethanol increases the polarity of the solvent. Ethanol is the solvent used for extracting active compounds such as tannins, polyphenol, flavonoids, terpenoids, steroids, and alkaloids (Tiwari et al., 2011).

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The yields of the *n*-hexane, ethyl acetate, and water fractions of the resultant ethanol extract using the liquid-liquid extraction method were 1.74%, 5.29 %, and 52.94% (w/w), respectively. The *n*-hexane can dissolve non-polar compounds such as fixed oils, volatile oils, steroids, and several flavonoids aglycon. Semi-polar solvents such as ethyl acetate can dissolve alkaloids and the other aglycon like flavonoids (Houghton & Raman, 1998). Water as an extraction solvent can extract polar metabolite materials such as quaternary alkaloids, phenolic, carotenoids, tannins, glycoside flavonoids, sugar, amino acids, and the other glycosides (Hanani, 2015).

The sperm count, motility, and viability in male rats receiving *n*-hexane and ethyl acetate fractions (Groups II and III) at the dose of 11.85 mg.Kg^{-1} BW per day for seven days increased more significantly ($P<0.05$) compared with the normal control group (Group I) (**Figures 1-3**). The presence of steroids in the *n*-hexane fraction and flavonoids in the ethyl acetate fraction was identified using the TLC method (**Figure 4**).

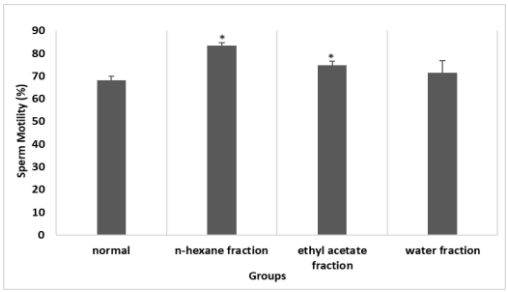

Figure 1. Effects of *Sauropus androgynous* leaf fractions on sperm motility in male rats (Note: * = a significant difference with the normal group (α =0.05))

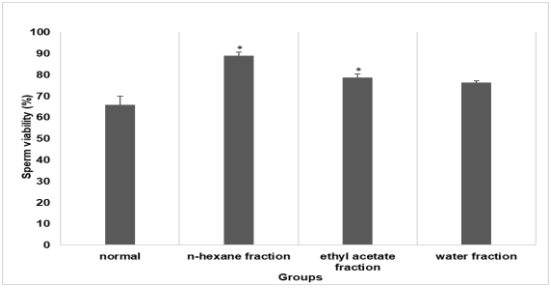

Figure 2. Effects of *Sauropus androgynous* leaf fractions on sperm viability in male rats (Note: * = a significant difference with the normal group (α =0.05))

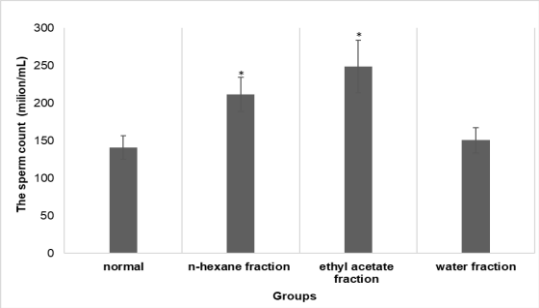

Figure 3. Effects of *Sauropus androgynous* leaf fractions on sperm count in male rats (Note: * = a significant difference with the normal group (α =0.05))

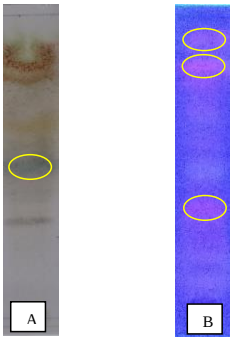

Figure 4. The presence of steroid compounds in the *n*-hexane fraction (A) and flavonoid compounds in the ethyl acetate fraction (B) of *Sauropus androgynous* leaves, identified using the TLC method.

DNA fragmentation is now considered an important factor in the aetiology of male infertility. DNA damage may be present in men with both abnormal and normal semen parameters and routine semen parameters are not robustly predictive of infertility or outcome of assisted reproduction treatment. The most common cause of DNA fragmentation in spermatozoa is reactive oxygen species (ROS) and oxidative stress. In spermatozoa, ROS are required for a number of specific and essential functions, which explains why they produce ROS themselves. Oxidative stress occurs when the concentration of ROS becomes too high and/or antioxidant defenses become overwhelmed. Highly oxidative ROS causes damage to cell components, particularly lipids, proteins, and DNA. Two major effects of oxidative stress impacting fertility are lipid peroxidation and DNA damage (Wright *et al*., 2014).

Generally, antioxidants are compounds that characteristically dispose of, scavenge, and halt the production of ROS or neutralize their actions (Adewoyin *et al*., 2017). Primary antioxidants are mainly phenolics in structure and include the following: antioxidant minerals, antioxidant vitamins, and

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See:
Introduction paragraf 1 dan 2
Functions of ROS in spermatozoa p.1
Oxidative stress causes DNA damage in spermatozoa p.1

phytochemicals, such as flavonoids, catechins, carotenoids, *β*-carotene, and lycopene (Moharram & Youssef, 2014), all of which have been recognized as having the potential to reduce risks of diseases as they can scavenge free radicals. Flavonoids, mainly *p*-cymene and carvacrol, in the essential oil of *Satureja khuzestanica* have positive effects on …. due to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Also, carvacrol represents significant antioxidant properties (Safarnavadeh & Rastegarpanah, 2011).

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Several phytomedicines, including phenolic compounds (phenols, sterols, lignans, and flavonoids), volatile oils, polyphenols, and saponins, have positive effects on spermatogenesis and sperm parameters (sperm motility, count, and viability) (Khojasteh *et al*., 2016). Some food supplements have been shown to increase sperm count and motility (Adewoyin *et al*., 2017). Also, prior scholars have reported that the fractions of *Sauropus androgynous* leaves at the dose of 11.85 $mg.Kg^{-1}$ BW can increase the weight of testicular and seminal vesicle (Rusdi *et al*., 2018). In this study, *n*-hexane and ethyl acetate fractions induced improvements in semen quality and quantity. This activity probably related to the presence of flavonoids and steroids as active compounds in both fractions (see **Figure 4**). Flavonoids can increase the levels of dehydroepiandrosterone (DHEA), which plays a role in increasing testosterone levels. Saponins, in the form of steroid glycosides, contribute to the DHEA biosynthesis, hence, increasing testosterone levels in the body (Andini, 2014). Multiplied sperm number in the treatment groups is due to increased production of testosterone and probably of follicle-stimulating hormone (FSH) (Al-Sa'aidi *et al*., 2009). Testosterone and FSH are reported to be responsible for spermatogenesis (McLachlan, 2004). Further studies should focus on the cellular and intracellular signaling pathways to understand the mechanism by which fractions improve spermatogenesis.

