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# Effect of *Phaleria macrocarpa* on Sperm Characteristics in Adult Rats

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**ABSTRACT**

***Purpose:*** The purpose of this study was to determine the effects of *Phaleria macrocarpa* (PM) on male fertility by assessing its effect on the sperm characteristics which included the sperm count, motility, viability and morphology. ***Methods:*** Eighteen male rats were equally divided into three groups. Each group of rats was orally supplemented for 7 weeks either with PM aqueous extract (240 mg/kg), distilled water (0 mg/kg) or testosterone hormone, Andriol® Testocaps™ (4 mg/kg) respectively. On the last day of supplementation period, the rats were sacrificed and sperm was obtained from cauda epididymis via orchidectomy. The sperm count, motility, viability and morphology were determined. ***Results:*** PM aqueous extract significantly increased ($p<0.05$) the percentage of sperm viability. However, there was no significant effect of PM on the percentage of both sperm motility and morphology. The mean of body weight declined significantly in rats supplemented with PM aqueous extract compared to control groups ($p<0.05$). ***Conclusion:*** The results showed that PM significantly increased sperm viability without changing the sperm motility and morphology. Hence, this study suggests that PM offers an alternative way to improve male fertility by improving the sperm quality.

## Introduction

Infertility is one of the most serious problems faced by some people around the world and the male counterpart contributes half of the infertility cases.[1] This problem is identifiable in about one out of thirteen couples who attempt to conceive.[2] According to United States Food and Drug Administration (FDA), infertility can be caused by androgen deficiency or low testosterone level. Testosterone deficit in men may exhibit symptoms such as decrease libido and erectile quality, low or zero sperm in semen, decrease body hair, decrease lean body mass and changes in mood.[3] The diagnostic testing can be done from the history, physical examination and of course, semen analysis.[2] The evaluation of infertility can aid in determining the underlying cause of infertility as well as giving treatment to allow conception to occur.[4] According to Schulte *et al*.,[5] sperm characteristics assessment has been increasingly important in reproductive studies. According to Concept Fertility Centre Kuala Lumpur,[6] approximately 13% of men have untreatable sterility, 11% have treatable conditions and 76% have disorders of sperm production or function which do not usually have clearly defined effective treatments. Medical practitioner initiates Testosterone Replacement Therapy (TRT) when clinical complaints are accompanied by testosterone decline. But, side effects may take place if TRT is used in excess amount. Some examples are nausea, acne, headache, fluid retention, liver toxicity, sleep apnea, tender breasts, polycytemia and prostate hyperplasia.[3,7] Over the past decades, herbal medicines have been accepted universally due to the various adverse reactions of hormone therapy. Traditional medicines continue to play an important role in healthcare system of a large number of world's population including Indian, Chinese, African, American and other people.[8-10] A National Centre for Complementary and Alternative Medicine has also been established in USA.[11]

*Phaleria macrocarpa* which is also known as mahkota dewa is an Indonesian herbal plant (Figure 1a, 1b) that was claimed to have various medicinal properties. Traditionally, Phaleria macrocarpa has been used to treat impotency, control cancer, haemorrhoids, diabetes mellitus, allergies, liver and heart disease, kidney disorder, blood diseases, acne, stroke, migraine and various skin diseases.[12] *Phaleria macrocarpa* has also been claimed to improve fertility in man, but its potential is still unknown. Besides that, there is still not enough scientific data to prove the claim to be true. Therefore the purpose of current study was to determine the effects of PM on male fertility by assessing its effect on the sperm characteristics.

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## Materials and Methods

### *Extraction of Phaleria macrocarpa*

*Phaleria macrocarpa* (Voucher no. SK1929/11) fresh fruits were supplied by Associate Prof. Dr. Mohammad Aziz bin Dollah. 250 g of dried Phalera macrocarpa fruits slices were soaked in 4L of hot water boiled until the water become half. After that, the mixture solution was filtered and the filtrate was centrifuged at 3000rpm for 15minutes. The supernatant was freeze-dried to obtain crystal or powder form of the extract. The powder of the extract was weighted and kept in the freezer at -20 °C for later used. The extraction process was repeated till about 3kg of dried fruit slices was extracted.


