# The Effect of N-Hexane Extract of Sambiloto Leaves (*Andrographis paniculata*) on Spermatozoa Quality of Male ICR Mice (*Mus musculus*)



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# The Effect of N-Hexane Extract of Sambiloto Leaves (*Andrographis paniculata*) on Spermatozoa Quality of Male ICR Mice (*Mus musculus*)

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**Abstract**. The study aimed to determine the effect of N-Hexane extract of *Andrographis paniculata* leaves on testis weight of male mice (*Mus musculus*), along with the amount, motility, viability, and abnormality of its sperm. The tested animals used in this study were male *Mus musculus* aged 10-12 weeks with the average body weight (bw) of 21 g. The study was an experimental study with a Completely Randomized Design (CRD), which consists of one control group (A0, without extract) and three experimental groups (A1, A2, A3, with the extract concentration of 125 mg/kg bw, 250 mg/kg bw, and 500 mg/kg bw, respectively). The volume of extract given to each experimental mice was 1 cc per day, for a total of 18 days. The parameter of observation were the weight of testis, along with the amount, motility, viability, and the total of abnormal sperm. The data collected were analyzed by using F Test (α 0,05) and BNT Test (α 0,05). The result of data analysis showed that the N-Hexane extract of *A. paniculata* which was given for 18 days consecutively has affected the spermatozoa production of male mice. Each concentration of N-Hexane extract of *A. paniculata* (125, 250, and 500 mg/kg bw) had caused the decreasing of testis weight; the decreasing of spermatozoa production; the decreasing of motility and viability of spermatozoa; and the increasing of abnormal sperm. Hence, the study suggested that the N-hexane extract of *A. paniculata* has the potential to be used as antifertility treatment as it decreased the reproductive fuction of male *Mus musculus*. The extract concentration which cause the biggest effect is 500 mg/kg bw.

## 1. Introduction

Indonesia is a country rich in natural resources. The natural resources are mostly efficacious as medicine. Since the 19th century, this type of medicinal plant had begun to give a scientific touch, both in terms of planting and in terms of research on its content and properties. Indonesia has abundant natural resources. Many plants contain bioactive compounds which can be used as medicines. Generally, these compounds originate from steroids, isoflavonoids, alkaloids, and xanthons, all of which can affect reproductive function in mice [1].

Phytosterol is a sterol which is naturally obtained from plants. Chemically, phytosterol is similar to cholesterol obtained from animals. Sterol consists of three cyclohesan ring combinations with various types of sterols (more than 40 phytosterol). In plants, there are more than 40 compounds dominated by the phytosterol group [2]. Phytosterol (also known as plant sterol) is a cholesterol-like molecule found in plants. Phytosterol is a group of steroid alcohols, phytochemicals that occur naturally in plants. Plants contain a number of phytosterols which act as structural components of cell membranes. In

mammalian cells, this role is replaced by cholesterol. Three types of compounds commonly referred to as "phytosterol" are sitosterol (better known as beta-sitosterol), stigmasterol and campestero [3].

Plants have a great potential to be used as a source of fertility regulating compunds. Various bioactive compounds in plants, especially groups of steroid compounds, alkaloids, isoflafonoid, triterpenoids and xanthons have properties as a regulator of fertility. Preclinical tests conducted by researchers showed that bioactive compounds in plants affect the fertility of test animals, such as disturbing spermatogenesis, reducing the power of conception, increasing the percentage of gestation loss, preventing/ inhibiting implantation and reducing the number of progeny. Bioactive ingredients from plants have activities that can be reversed, and if their use is stopped, fertility returns to recovery [4].

Today, many researchers in the world are beginning to highlight medicinal plants to be used as alternatives to overcome reproductive problems, since the use of medicinal plants (herbs) is considered as natural and not harmful. One example is Sambiloto (*Andrographis paniculata)*, a bitter plant which is suspected as a plant that have the potential to overcome reproductive problems. *A. paniculata* is one of the plants that can be found easily in Indonesia, as it is high in abundance and has been widely known as a traditional medicine. The use of the plant is pervasive, since people in Indonesia already familiar with its use [5].