CONCLUSION

The *n*-hexane and ethyl acetate fractions of *Sauropus androgynous* leaves have the potential to be developed into natural male fertility-enhancing agents.

ACKNOWLEDGEMENT

We would like to thank the Research and Development Institute (Lembaga Penelitian dan Pengembangan) of Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta, for financially assisting this research under the Funding Grant Scheme of Science and Technology Development Research Batch II 2018.

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Pharmaciana: Jurnal Kefarmasian
ISSN 2088-4559 and e-ISSN 2477-0256
Accredited by ministry of Highereducation No: 32a/E/KPT/2017
Sekretariat : Fakultas Farmasi Universitas Ahmad Dahlan
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Posisi (judul/abstrak abstrak/pendahuluan dst)	Halaman, Baris	Tertulis	Perbaikan
Abstract	1,10	measured on Day 8 …	measured on day 8 …
Abstract	1,5	liquid-liquid technique	liquid-liquid extraction
Introduction	2,2	(World Health Organisation, 2016).	(World Health Organisation, 2020).
Introduction	2,5	(World Health Organisation, 1992).	(World Health Organisation, 2010)
Introduction	2,10	… and folate.	and folate
Introduction	2,14	*Sauropus androgynous*	*Sauropus androgynus*

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Introduction	2,14	… S. androgynous	… *S. androgynus*
Introduction	2,16	*androgynus….*	*androgynus…*
Introduction	2,21	*… S. androgynus…*	*… S. androgynus..*
Materials: Plant sample and authentication	2,1	*S. androgynus*	*S. androgynus*
Methods: Preparation of fractions	2,1	*S. androgynus*	*S. androgynus*
Methods: Test animal treatment	3,3	On Day 8…	On day 8…
Results and Discussion	4,1	*S. androgynus*	*S. androgynus*
Figure 1	4,1	*S. androgynus*	*S. androgynus*
Figure 2	4,1	*S. androgynus*	*S. androgynus*
Figure 3	5,1	*S. androgynus*	*S. androgynus*

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Figure 4	5,2	*S. androgynous*	*S. androgynus*
Results and Discussion	5,2	oxidant stress	oxidative stress
Results and Discussion	6,5	*S. androgynous*	*S. androgynus*
Conclusion	6,1	*S. androgynous*	*S. androgynus*
References	7,1	Organisation, W. H. (2016). Sexual and reroductive Health: multiple definitions of fertility	Dihapus, ganti dengan: World Health Organisation. (2020). Sexual and Reroductive Health: Multiple Definitions of Fertility. Retrieved from https://www.who.int/reproductivehealth/topics/infertility/multiple-definitions/en/

Jakarta, 8 Agustus 2020
Penulis

(Numlil Khaira Rusdi)

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WORD COUNT 2691
TIME SUBMITTED 06-AUG-2020 03:01AM
PAPER ID 61915758

Pharmaciana
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Phytochemical Screening of Active Fractions using TLC method

Phytochemical screening was carried out by the TLC method to examine the presence of steroids and flavonoids in the active fraction that, in this procedure, was dissolved in ethanol. The TLC method used silica gel GF254 (Merck) as the stationary phase, while two different mobile phases were used to each detect the presence of steroid and flavonoid compounds. The mobile phase used to identify the presence of steroid compounds was chloroform:methanol (10:1), with vanillin-sulfuric acid as the detection reagent. Meanwhile, the mobile phase used to detect flavonoid compounds was *n*-hexane:ethyl acetate (1:1), with ammonia vapor as the detection reagent (Hanani, 2015).

Test Animal Preparation

The test animals were divided into four groups, each consisting of six rats. Before receiving the treatment, they were acclimatized for seven days, fed with pelleted rat feed, and allowed access to water ad libitum. Protocol no. 17-05-0438 was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia no. 631/UN2.F1/ETIK/2017.

Test Animal Treatment

All four groups received different treatments for seven days: Group I (normal control group) was given Na-CMC 0.5% each day, while Groups II, III, and IV were given the *n*-hexane, ethyl acetate, and water fractions, respectively at the dose of 11.85 mg.Kg^{-1} BW per day. On Day 8, they were injected with ketamine at the dose of 33.35 mg.Kg^{-1} BW i.m. and sacrificed by cervical dislocation. The epididymis was carefully separated from the testis, and the caudal epididymis was lacerated with scissors and collected for sperm count, motility, and viability analyses.

Sperm Count Analysis

The sperm count was evaluated by removing the caudal epididymis from the left and right testes. Then, it was cut into pieces, mixed with Ringer's solution, and left for 2 minutes. Ten µL of the semen suspension was collected and inserted into the hemocytometer, then immediately examined under the Olympus Microscope at ×40 magnification (Quadri & Yakubu, 2017).

Sperm Motility Test

Ten µL of the semen suspension in Ringer's solution was collected and inserted into the hemocytometer, then any moving and immovable spermatozoa were immediately examined under the Olympus Microscope at ×40 magnification. The percentage of motile sperm cells was determined by the progressive and nonprogressive movement of the sperm cells under the microscope. It was defined as the number of motile sperm cells divided by the total number of counted sperm cells (Quadri & Yakubu, 2017).

Sperm Viability Test

Ten µL of the semen suspension in Ringer's solution was collected, dropped into the object's glass, then added with one drop of 2% eosin. After homogenized, it was covered with a cover glass. The stained slide was immediately examined under the Olympus Microscope at ×40 magnification. The live sperm cells were unstained (transparent), while the dead ones absorbed the stain (red color). Sperm viability is expressed in percent (Quadri & Yakubu, 2017).

Data Analysis

The data were statistically analyzed using one-way ANOVA, followed by a Tukey's test to find significantly different means. A statistical significance was identified from $p<0.05$.