**Figure 1.** (a): *P. macrocarpa* fresh fruit. (b): *P. macrocarpa* dried fruit slices.

### *Working Solutions*

There are three treatment groups in this study; negative control, positive control and supplemented with aqueous extract of *Phaleria macrocarpa*. In negative control, distilled water was used as supplement and in positive control; commercial testosterone drug (Andriol® Testocap™) was used as supplement. The *Phaleria macrocarpa* supplemented groups was given 240 mg/kg of aqueous extract of *Phaleria macrocarpa.* The *Phaleria macrocarpa* extract was weighted using electronic balance (AND GF3000) and reconstitute in distilled water. While the working solution for the commercial drug was used directly from the original product that was purchased from Schering-plough Sdn. Bhd. All of the working solutions were kept at -4 °C. The working solutions were prepared once a week to prevent any deactivation of the active compound in the extract and to maintain the quality of the working solution.

### *Experimental Animals*

Eighteen Sprague Dawley male rats and ninety Sprague Dawley female rats with body weight 250-300 g, and two months old were used. They were kept in the animal house of Faculty Medicine and Health Sciences, University Putra Malaysia, under room temperature (29-32 °C), with 70-80% humidity, and automatic 12 hours light-dark cycle. The rats evaluated to be free from diseases and deformities. The rats were acclimatized for one week before starting treatments. They were group fed with pellet and drinking water was given *ad libitum*.

### *Experimental Design*

Randomized experimental design with 3 supplementations was used for this study. Experiments were carried out according to the guidelines for the use of animals and approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, University Putra Malaysia. The rats in each group were force fed with working solution according to their treatment groups (distilled water, 240 mg/kg Phaleria macrocarpa extract, Andriol® Testocap™) for seven weeks.

### *Parameters*

#### *Body Weight Measurement*

The body weight of male rats were measured weekly by using electronic balance (Scaltec SBA5T) throughout the experiment period.

#### *Cauda Epididymal Sperm Collection*

Animals were killed after ether anaesthesia and the cauda epididymidis was quickly removed. The adherent fat, blood vessels and connective tissue were cut away and the organ from each animal was placed in a hollow plate that contained normal saline to wash out the blood. The cauda epididymidis were cut longitudinally with a pair of fine-pointed scissors and compressing with forceps. The sperm were released by mincing the cauda epididymis into pieces on the Petri dishes that contained phosphate buffer saline (PBS) for sperm characteristics analysis. Since epididymis came in pairs, one cauda epididymis was put in a Petri dish

containing 10 ml of 0.1M PBS specifically for sperm count and sperm motility analysis while the other cauda epididymis was put in another Petri dish containing 1 ml 0.1M PBS for sperm viability and sperm morphology. The spermatozoa were allowed to flow out from cauda epididymis into the buffer. Then, the sperm suspensions were left at room temperature for 10 minutes for the suspension to allow sperm to swim out of the lumen of the cauda epididymidis for sperm characteristics analysis.

### *Sperm Characteristic Analysis*

*Sperm count analysis:* Sperm count was determined using the haemocytometer under light microscope. A cover slip was placed on the haemocytometer before a drop with 10 µl of caudal epididymal sperm solution was loaded under the cover slip. The haemocytometer was placed under the light microscope and viewed under x400 magnification. Sperm count was done by counting 4×4 squares (horizontally or vertically) as shown in Figure 2. Sperm count was determined using the formula below as described previously by Rathje *et al.*[13]

$$\text{Sperm count} = \text{total no. of sperm in 5 squares} \times 50{,}000 \times 100 \ (\text{cells/ml})$$