The testing of various types of drugs is usually conducted prior to its application for livestock or human. Thus, tested animals are needed for investigating the effect of a certain drugs. For this purpose, mice are commonly used as tested animals. A study on oral administration of *A. paniculata* have shown an antifertility effect as it decreases the quality of the ejaculated sperm on male mice [6]. A study conducted by Akbarsha and Murugaian [7] also found that the treatment of *A. paniculata* leaf to male mouse has caused the seminiferous epithelium was thoroughly disrupted and the fully differentiated spermatozoa were far too limited. Thus, it supports the possible prospective use of *A. paniculata* leaf as as antifertility compound.

The potential benefits of *A. paniculata* to be used as antifertility, along with the scientific objectives to strengthen previous researches, has put an urge to conduct further research on the effect of *A. Paniculata* leaf extract on the quality of spermatozoa, particularly on mice (*Mus musculus*).

## 2. Research Method

### *2.1. Time and Place of Research*

The research was conducted from May to June 2013. The implementation of this study took place at the Biology Microtechnics Laboratory, Faculty of Mathematics and Natural Sciences, UNM.

### *2.2. Type of Research*

The research is an experimental research which consists of two stages, namely (i) the preparation stage, which included the maintenance of test animals, the copulation of test animals, the making of extracts; and (ii) the implementation stage, which included the treatments, surgery, and the preparation of wet mount slide to be observed under the microscope.

### *2.3. Research Design*

The research design used was a Completely Randomized Design (CRD) consisting of four treatment groups with five replications to obtain twenty combinations. Group I (was given the symbol A0) is a control group that was not given the n-hexane extract of *A. paniculata* leaves. Group II, III, and IV (were given the symbols A1, A2, and A3, respectively) are the groups which were given the n-hexane extract of *A. paniculata* leaves based on the prescribed dose. Group II was given a dose of 125 mg/ kg bw. Meanwhile, group III and IV was given a dose of 250 mg/ kg bw and 500 mg/ kg bw, respectively.

### *2.4. Research Procedure*

*2.4.1. Test Animals*

The test animals used in this study were male ICR (*Mus musculus*) which were obtained from the Maros Regency Animal Research Agency (BPH). The age of the mice were 10-12 weeks with a body weight of 21 g. The food given for the test animals were AD2 which was obtained from Sinar Harapan store in Veteran Selatan and the drinking water used was tap water.

The handling of the test animals was carried out in the microtechnical laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, UNM with 12 hours of lighting and 12 hours of dark conditions. The mice were placed in a rectangular box that has been given husk to prevent the base from the dirt and also a cover made from a wire mesh. The husk is replaced twice a week.

*2.4.2. Extraction*

The material to be extracted was the *A. paniculata* leaves obtained from the Green House experimental garden, Faculty of Mathematics and Natural Sciences, UNM. The extraction process was carried out at the Microbiology Laboratory of Biology Department. In order to prepare the leaf extract of *A. paniculata* leaf extract, the leaves of *A. paniculata* was washed with a wet weight of 2000 g, then dried (aerated) until it reached a dry weight of 1700 g. Dried leaves were then mashed and soaked with n-hexan for 24 hours, then could be filtered with filter paper. The extract was then evaporated for 6-8 hours to obtain a pure extract. The result of extraction was 1 gram of a semi-liquid extract with a deep green colour and a bitter stinging smell

*2.5 Implementation of Research.*

*2.5.1. Preparation*

Before being given to the tested animals, the extract was weighed using an analytical balance based on the required dose, and then diluted with 0.5% Carboxil Methyl Cellulosa (CMC). Prior to the dilution, 0.5 g of CMC was weighed with an analytical balance and dissolved in 100 ml of distilled water. The mixture of CMC and extract were homogenized by using Hot Plate Magnetic stirrer and stirring rod so that the solution becomes homogeneous and ready to be used. The dosage of solution for each treatment was made once for three days. The volume of n-Hexan extract from *A. paniculata* leaves given to each mouse was 1 cc.

*2.5.2. Treatment*

Each experimental group (A1, A2, and A3) was treated by giving n-Hexan extract of *A. paniculata* leaves once a day. Each experimental group was given different doses, i.e. A1 = 125 mg / kg bw, A2 = 250 mg / kg bw, and A3 = 500 mg / kg bw. All male mice were weighed using Ohaus balance from the first day of handling until the 18th day of extract treatment. The extract was given every day at 08.00 – 10.00, by using Feeding Tube No.4 and 1 cc size spoit.