Results and Discussion

The extraction yield of powdered dried *Sauropus androgynous* leaves by maceration with 70% ethanol was 21.99% (w/w). The higher the concentration of ethanol as extraction solvent, the more the bioactive flavonoid compounds detected with 70% ethanol, mainly due to its higher polarity than pure ethanol. Adding water to the pure ethanol up to 30% in the preparation of 70% ethanol increases the polarity of the solvent. Ethanol is the solvent used for extracting active compounds such as tannins, polyphenol, flavonoids, terpenoids, steroids, and alkaloids (Tiwari et al., 2011).

The yields of the *n*-hexane, ethyl acetate, and water fractions of the resultant ethanol extract using the liquid-liquid extraction method were 1.74%, 5.29 %, and 52.94% (w/w), respectively. The *n*-hexane can dissolve non-polar compounds such as fixed oils, volatile oils, steroids, and several flavonoids aglycon. Semi-polar solvents such as ethyl acetate can dissolve alkaloids and the other aglycon like flavonoids (Houghton & Raman, 1998). Water as an extraction solvent can extract polar metabolite materials such as quaternary alkaloids, phenolic, carotenoids, tannins, glycoside flavonoids, sugar, amino acids, and the other glycosides (Hanani, 2015).

The sperm count, motility, and viability in male rats receiving *n*-hexane and ethyl acetate fractions (Groups II and III) at the dose of 11.85 mg.Kg^{-1} BW per day for seven days increased more significantly ($P<0.05$) compared with the normal control group (Group I) (**Figures 1-3**). The presence of steroids in the *n*-hexane fraction and flavonoids in the ethyl acetate fraction was identified using the TLC method (**Figure 4**).

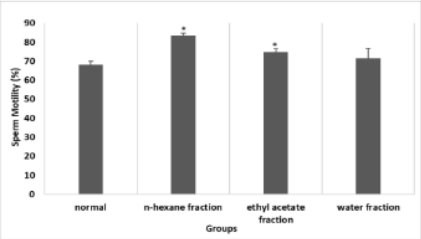

Figure 1. Effects of *Sauropus androgynous* leaf fractions on sperm motility in male rats (Note: * = a significant difference with the normal group (α =0.05))

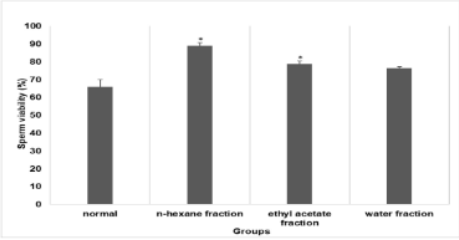

Figure 2. Effects of *Sauropus androgynous* leaf fractions on sperm viability in male rats (Note: * = a significant difference with the normal group (α =0.05))

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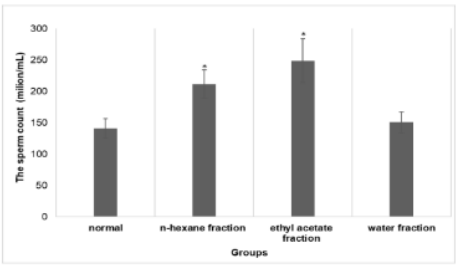

Figure 3. Effects of *Sauropus androgynous* leaf fractions on sperm count in male rats (Note: * = a significant difference with the normal group (α =0.05))

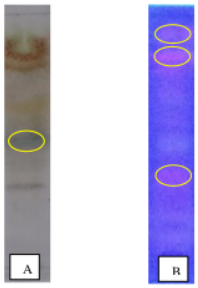

Figure 4. The presence of steroid compounds in the *n*-hexane fraction (A) and flavonoid compounds in the ethyl acetate fraction (B) of *Sauropus androgynous* leaves, identified using the TLC method.

Fragmentation of DNA is now recognized as an important factor in male infertility aetiology. DNA damage occurs in men with both abnormal and normal semen parameters, and parameters of routine semen are not vigorously predictive of infertility or even the outcome of assisted reproduction. Reactive oxygen species (ROS) and oxidant stress are the most common cause of DNA fragmentation in spermatozoa. ROS are required in spermatozoa for a number of specific and essential functions, which explains why they themselves produce ROS. Oxidative stress occurs when ROS concentrations get too high and/or antioxidant defenses get overwhelmed. Highly oxidative ROS causes damage to components of the cells, especially lipids, proteins, and DNA. The lipid peroxidation and DNA damage are two major effects of oxidative stress affecting fertility (Wright *et al*., 2014).

Generally, antioxidants are compounds that characteristically dispose of, scavenge, and halt the production of ROS or neutralize their actions (Adewoyin *et al*., 2017). Primary antioxidants are mainly phenolics in structure and include the following: antioxidant minerals, antioxidant vitamins, and phytochemicals, such as flavonoids, catechins, carotenoids, β-carotene and lycopene (Moharram &

Youssef, 2014), all of which have recognized as having the potential to reduce risks of diseases as they can scavenge free radicals. Flavonoids, mainly *p*-cymene and carvacrol, in the essential oil of *Satureja khuzestanica* have positive effects on antioxidant due to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Also, carvacrol represents significant antioxidant properties (Safamavadeh & Rastegarpanah, 2011).

Several phytomedicines, including phenolic compounds (phenols, sterols, lignans, and flavonoids), volatile oils, polyphenols, and saponins, have positive effects on spermatogenesis and sperm parameters (sperm motility, count, and viability) (Khojasteh *et al*., 2016). Some food supplements have been shown to increase sperm count and motility (Adewoyin *et al*., 2017). Also, prior scholars have reported that the fractions of *Sauropus androgynous* leaves at the dose of 11.85 mg.Kg^{-1} BW can increase the weight of testicular and seminal vesicle (Rusdi *et al*., 2018). In this study, *n*-hexane and ethyl acetate fractions induced improvements in semen quality and quantity. This activity probably related to the presence of flavonoids and steroids as active compounds in both fractions (see **Figure 4**). Flavonoids can increase the levels of dehydroepiandrosterone (DHEA), which plays a role in increasing testosterone levels. Saponins, in the form of steroid glycosides, contribute to the DHEA biosynthesis, hence, increasing testosterone levels in the body (Andini, 2014). Multiplied sperm number in the treatment groups is due to increased production of testosterone and probably of follicle-stimulating hormone (FSH) (Al-Sa'aidi *et al*., 2009). Testosterone and FSH are reported to be responsible for spermatogenesis (McLachlan, 2004). Further studies should focus on the cellular and intracellular signaling pathways to understand the mechanism by which fractions improve spermatogenesis.