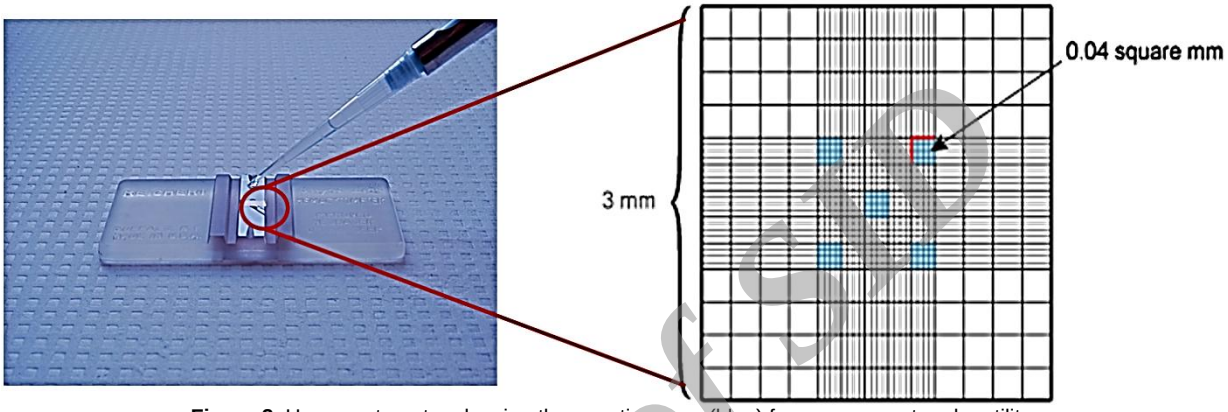


**Figure 2.** Haemocytometer showing the counting area (blue) for sperm count and motility.

Counting was only done for sperm heads that was found within the squares areas.

*Sperm motility analysis:* Haemocytometer was again used for sperm motility analysis. A cover slip was placed on the haemocytometer before a drop with 10 µL of caudal epididymal sperm solution was loaded under the cover slip. The haemocytometer was placed under the light microscope and viewed under x400. The light from the microscope was kept dim to reduce the heat effect on the sperm which can reduce its motility and kill them. The sperm was counted when it entered the 4×4 squares (horizontally or vertically) according to its motility grade as shown in Table 1. Before statistically analysed, the raw data were tabulated in the form of percentage using the formula:

$$\text{Percentage of sperm for particular grade} = \frac{\text{no.of sperm for particular grade} \times 100\%}{\text{Total no of sperm from all grades}}$$

**Table 1.** Sperm Motility Characteristics.

| Grade | characteristic |
|---|---|
| **Grade 1** | Sperm are immotile and fail to move. |
| **Grade 2** | Sperm known as non-progressive motility. They do not move forward despite the fact that they move their tail (vibrating-like movement). |
| **Grade 3** | Sperm known to have non-linear motility. They also move forward but tend to travel in a curved or crooked motion. |
| **Grade 4** | Sperm have progressive motility meaning that they are the strongest and swim fast in a straight line. |

*Sperm viability analysis:* This analysis used the sperm from the other cauda epididymis that was put in a Petri dish with 1 ml 0.1M PBS. On a clean glass slide, 1 drop of sperm suspension was gently mixed with 3 drops of eosin using the sharp glass slide end. After 30 seconds, 1 drop of nigrosin was mixed together with the solution and a smear was made. The smear was then air-dried and observed under x200 magnification of imaging microscope. The sperm was counted based on the degree of membrane permeability. The dead sperm showed pink colouration of the head whereas the viable sperm showed whitish or colourless head. Before statistically analysed, the raw data were tabulated in the form of percentage using the following formula:

$$\text{Percentage of viable} = \frac{\text{No.of viable sperm} \times 100\%}{\text{Total no of dead and viable sperm}x}$$

*Sperm morphology analysis:* Sperm morphology analysis used the same sperm smear made for sperm viability analysis. This time, the sperm were observed under x400 magnification of imaging microscope to clearly evaluate the morphology of the sperm head, neck and tail. The sperm were generally classified as normal or abnormal without further characterized the types of abnormality found on the sperm. The normal sperm was given a score of 100 and the abnormal one will be given score of 0 to enable statistical analysis by

using Statistical Analysis System (SAS) to be carried out easily.