*2.5.3. Surgery*

On the 18th day, the injection of n-hexane extract of *A. paniculata* was stopped. The tested animals were then subjected to euthanasia through cervical dislocation. In order to produce the dislocation, the mice were restrained in a normal standing position on a flat surface. The right hand then quickly pushed the mice head forward and down, while the left hand pulling the tail backward. The surgery was performed by stretching the mice body on a surgical board, holding the leg by using a pin, then cutting it through the abdomen slowly.

*2.5.4. Observation*

The observational parameters of this study, including: the weight of testis, along with the amount, motility, viability, and the total of abnormal sperm.

a). The Weight of Testis
The male mice were euthanized through cervical dislocation, then were subjected to surgery by using surgical instruments, such as surgical scissors, surgical tweezers, and pins. The reproductive organs were cut then stored in a petri dish containing 2 ml of NaCL 0.9%. The testis was then cleaned from fat, dried with tissue, and then weighed using an electronic balance scale. The reproductive organ that have been taken must be stored in 0.9% physiological NaCl solution as soon as possible, so that the spermatozoa inside the testicles do not die and the consistency of the organ could be maintained.

b) Total Sperm
Spermatozoa were obtained from the cauda epididymis. Both Cauda epididimymis squeezed into a watch glass containing 1 ml of 0.9% NaCl. It was cut into smaller parts by using scissors until it reached a smooth consistency, and then stirred with a stirring glass. This solution is called a spermatozoa suspension. The number of spermatozoa was calculated by using an improved Neubauer count chamber (haemocytometer). The spermatozoa suspension that has been diluted with 1 mL of physiological saline (0.9% NaCl) was taken by using the haemacytometer pipette until it reached 0.5 scale and then NaCl was taken using the same pipette until it reached the scale 11, and shaken until homogenous then put into counting chamber (heemocytometer), after which be closed with a cover glass. While closing with a cover glass, the air bubble should not be formed. The haemocytometer which contained a suspension of spermatozoa was then observed under a light microscope with a magnification of 400x. The result of calculation was then entered into the formula of sperm count = N/ 2 spermatozoa/ ml suspension. In which, N = number of sperm counted in boxes 1, 2, 3, 4 and 5 (middle part).

c) Sperm Motility
According to WHO, sperm motility is classified into four levels, namely: Class A, sperm that moves forward quickly in a straight line; Class B, sperm that moves forward but in curved or wavy lines or in a straight line but slowly; Class C: sperm that moves its tail but does not move; and Class D, sperm that doesn't move at all. In accordance with WHO motility assessment standards, spermatozoa are said to be normal if the sperm percentage of criteria A and B is greater or equal to 50%. For this reason, the data analysis in this study only used the percentage of sperm criteria A + B. The total number of Class A and Class B sperm were then analysed for its average along with its percentage.

d). Sperm Viability
The spermatozoa suspension obtained can be used to analyse the viability of the sperm. Sperm viability analysis was carried out with supravital staining, through which 1 drop of sperm was placed on the glass object, added with 1 drop of eosin solution, then stirred. After that, a smear preparation was made and dried in air. The preparation was observed under a microscope with magnification of 400 x. Living spermatozoa are colorless, while dead ones are red. A total of 100 spermatozoa were calculated, and the results are presented in percent.

e). Sperm Abnormalities
Observations were carried out by looking at the forms of abnormalities that exist. Observation on the abnormal characteristics of sperm were conducted by calculating the number of abnormal spermatozoa from 100 observed spermatozoa. The characteristics of abnormal spermatozoa including double heads, large heads, small, broken body, broken heads, curly tail, and so on.

*2.6 Data Analysis Techniques*

Data obtained from the observation were analysed by using inferential analysis of F test with the significance of α 0.05, followed with LSD test with the significance of α 0.05.

## 3. Result and Discussion

### *3.1. Research Result*

The results of observation on the average body weight (g), testicular weight (g), sperm count (%), sperm motility (%), sperm viability (%), and the average number of abnormal sperm (%) in control mice and in mice treated with n-hexane extract of *A. paniculata* for 18 days at a dose of 125 mg/ kg bw, 250 mg/ kg bw, and 500 mg/ kg bw respectively are presented in Table 1 to Table 6.