CONCLUSION

The *n*-hexane and ethyl acetate fractions of *Sauropus androgynous* leaves have the potential to be developed into natural male fertility-enhancing agents.

ACKNOWLEDGEMENT

We would like to thank the Research and Development Institute (Lembaga Penelitian dan Pengembangan) of Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta, for financially assisting this research under the Funding Grant Scheme of Science and Technology Development Research Batch II 2018.

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By Ni Putu Ermi Hikmawanti

WORD COUNT 2608 TIME SUBMITTED 06-AUG-2020 10:58PM
PAPER ID 61947103

Pharmaciana
Vol.x, No.x, Bulan 201x, Hal. xx-xx
ISSN: 2088 4559; e-ISSN: 2477 0256
DOI: 10.12928/pharmaciana.xxxx.xxxx

Evaluation of Sperm Quality in Male Rats Treated with *Sauropus androgynus* (L.) Merr. Leaf Fractions

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Submitted :..... Reviewed :..... Accepted:.....

ABSTRACT

Sauropus androgynus (L.) Merr. or katuk is one of the medicinal plants broadly used in Indonesia as it has active compounds that can, among others, stimulate reproductive hormones. This study was aimed to determine which active fraction of *S. androgynus* leaves that have the potential to improve the sperm quality of male rats based on three parameters, namely sperm count, viability, and motility. It employed fractionation using the liquid-liquid technique with *n*-hexane, ethyl acetate, and water solvents to obtain the fractions. Twenty-four mature Sprague-Dawley male rats were divided into four equal groups: the normal group (untreated group) received 0.5% Na-CMC suspense, and the three fraction groups were given the *n*-hexane, ethyl acetate, and water fraction p.o., respectively, at the dose of 11.85 mg.Kg^{-1} BW daily for seven days. Sperm count, viability, and motility were measured on Day 8 (after treatment) from the sperm samples collected at the cauda epididymis of the sacrificed test rats. The results showed that compared with the normal group, the *n*-hexane and ethyl acetate fractions significantly increased the three parameters ($p<0.05$). Therefore, the *S. androgynus* leaf fractions have the potential as a natural material that can increase the fertility of male rats.

Keywords: fertility, fractions, katuk, *Sauropus androgynus,* sperm

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INTRODUCTION

Infertility is defined as "Clinical pregnancy failure after 12 months or more of regular unprotected sexual intercourse" (World Health Organisation, 2016). No less than 50 percent of cases are responsible for male infertility. Essentially, identified contributors to sperm dysfunction and male infertility factor are primarily environmental, physiological, and genetic (World Health Organisation, 1992). Analysis of semen is still the first step in male infertility diagnosis, which is commonly perceived as a change in sperm concentration, motility, morphology, or its combination.

Among the proposed mechanism which lead to impaired spermatogenesis are increased ROS and oxidative levels (oxidative stress). Lack of vitamin A, elements such as flavonoids, folate, and overall dietary reduction of antioxidants may be the cause of infertility, particularly oligospermia and asthenozoospermia in humans (Ouladsahebmadarek *et al*., 2016). It has long been considered a practical solution to use herbal medicine to increase fertility and improve its factors such as hormonal imbalance, oligospermia, and low sperm motility. Indonesia is a country rich in plants that can be made as traditional medicine, one of which is *Sauropus androgynous* (L.) Merr. or they are known as katuk. The ethanol extract of S. androgynous leaves contains steroids, tannins, alkaloids, flavonoids, and terpenoids (Rusdi *et al*., 2018). *S. androgynous* leaf extract can increase viability, motility, and concentration of spermatozoa in male mice exposed to cigarette smoke when administered the dose of 6mg/20g BW (Khoironi, 2015). Rusdi *et al*., (2018) reported that the *n*-hexane fraction at a dose of 11.85 $mg.Kg^{-1}$ BW can increase the sexual desire of male rats toward female rats, as observed from the number of mounts and precopulatory investigations, and the weight of testicular and vesicular of male rats.

Thus, an active fraction of 70% ethanol extract of *S. androgynous* leaves was analysed for its effectiveness as medicinal ingredients in increasing sperm count, viability, and motility in male rats.

MATERIALS AND METHOD

Materials

Plant sample and authentication

The fresh *S. androgynous* leaves were obtained from the Indonesian Medicinal and Aromatic Crops Research Institute (BALITTRO), Bogor. It was authenticated at the Botanical Research and Development Centre "Herbarium Bogoriense" LIPI, Bogor.

Experimental animals

Twenty-four male Sprague Dawley sexually mature rats aged 2-3 months and weighing around 150-250 g were obtained from Research Animal Breeder, Bekasi. These rats were kept in a well-ventilated house with 12-hour light and 12-hour dark period. Rats were given pelleted feed and ad libitum access to water.

Methods

Preparation of Fractions

The dried powder of *S. androgynous* leaves (1.43 Kg) was macerated using ethanolic solvent for 24 hours. The filtrate was separated from the pulp by filtration. While the pulp was extracted again with 70% ethanol using the same procedure up to three times, the filtrate was evaporated using a vacuum rotary evaporator N-1200 BS series (EYELA, Shanghai, China) until thick extract was obtained. A known quantity (306.52 g) of the thick ethanol extract was fractionated with water and *n*-hexane solvents in a separating funnel and left to form two phases. Afterwards, the two phases were separated. This procedure was also applied to fractionation with ethyl acetate. Finally, the *n*-hexane, ethyl acetate, and water fractions were evaporated to produce fractions in their thick forms (Suprayogi *et al*., 2015).

Phytochemical Screening of Active Fractions using TLC method

Phytochemical screening was carried out by the TLC method to examine the presence of steroids and flavonoids in the active fraction that, in this procedure, was dissolved in ethanol. The TLC method used silica gel GF254 (Merck) as the stationary phase, while two different mobile phases were used to each detect the presence of steroid and flavonoid compounds. The mobile phase used to identify the presence of steroid compounds was chloroform:methanol (10:1), with vanillin-sulfuric acid as the detection reagent. Meanwhile, the mobile phase used to detect flavonoid compounds was *n*-hexane:ethyl acetate (1:1), with ammonia vapour as the detection reagent (Hanani, 2015).