### *Statistical Analysis*

Data analysis was performed using Statistical Analysis System (SAS) version 9.2. Data of body weight, serum testosterone, mounting latency and mounting frequency were subjected to analysis of variance (ANOVA) to analyze the significant treatment effect and the mean between group was compared using Duncan Multiple Range Test if F value was significant at $p<0.05$.

## Results

### *Body Weight*

The means of rats' body weight supplemented with aqueous extract of *Phaleria macrocarpa* for 6 weeks period was shown in Table 2. The mean of body weights of the negative control, *Phaleria macrocarpa* and positive control groups before the study was 344, 317 and 310 g respectively, while at the end of the study, their mean body weights were 341, 321 and 342 g respectively. The ANOVA showed that the body weight was significantly affected ($p<0.05$) by the treatment. The mean of body weight for the rats supplemented with *P.macrocarpa* aqueous extract was significantly lower ($p<0.05$) than the control groups.

**Table 2.** Effects of distilled water, *P. macrocarpa* aqueous extract and Andriol® Testocaps™ supplementation on the body weight (mean ± SE) (g) of adult male rats.

| Weeks | Treatment | | |
|---|---|---|---|
| | Negative Control ( Distilled water) (0 mg/kg) | Supplementation (*P.macrocarpa* aqueous extract) (240 mg/kg) | Positive Control ( Andriol® Testocaps™) ( 4 mg/kg) |
| 1 | 344.00 ± 7.61 | 317.00 ± 6.40 | 310.00 ± 9.68* |
| 2 | 324.00 ± 7.68 | 287.00 ± 8.59** | 359.00 ± 10.29* |
| 3 | 305.00 ± 8.07* | 342.00 ± 7.99 | 339.00 ± 8.92 |
| 4 | 368.00 ± 12.81 | 329.00 ± 5.68* | 347.00 ± 8.49 |
| 5 | 343.00 ± 9.50 | 330.00 ± 6.56 | 323.00 ± 4.28 |
| 6 | 341.00 ± 10.00 | 321.00 ± 5.07* | 342.00 ± 5.56 |
| **Total** | 337.00 ± 4.11 | 321.00 ± 3.88* | 337.00 ± 4.87 |

Data are presented as Mean ±Standard Error of Mean
* indicate significant variation at $p < 0.05$
** indicate significant variation at $p < 0.01$

### *Sperm Count*

The mean value of sperm count treated with distilled water (0 mg/kg), *P. Macrocarpa* aqueous extract (240 mg/kg) and Andriol® Testocaps™ (4 mg/kg) illustrated in Figure 3. The mean of sperm count is significantly highest in rats treated with 4 mg/kg Andriol® Testocaps™ (1112 million cells/ ml), followed by distilled water (712 million cells/ ml) and 240 mg/kg *P. macrocarpa* aqueous extract (707 million cells/ml). The results indicated that there was no significant difference between *P. macrocarpa* aqueous extract and negative control, while sperm count in positive control group was significantly different from those of *P. macrocarpa* aqueous extract and negative control.

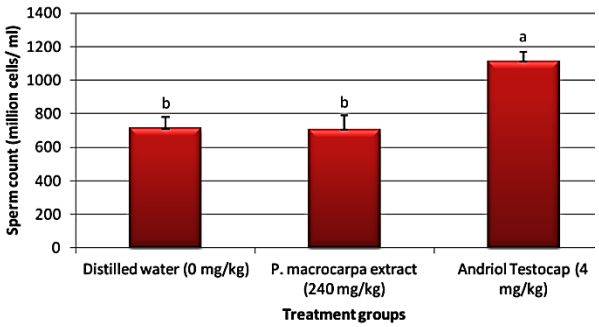


**Figure 3.** Means of sperm count in rats supplemented with *P.macrocarpa* aqueous extract and control groups for 6 weeks.
abc: different superscript indicated significance at $p<0.05$

### *Sperm Motility*

Generally the percentage of motile sperm following treatment period did not change from baseline in all groups which illustrated graphically in Figure 4.