**Table 1.** The average body weight (g) on various treatment with n-hexane extract of *A. paniculata* for 18 days.

| No. | Dosage (mg/bw) | Average ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 24,546 ± 0,3242c | |
| 2 | 125 | 22,622 ± 1,2726b | 1,527 |
| 3 | 250 | 21,083 ± 1,1403b | |
| 4 | 500 | 22,483 ± 0,9538a | |

Note: the numbers followed by the same letter are not statistically different on $\alpha$ 0,05 of LSD test.

**Table 2.** The average weight of mice testis (g) on various treatment with n-hexane extract of *A. paniculata for* 18 days

| No. | Dosage (mg/bw) | Average (g) ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 0,609 ± 0,075b | |
| 2 | 125 | 0,567 ± 0,009ab | 0,108 |
| 3 | 250 | 0,515 ± 0,396a | |
| 4 | 500 | 0,500 ± 0,062a | |

**Table 3.** The average number of mice spermatozoa (105 million/ml) on various treatment with n-hexane extract of *A. paniculata* for 18 days

| No. | Dosage (mg/bw) | Average ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 59,863 ± 2,9744d | |
| 2 | 125 | 41,162 ± 1,6996c | 9,354 |
| 3 | 250 | 26,275 ± 2,8712b | |
| 4 | 500 | 20,790 ± 3,1819a | |

Note: the numbers followed by the same letter are not statistically different on $\alpha$ 0,05 of LSD test.

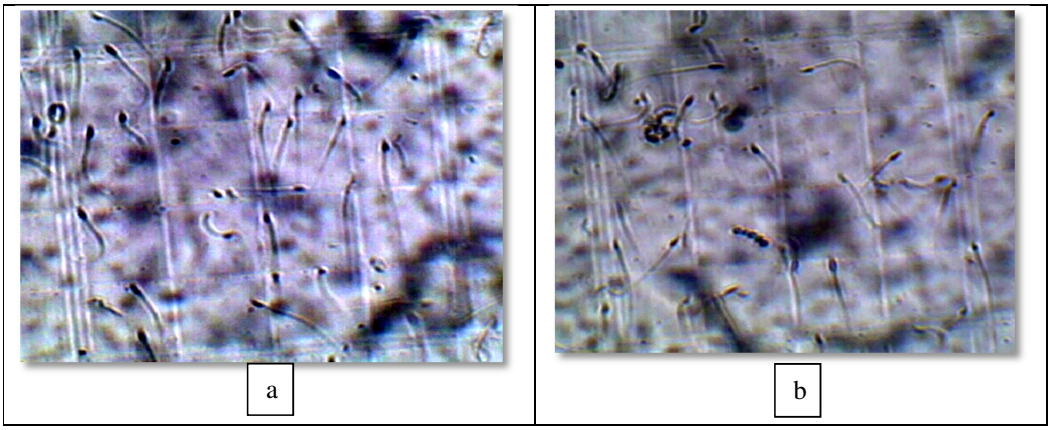


**Figure 1.** The counting of total number of spermatozoa on haemocytometer with the magnification of 400 x: a. control group, b. experimental group.

**Table 4.** The average motility of spermatozoa (%) on various treatment with n-hexane extract of *A. paniculata* for 18 days

| No. | Dosage (mg/bw) | Average (%) ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 34,662 ± 3,4119d | |
| 2 | 125 | 24,490 ± 1,0464c | 2,29 |
| 3 | 250 | 16,832 ± 0,3350b | |
| 4 | 500 | 12,997 ± 0,2735a | |

Note: the numbers followed by the same letter are not statistically different on $\alpha$ 0,05 of LSD test.

**Table 5.** The average viability of spermatozoa (%) on various treatment with n-hexane extract of *A. paniculata* for 18 days

| No. | Dosage (mg/bw) | Average (%) ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 18,330 ± 1,1885a | |
| 2 | 125 | 36.162 ± 3,5559b | |
| 3 | 250 | 49,495 ± 3,4179c | 4,484 |
| 4 | 500 | 46,283 ± 1,1940c | |

Note: the numbers followed by the same letter are not statistically different on $\alpha$ 0,05 of LSD test.