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The animals under test were divided into four groups, each consisting of six rats. Before receiving the treatment, they were acclimatized for seven da fed with pelleted rat feed, and allowed access to water ad libitum. Protocol no. 17-05-0438 was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia no. 631/UN2.F1/ETIK/2017.

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All four groups received different treatments for seven days: Group I (normal control group) was given Na-CMC 0.5% each day, while Groups II, III, and IV were given the fractions (*n*-hexane, ethyl acetate, and water) respectively at the dose of 11.85 $mg.Kg^{-1}$ BW per day. On Day 8, they were injected with ketamine at the dose of 33.35 $mg.Kg^{-1}$ BW i.m. furthermore sacrificed by cervical dislocation. The caudal epididymis was separated carefully from the testis. It was lacerated with scissors and was collected for analysis of sperm counts, motility and viability

Sperm Count Analysis

After removing the caudal epididymis from the left and right samples, the sperm count was evaluated. Then, it was cut into pieces, mixed with Ringer's solution, and left for 2 minutes. Ten µL of the semen suspension was collected and inserted into the haemocytometer. It was immediately examined at x40 magnification under the microscope (Olympus Co., Tokyo, Japan) (Quadri & Yakubu, 2017).

Sperm Motility Test

Ten µL of the semen suspension in Ringer's solution was collected and inserted into the hemocytometer. Any moving and immovable spermatozoa under the microscope at ×40 magnification was immediately examined. The percentage of motile sperm cells was determined under the microscope by the progressive and non-progressive movement of the sperm cells. It was calculated according to the number of motile sperm cells divided by the total number of sperm cells counted (Quadri & Yakubu, 2017).

Sperm Viability Test

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Data Analysis

The data were analysed statistically using one-way ANOVA, followed by a Tukey 's test to find significantly different means. A statistical significance was identified from $p<0.05$.

Results and Discussion

The extraction yield of *S. androgynous* leaves dried powder by cold maceration with 70% ethanol was 21.99% (w/w). In extracts obtained with 70% ethanol, more bioactive flavonoids were detected. It is because of its higher polarity compared to absolute ethanol as an extraction solvent. Ethanol is the solvent used for extracting active compounds such as tannins, polyphenol, flavonoids, terpenoids, steroids, and alkaloids (Tiwari et al., 2011).

The percentage yields of the *n*-hexane, ethyl acetate, and water fractions from ethanolic extract using the liquid-liquid extraction method were 1.74%, 5.29 %, and 52.94% (w/w), respectively. The *n*-hexane can dissolve non-polar compounds such as fixed oils, volatile oils, steroids, and several flavonoids aglycon. Semi-polar solvents such as ethyl acetate can dissolve alkaloids and the other aglycon like flavonoids (Houghton & Raman, 1998). Water as an extraction solvent can extract polar metabolite materials such as quaternary alkaloids, phenolic, carotenoids, tannins, glycoside flavonoids, sugar, amino acids, and the other glycosides (Hanani, 2015).

Male rats were receiving *n*-hexane and ethyl acetate fractions (Groups II and III) at the dose of 11.85 mg.Kg BW per day for seven days showed a significant increase in the sperm count, motility, and viability ($P<0.05$) compared to the normal control group (Group I) (**Figures 1-3**). The presence of steroids in the *n*-hexane fraction and flavonoids in the ethyl acetate fraction was identified using the TLC method (**Figure 4**).

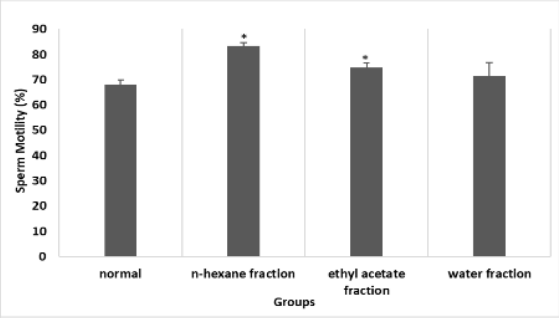

Figure 1. Effects of *S. androgynous* leaf fractions on sperm motility in male rats (Note: * = a significant difference with the normal group (α =0.05))

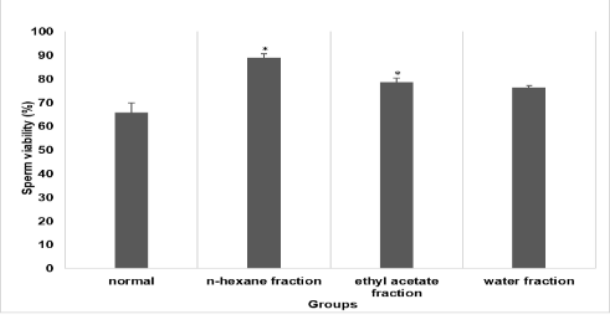

Figure 2. Effects of *S. androgynous* leaf fractions on sperm viability in male rats (Note: * = a significant difference with the normal group (α =0.05))

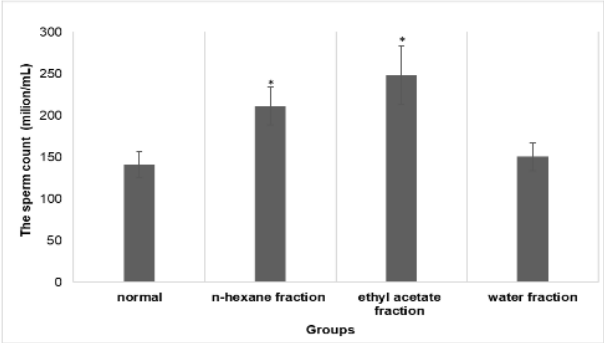

Figure 3. Effect of *S. androgynous* leaf fractions on sperm count in male rats (Note: * = a significant difference with the normal group (α =0.05))

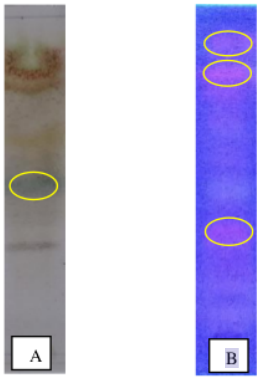

Figure 4. The presence of steroid compounds in the *n*-hexane fraction (A) and flavonoid compounds in the ethyl acetate fraction (B) of *S. androgynous* leaves, identified using the TLC method.