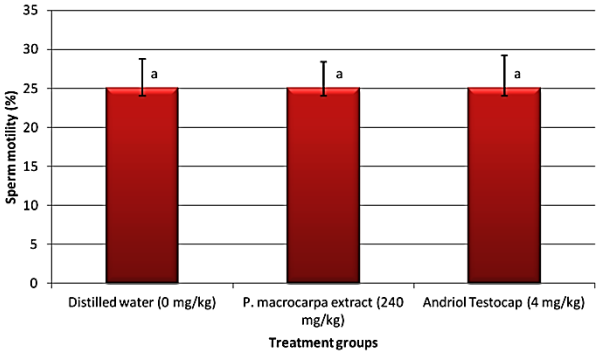


**Figure 4.** Means of motile sperm percentage in rats supplemented with *P.macrocarpa* aqueous extract and control groups for 6 weeks.
abc: different superscript indicated significance at $p<0.05$

However, grading of motility was done; the result showed that percentage of rats' motile sperm that treated with 240 mg/kg *P. macrocarpa* aqueous extract detected an increasing pattern from grade 1 to grade 3. Percentage of motile sperm with grade 3 from this group also was significantly higher than those control groups ($p < 0.05$). For grade 4 motility; there was no

significant difference between motile sperm percentage of *P. macrocarpa* aqueous extract and positive control group, but when compared with negative control group, the percentage of sperm treated with *P. macrocarpa* showed significant difference ($p < 0.05$).

### *Sperm Viability*

The sperm viability in rats treated with *P. macrocarpa* aqueous extract (240 mg/kg) was significantly higher ($p < 0.05$) as compared to rats treated with distilled water (0 mg/kg). Whereas, there was no significant difference between rats treated with *P. macrocarpa* aqueous extract (240 mg/kg) and Andriol® Testocaps™ (4 mg/kg) (Figure 5). The percentage of sperm viability was significantly increased ($p < 0.05$) in response to 6 weeks treatment with *P. macrocarpa* aqueous extract and Andriol® Testocaps™ (65.26%, 37.22% and 70.46% for *P. macrocarpa*, distilled water and Andriol® Testocaps™ respectively) (Figure 6).

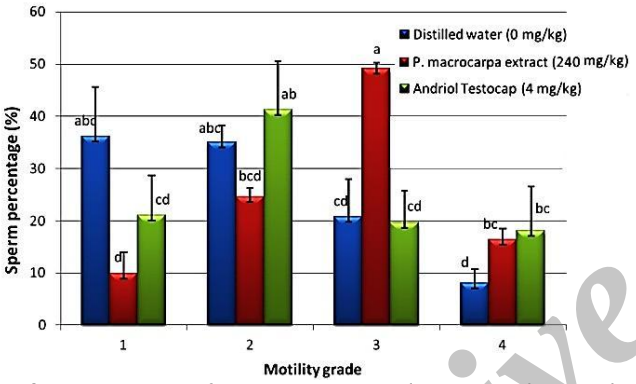


**Figure 5.** Means of sperm percentage in rats supplemented with P.macrocarpa aqueous extract and control groups according to motility grade.
abc: different superscript indicated significance at p<0.05

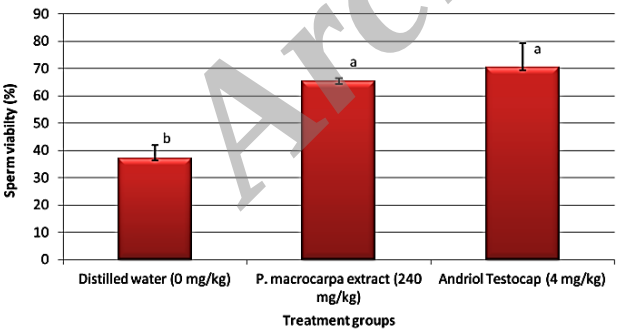


**Figure 6.** Means of viable sperm percentage in rats supplemented with *P.macrocarpa* aqueous extract and control groups for 6 weeks.
abc: different superscript indicated significance at p<0.05.