**Table 6.** The average abnormal spermatozoa (%) on various treatment with n-hexane extract of *A. paniculata* for 18 days

| No. | Dosage (mg/bw) | Average (%) ± SD | LSD α 0,05 |
|---|---|---|---|
| 1 | 0 | 16,332 ± 1,4150a | |
| 2 | 125 | 30,662 ± 1,6557b | 3,872 |
| 3 | 250 | 36,165 ± 3,7170c | |
| 4 | 500 | 44,580 ± 2,6017d | |

Note: the numbers followed by the same letter are not statistically different on $\alpha$ 0,05 of LSD test.

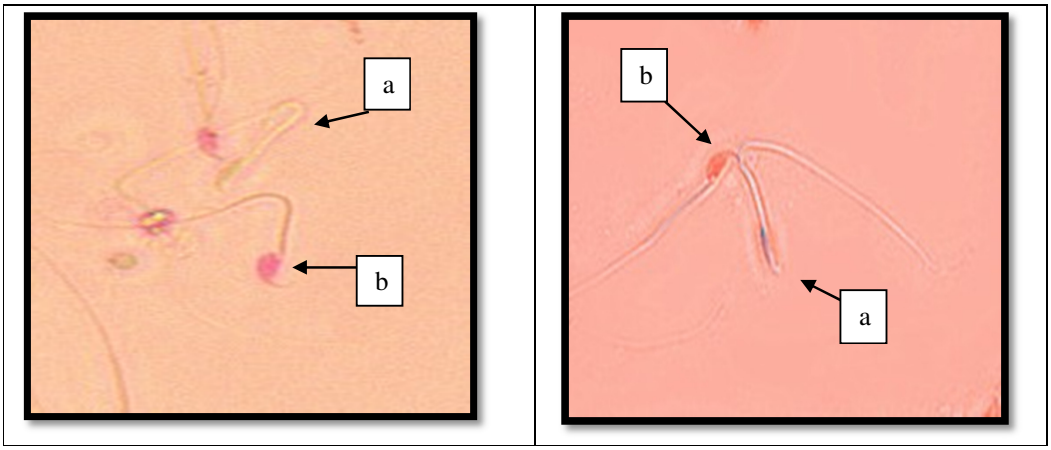


**Figure 2.** Observation of spermatozoa viability, a. alive spermatozoa = do not absorb dye, b. dead spermatozoa = absorb dye.

*3.2. Discussion*

The present study indicates that the treatment of N-Hexane *A. paniculata* extract results in the decreasing of spermatozoa quality in the treated *Mus musculus*, as it caused the decreasing of testical weight, spermatozoa counts, motility, and viability of spermatozoa, while increasing the total number of abnormal sperm. The extract concentration of *A. paniculata* which cause the biggest effect is 500 mg/kg bw.

The decreasing weight of testis along with the decreasing quality of spermatozoa after the treatment with N-Hexane *A. paniculata* extract for 18 consecutive days could occur due to several factors, including (i) the androgenic and cytotoxic characteristics of *A. paniculata* extract, (ii) *A. paniculata* extract works directly on germinal cells, and (iii) *A. paniculata* extract works to interfere with steroidogenesis. The decreasing of mice body weight along with the shrinkage of its testis may occur due to the presence of androgenic and cytotoxic compounds in *A. paniculata* extract. The compound is β-sitosterol which belong to the group of phytosterol and cytotoxic saponins. Sterols are derivatives of steroid compounds. The excessive use of steroids can cause side effects, such as the increasing risk of infertility and the shrinkage of testis.

A decreased in testicular size occurs due to the loss of germinal epithellial cells which cannot undergo regeneration. It can be seen from testicular size and weight of test treated and control mice which is influenced by the level of estrogen hormone in male mice. It is argued that cytotoxic effects of a substance can cause the death of spermatogenic cells in seminiferus tubule, thus causing a decrease in testicular weight.

The decrease in counts, motility, and viability of sperm is caused by the presence of saponin compounds, β-sitosterol, in *A. paniculata* extract which can cause the increase of testosterone levels along with the inhibition of energy formation in the body of tested animals. The increase of testosterone levels has a negative feedback effect on hypothalamus and anterior pituitary. The negative feedback effect on hypothalamus will stop the secretion of GnRH (Gonadothropins Releasing Hormone), thus inhibiting gonadotropin secretion, i.e. Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), by the anterior pituitary. LH functions to stimulate Leydig cells to produce testosterone, while FSH functions to stimulate spermatogenesis [8].