Fragmentation of DNA is now recognized as an important factor in male infertility aetiology. DNA damage occurs in men with both abnormal and normal semen parameters. Reactive oxygen species (ROS) and oxidant stress are the most common cause of DNA fragmentation in spermatozoa. ROS are produced and required in spermatozoa for several specific and essential functions. when ROS concentrations increase and / or antioxidant defenses get overwhelmed, oxidative stress occurred. It causes damage to components of the cells, especially lipids, proteins, and DNA. The lipid peroxidation and DNA damage two major effects of oxidative stress affecting fertility (Wright *et al*., 2014).

In general, antioxidants are compounds that remove, scavenge and hold back the production of ROS or neutralize their actions (Adewoyin *et al*., 2017). Primary antioxidants are mainly phenolics in structure and include the following: antioxidant minerals, antioxidant vitamins, and phytochemicals, such as flavonoids, catechins, carotenoids, *β*-carotene, and lycopene (Moharram & Youssef, 2014). All of which have been described as having the effect of reducing risk of disease, as free radicals can be scavenged. Effects on flavonoids antioxidants, mainly *p*-cymene and carvacrol, in the essential oil of

Satureja khuzestanica due to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species (Safarnavadeh & Rastegarpanah, 2011).

Several phytomedicines, including phenolic compounds (phenols, sterols, lignans, and flavonoids), volatile oils, polyphenols, and saponins, have positive effects on spermatogenesis and sperm parameters (sperm motility, count, and viability) (Khojasteh *et al*., 2016). Some food supplements have been shown to increase sperm count and motility (Adewoyin *et al*., 2017). Also, previous study have reported that the fractions of *S. androgynous* leaves can increase the weight of testicular and seminal vesicle (Rusdi *et al*., 2018). In this study, *n*-hexane and ethyl acetate fractions induced improvements in semen quality and quantity. This activity probably related to the presence of flavonoids and steroids as active compounds in both fractions (see **Figure 4**). Flavonoids can increase dehydroepiandrosterone (DHEA) levels, which plays a role in increasing testosterone levels. Saponins, in the form of steroid glycosides, contribute the DHEA biosynthesis, hence, increasing testosterone levels in the body (Andini, 2014). Multiplied sperm number in the treatment groups is due to increased production of testosterone and probably of follicle-stimulating hormone (FSH) (Al-Sa'aidi *et al*., 2009). The process of spermatogenesis is associated with testosterone and FSH levels (McLachlan, 2004). Further studies should focus on mechanism by which fractions improve spermatogenesis through the cellular and intracellular signalling pathways.

CONCLUSION

The *n*-hexane and ethyl acetate fractions of *S. androgynous* leaves have the potential to be developed into natural male fertility-enhancing agents.

ACKNOWLEDGEMENT

We would like to thank the Research and Development Institute (Lembaga Penelitian dan Pengembangan) of Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta, for financially assisting this research under the Funding Grant Scheme of Science and Technology Development Research Batch II 2018.

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Pharmaciana
ISSN Print: 2088-4559 | ISSN Online: 2477-0256
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Kode pos 55164
Email: pharmaciana@pharm.uad.ac.id

Pharmaciana
Vol.10, No.2, July 2020, Page. 193 – 200
ISSN: 2088 4559; e-ISSN: 2477 0256
DOI: 10.12928/pharmaciana.v10i2.13514

Evaluation of sperm quality in male rats treated with *Sauropus androgynus* (L.) merr. leaf fractions

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Submitted: 05-07-2020 ***Reviewed: 05-07-2020*** ***Accepted: 07-08-2020***

ABSTRACT

Sauropus androgynus (L.) Merr. or katuk is one of the medicinal plants broadly used in Indonesia as it has active compounds that can, among others, stimulate reproductive hormones. This study was aimed to determine which active fraction of *S. androgynus* leaves that have the potential to improve the sperm quality of male rats based on three parameters, namely sperm count, viability, and motility. It employed fractionation using the liquid-liquid extraction with *n*-hexane, ethyl acetate, and water solvents to obtain the fractions. Twenty-four mature Sprague-Dawley male rats were divided into four equal groups: the normal group (untreated group) received 0.5% Na-CMC suspense, and the three fraction groups were given the *n*-hexane, ethyl acetate, and water fraction p.o., respectively, at the dose of 11.85 $mg.Kg^{-1}$ BW daily for seven days. Sperm count, viability, and motility were measured on day 8 (after treatment) from the sperm samples collected at the cauda epididymis of the sacrificed test rats. The results showed that compared with the normal group, the *n*-hexane and ethyl acetate fractions significantly increased the three parameters ($p<0.05$). Therefore, the *S. androgynus* leaf fractions have the potential as a natural material that can increase the fertility of male rats.

Keywords: fertility, fractions, katuk, *Sauropus androgynus*, sperm

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INTRODUCTION

Infertility is defined as "Clinical pregnancy failure after 12 months or more of regular unprotected sexual intercourse" (World Health Organization, 2020). No less than 50 percent of cases are responsible for male infertility. Essentially, identified contributors to sperm dysfunction and male infertility factor are primarily environmental, physiological, and genetic (World Health Organization, 2010). Analysis of semen is still the first step in male infertility diagnosis, which is commonly perceived as a change in sperm concentration, motility, morphology, or its combination.

Among the proposed mechanisms which lead to impaired spermatogenesis are increased ROS and oxidative levels (oxidative stress). Conditions of male infertility in humans primarily oligospermia and asthenozoospermia. This can be caused by a complete dietary loss of antioxidants and a vitamin A deficiency or other elements such as flavonoids, phenolic and folate (Ouladsahebmadarek et al., 2016). It has long been thought that the use of herbs by men with infertility could improve the sperm parameters or regulate male reproductive hormones associated with the spermatogenesis process. Indonesia is a country rich in plants that can be made as traditional medicine, one of which is *Sauropus androgynus* (L.) Merr. or they are known as katuk. The ethanol extract of S. androgynus leaves contains steroids, tannins, alkaloids, flavonoids, and terpenoids (Rusdi et al., 2018). *S. androgynus* leaf extract can increase viability, motility, and concentration of spermatozoa in male mice exposed to cigarette smoke when administered at the dose of 6mg/20g BW (Khoironi, 2015). Rusdi et al., (2018) reported that the *n*-hexane fraction at a dose of 11.85 $mg.Kg^{-1}$ BW can increase the sexual desire of male rats toward female rats, as observed from the number of mounts and precopulatory investigations, and the weight of testicular and vesicular of male rats.