### *Sperm Morphology*

The study showed that sperm morphology did not affect by treatment protocol and there was no difference in percentage of sperm morphology after 6 weeks supplementation by either *P. macrocarpa or* distilled water and Andriol® Testocaps™ ( Figure 7 and Figure 8 a, b, c) and the percentage of normal sperm morphology were 50% across all supplementation groups.

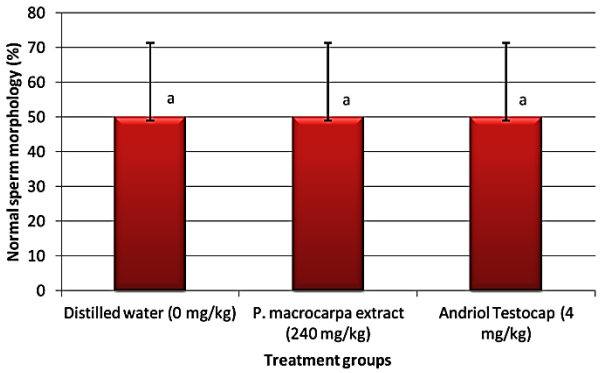


**Figure 7.** Percentage of normal sperm morphology in rats supplemented with *P.macrocarpa* aqueous extract and control groups for 6 weeks.
abc: different superscript indicated significance at p<0.05

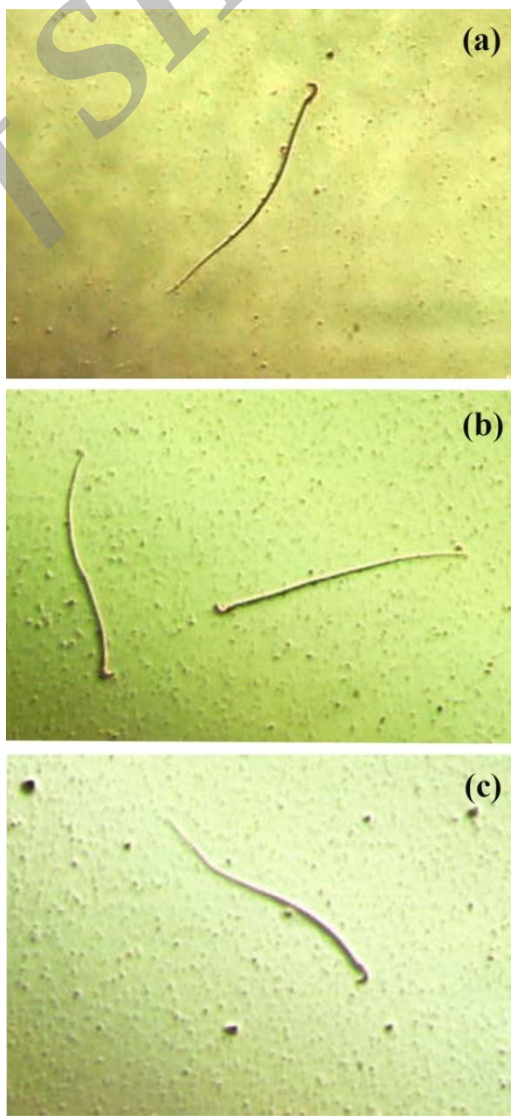


**Figure 8.** Normal sperm morphology observed (x 400) in male rats supplemented with **a)** distilled water **b)** *P. macrocarpa* (240 mg/kg) **c)** Andriol® Testocaps™ (4 mg/kg).

## Discussion

This study aimed to evaluate the effect of *Phaleria macrocarpa* on sperm characteristics of adult rats during 7 weeks supplementation in comparison to either testosterone hormone as positive control or distilled water as negative control. In order to monitor the health condition of the rats, their body weight was measured every week. After 6 weeks of treatment, the mean of body weight for rats given 240 mg/kg *Phaleria macrocarpa* fruit aqueous extract was decline significantly as compared to both control groups. The reduction of body weight of rats supplemented with *Phaleria macrocarpa* might be due to its saponin content that has stimulatory effect on testosterone hormone. Gray *et al.*[14] reported that long-term treatment with high dose of testosterone reduce body weight gain and carcass fat content. Besides that, a study by Chong[15] showed that *Phaleria macrocarpa* significantly reduce body weight gain, total cholesterol, triglyceride, HDL and LDL and up regulated hepatic LDL receptor.