The decrease in the number of motile sperm may occur due to the presence of compounds in *A. paniculata* n-hexane extract which can inhibit the formation of energy, namely β-sitosterol, which has a chemical structure similar to the hormone testosterone as a hydrocarbon compound. A substance can

work as a hormone because it contains substances whose molecular arrangement is similar to hormones. Thus, it is suspected that β-sitosterol also works like testosterone.

Testosterone is an essential hormone in spermatogenesis. However, it has a nature of negative feed back when it reaches a high level of concentration in the blood. It is suspected that β-sitosterol compounds contained in the extract of *A. paniculata* leaf also cause testosterone levels in the body of tested animals to increase.

The decreasing count of motile sperm is thought to be due to the presence of compounds in *A. paniculata* which can inhibit the formation of energy. Namely β-sitosterol, which has a chemical structure similar to the hormone testosterone which is a hydrocarbon compound. A substance can work as a hormone because it contains substances whose molecular arrangement is similar to hormones. Thus, it is suspected that β-sitosterol is also like testosterone. Testosterone is an essential hormone in spermatogenesis, but in high levels in the body it has the nature of negative feedback. It is suspected that β-sitosterol compounds contained in the extract of bitter leaf also cause testosterone levels in the body of test animals to increase [9]. The increasing level of testosterone in the blood, will cause a negative feedback to occur on the hypothalamus and the anterior pituitary. Thus, the secretion of gonadotropin will be inhibited. If FSH production is stopped or reduced due to the effect of negative feedback, then spermatogenesis will also stop [10].

Testosterone is the primary reproductive hormone that has a role in the process of spermatogenesis (sperm formation). During the process of spermatogenesis, testosterone in collaboration with FSH and LH will affect the formation of spermatozoa. However, if there is a significant increase of testosterone level in blood plasma, it will inhibit the release of FSH and LH. Accordingly, it is suggested that there must be a balance between the three groups of hormone. As a hormone that plays a role in the process of spermiogenesis, testosteron will show a different pattern if its concentration is low. In this case, the low level of testosterone will cause the process of spermiogenesis to be disrupted. Moreover, it may also lead to primary abnormalities, such as the size of spermatozoa's head is too big or too small, the tail is double or has a different appearance than the general structure of spermatozoa. Testosterone biosynthesis involves various substances, enzymes, and other steroid hormones, including hormones in the group of the estrogen and androgen. The presence of flavonoid inhibited the activation of kinase protein, thus inhibiting the testosteron production and the process of spermiogenesis [8, 11, 12].

The treatment of male mice with n-hexane extract of *A. paniculata* has affected the count of normal sperm. The higher the dosage given, the lower the count of normal sperm. The decrease in the number of normal sperm may occur due to the interference during spermatogenesis. As stated earlier, that the chemical content of *A. paniculata* leaf extract is antiproliferative (saponin and B-sitosterol), thus interferes with the spermiogenogenesis process which causes the spermiogenesis process to not proceed properly. Consequently, the counts of normal spermatozoa decreased.

The results of microscopic observation showed that there were several types of abnormal spermatozoa found on treated mice, including spermatozoa with branched head; branched tail; twisted tail; doubled head; enlarged body; folded body; broken tails; curly tails; and rolled tails. The additional parts of spermatozoa may inhibit its effectiveness to move toward the ovum. Thus, fertilization is not likely to occur. A good morphology of spermatozoa including a 'comma' shaped head with a normal magnitude and a long tail that is not circular or double. The tail is used by spermatozoa to do movements. The main part of the tail contains most of the motility power of the spermatozoa and has a vital role for motility [11].

## 4. Conclusions

Administration of n-hexane extract of *A. paniculata* leaf at a dose of 125 mg / kg bw, 250 mg / kg bw, and 500 mg / kg bw for 18 consecutive days has affected the spermatozoa quality of male mice (Mus musculus), as it causes the decrease of testis weight; the decrease of count, motility, and viability of spermatozoa; while increasing the number of abnormal spermatozoa.

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