Thus, an active fraction of 70% ethanol extract of *S. androgynus* leaves was analysed for its effectiveness as medicinal ingredients in increasing sperm count, viability, and motility in male rats.

MATERIALS AND METHOD

Materials

Plant sample and authentication

The fresh *S. androgynus* leaves were obtained from the Indonesian Medicinal and Aromatic Crops Research Institute (BALITTRO), Bogor. It was authenticated at the Botanical Research and Development Centre "Herbarium Bogoriense" LIPI, Bogor.

Experimental animals

Twenty-four male Sprague Dawley sexually mature rats aged 2-3 months and weighing around 150-250 g were obtained from Research Animal Breeder, Bekasi. These rats were kept in a well-ventilated house with 12-hour light and 12-hour dark period. Rats were given pelleted feed and ad libitum access to water.

Methods

Preparation of fractions

The dried powder of *S. androgynus* leaves (1.43 Kg) was macerated using ethanolic solvent for 24 hours. The filtrate was separated from the pulp by filtration. While the pulp was extracted again with 70% ethanol using the same procedure up to three times, the filtrate was evaporated using a vacuum rotary evaporator N-1200 BS series (EYELA, Shanghai, China) until thick extract was obtained. A known quantity (306.52 g) of the thick ethanol extract was fractionated with water and *n*-hexane solvents in a separating funnel and left to form two phases. Afterwards, the two phases were separated. This procedure was also applied to fractionation with ethyl acetate. Finally, the *n*-hexane, ethyl acetate, and water fractions were evaporated to produce fractions in their thick forms (Suprayogi et al., 2015).

Phytochemical screening of active fractions using TLC method

Phytochemical screening was carried out by the TLC method to examine the presence of steroids and flavonoids in the active fraction that, in this procedure, was dissolved in ethanol. The TLC method used silica gel GF254 (Merck) as the stationary phase, while two different mobile phases were used to each detect the presence of steroid and flavonoid compounds. The mobile phase used to identify the presence of steroid compounds was chloroform:methanol (10:1), with vanillin-sulfuric acid as the detection reagent. Meanwhile, the mobile phase used to detect flavonoid compounds was *n*-hexane:ethyl acetate (1:1), with ammonia vapour as the detection reagent (Hanani, 2015).

Test animal preparation

The animals under test were divided into four groups, each consisting of six rats. Before receiving the treatment, they were acclimatized for seven days, fed with pelleted rat feed, and allowed access to water ad libitum. Protocol no. 17-05-0438 was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia no. 631/UN2.F1/ETIK/2017.

Test animal treatment

All four groups received different treatments for seven days: Group I (normal control group) was given Na-CMC 0.5% each day, while Groups II, III, and IV were given the fractions (*n*-hexane, ethyl acetate, and water), respectively at the dose of 11.85 $mg.Kg^{-1}$ BW per day. On day 8, they were injected with ketamine at the dose of 33.35 $mg.Kg^{-1}$ BW i.m. furthermore sacrificed by cervical dislocation. The caudal epididdymis was separated carefully from the testis. It was lacerated with scissors and was collected for analysis of sperm counts, motility and viability

Sperm count analysis

After removing the caudal epididymis from the left and right samples, the sperm count was evaluated. Then, it was cut into pieces, mixed with Ringer's solution, and left for 2 minutes. Ten µL of the semen suspension was collected and inserted into the haemocytometer. It was immediately examined at x40 magnification under the microscope (Olympus Co., Tokyo, Japan) (Quadri and Yakubu, 2017).

Sperm motility test

Ten µL of the semen suspension in Ringer's solution was collected and inserted into the hemocytometer. Any moving and immovable spermatozoa under the microscope at ×40 magnification was immediately examined. Progressive and non-progressive movement of sperm cells under the microscope describes motile sperm cells. It was calculated according to the number of motile sperm cells divided by the total number of sperm cells counted (Quadri and Yakubu, 2017).

Sperm viability test

Ten µL of the semen suspension in Ringer's solution was collected, dropped into the object's glass, then added with one drop of 2% eosin. After homogenized, it was covered with a cover glass. The stained cells were observed under a microscope at 40x magnification. The live sperm cells were unstained (transparent), while the dead ones absorbed the stain (red colour). Sperm viability is expressed in percentage (Quadri and Yakubu, 2017).

Data Analysis

One-way ANOVA followed by a Tukey 's test was used to analyzed data to find significantly different means ($p<0.05$).

RESULTS AND DISCUSSION

The extraction yield of *S. androgynus* leaves dried powder by cold maceration with 70% ethanol was 21.99% (w/w). In extracts obtained with 70% ethanol, more bioactive flavonoids were detected. It is because of its higher polarity compared to absolute ethanol as an extraction solvent. Ethanol is the solvent used for extracting active compounds such as tannins, polyphenol, flavonoids, terpenoids, steroids, and alkaloids (Tiwari et al., 2011).

The percentage yields of the *n*-hexane, ethyl acetate, and water fractions from ethanolic extract using the liquid-liquid extraction method were 1.74%, 5.29 %, and 52.94% (w/w), respectively. The *n*-hexane can dissolve non-polar compounds such as fixed oils, volatile oils, steroids, and several flavonoids aglycon. Semi-polar solvents such as ethyl acetate can dissolve alkaloids and the other aglycon like flavonoids (Houghton and Raman, 1998). Water as an extraction solvent can extract polar metabolite materials such as quaternary alkaloids, phenolic, carotenoids, tannins, glycoside flavonoids, sugar, amino acids, and the other glycosides (Hanani, 2015).

Male rats were receiving *n*-hexane and ethyl acetate fractions (Groups II and III) showed a significant increase in the sperm count, motility, and viability ($P<0.05$) compared to the normal control group (Group I) (Figures 1-3). The presence of steroids in the *n*-hexane fraction and flavonoids in the ethyl acetate fraction was identified using the TLC method (Figure 4).

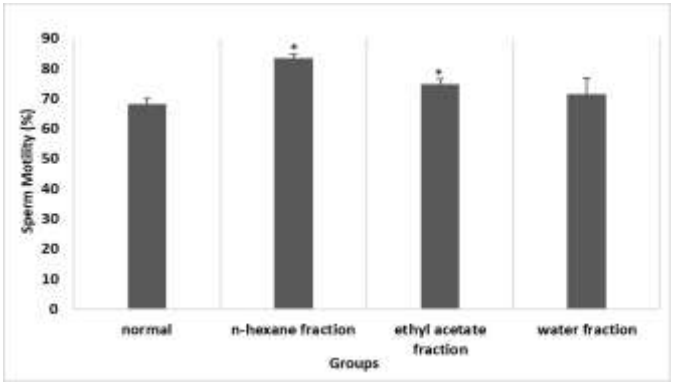

Figure 1. Effects of *S. androgynus* leaf fractions on sperm motility in male rats (Note: * = a significant difference with the normal group (α =0.05))

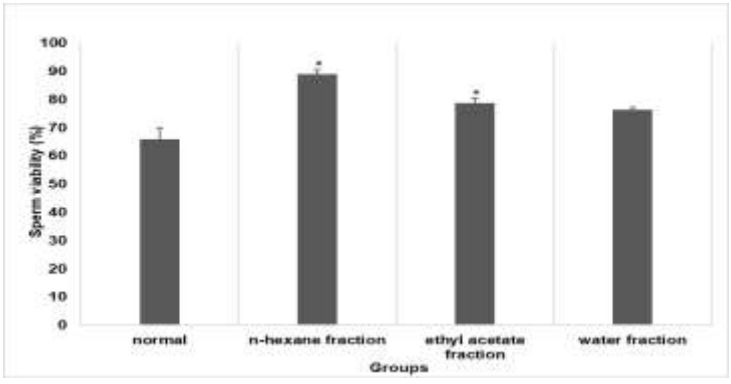

Figure 2. Effects of *S. androgynus* leaf fractions on sperm viability in male rats (Note: * = a significant difference with the normal group (α =0.05))

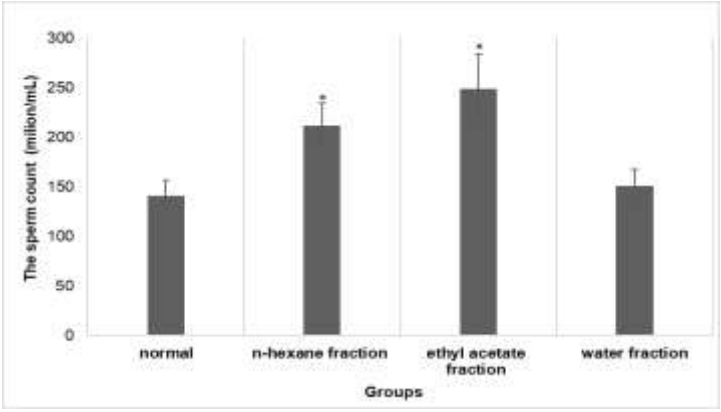

Figure 3. Effects of *S. androgynus* leaf fractions on sperm count in male rats (Note: * = a significant difference with the normal group (α =0.05))

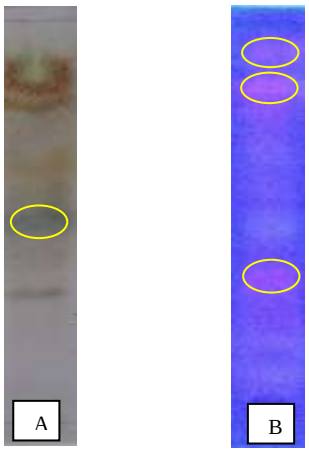

Figure 4. The presence of steroid compounds in the *n*-hexane fraction (A) and flavonoid compounds in the ethyl acetate fraction (B) of *S. androgynus* leaves, identified using the TLC method

Fragmentation of DNA in spermatozoa induced by Reactive Oxygen Species (ROS) and oxidative stress is now identified as a significant factor in male infertility aetiology. DNA damage occurs in men with both abnormal and normal semen parameters. ROS are produced and required in spermatozoa for several specific and essential functions. when ROS concentrations increase and/or antioxidant defenses get overwhelmed, oxidative stress occurred. It causes damage to components of the cells, especially lipids, proteins, and DNA. The lipid peroxidation and DNA damage are two major effects of oxidative stress affecting fertility (Wright et al., 2014).

In general, antioxidants can remove, scavenge and hold back the production of ROS or neutralize their actions (Adewoyin et al., 2017). Primary antioxidants are mainly phenolics in structure and include the following: antioxidant minerals, antioxidant vitamins, and phytochemicals, such as flavonoids, catechins, carotenoids, *β*-carotene, and lycopene (Moharram and Youssef, 2014). All of which have been described as having the effect of reducing risk of disease, as free radicals can be

scavenged. Effects on flavonoids antioxidants, mainly *p*-cymene and carvacrol, in the essential oil of *Satureja khuzestanica* due to their ability to decrease ROS (Safarnavadeh and Rastegarpanah, 2011).

Several phytomedicines, including phenolic compounds (phenolics, sterols, lignans, and flavonoids), volatile oils, and saponins, are found in dietary supplements. They have been shown to increase sperm parameters (sperm motility, count and viability) and have positive effects on spermatogenesis (Khojasteh et al., 2016; Adewoyin et al., 2017). Also, previous study have reported that the fractions of *S. androgynus* leaves can increase the weight of testicular and seminal vesicle (Rusdi et al., 2018). In this study, *n*-hexane and ethyl acetate fractions induced improvements in semen quality and quantity. This activity probably related to the presence of flavonoids and steroids as active compounds in both fractions (see Figure 4). Flavonoids can increase dehydroepiandrosterone (DHEA) levels, which plays a role in increasing testosterone levels. Saponins, in the form of steroid glycosides, contribute to the DHEA biosynthesis, hence, increasing testosterone levels in the body (Andini, 2014). Multiplied sperm number in treated rats groups is due to increased production of male reproduction hormone (Al-Sa'aidi et al., 2009). The process of spermatogenesis is associated with testosterone and follicle-stimulating hormone (FSH) levels (McLachlan, 2004). Further studies should focus on mechanism by which fractions improve spermatogenesis through the cellular and intracellular signalling pathways.

CONCLUSION

The *n*-hexane and ethyl acetate fractions of *S. androgynus* leaves have the potential to be developed into natural male fertility-enhancing agents.

ACKNOWLEDGEMENT

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