For the purpose of monitoring the fertility of rats, some andrological parameters f the Sprague Dawley rats were evaluated including sperm count, sperm motility, viability and morphology. The mean of sperm count of rats from *Phaleria macrocarpa* treated group showed no significant difference when compared with negative control group. The reduction seen in sperm count of rats treated with *Phaleria macrocarpa* can be due to the increase in mounting frequency in rats of the same group. This result is consistence with result found by Che Zairieha[16] who observed that the testosterone concentration level and mounting frequency was the highest in rats supplemented with *Phaleria macrocarpa.* Mounting frequency is a strong indicator of the occurrence of sexual performance. In addition, *Phaleria macrocarpa* contain saponin as one of its active compound. Saponin-rich extracts are able to improve the sexual performance as seen in rat model.[17]

In this study, sperm was collected from cauda epididymis, which is the primary sperm storage site prior to ejaculation. During the storage period, the cauda epididymis accumulates sperm to ensure that a sufficient number is available at the time of ejaculation.[18] During the seventh week of the treatment, the female rats were put in the cages of the male rats to enable the assessment of libido behavior. This assessment was carried out for five consecutive days. After 24 hours, the rats were sacrificed to obtain the sperm. This study design did not give the rats any sexual rest to allow the cauda epididymis to be sufficiently filled with mature sperm. Gloria *et al.*[19] also reported that sperm concentration in the cauda epididymis could depend on factors such as sexual rest and semen collection frequency. Besides that, there is a general agreement that semen volume and sperm concentration increase with prolonged sexual abstinence as reported by some scholars.[20-22] This explained the lower sperm count retained in the cauda epididymis in *Phaleria macrocarpa* treated rats.

According to Oyeyemi *et al.*,[23] sperm motility is one of the most important indices determining the ability of a male to produce viable sperm. It is expressed in a percentage of all moving sperm in a sample. In this study, total mean percentage of motile sperm was not changed in all groups of treatments. The mean value of percentage of motile sperm was 25% across all treatment groups. This might be due to the sperm sampling which was collected from cauda epididymis. A study conducted by Gloria *et al.*,[19] reported that progressive motility for epididymal sperm was lower than ejaculated sperm. A correlation between mitochondrial activities and motile sperm has been shown using cytochemical techniques and flow cytometry. Hung *et al.*,[24] also showed rhesus macaques that ATP from mitochondria contributes to sperm motility. Besides that, the ejaculated sperm may have better progressive motility due to the presence of fructose in the fluid secreted by seminal vesicle. Fructose is the simple sugar that acts as the energy source for the sperm to keep swimming in its journey to fertilize an ovum. To compare, fluid from epididymis only allow sperm to survive for a few weeks instead of keeping them motile.

However, the increasing pattern of the percentage of motile sperm from grade 1 to grade 3 motility for rats supplemented with *Phaleria macrocarpa* indicated favorable effect of *Phaleria macrocarpa* in exerting motility of sperm compared to the negative and positive control groups. In order to fertilize an ovum, an ideal sperm should at least have graded as 3. For grade 4 motility, the percentage of sperm for rats treated with *Phaleria macrocarpa* was significantly higher than distilled water group. This result indicated that *Phaleria macrocarpa* supplementation increased the sperm motility by increasing the percentage of the sperm with higher motility grade.

Viable sperm can be defined as sperm that is alive and capable of fertilizing an ovum. In motility analysis, grade 1 sperm are immotile and fail to move. But, in certain cases, immotile sperm are still viable. Therefore, in cases of low motility as well as to further confirm the status of viability of the sperm, viability analysis was carried out. By using the eosin-nigrosin staining method, the viable and dead sperm was able to be differentiated. According to Björndahl *et al.*,[25] this classification was based on the degree of membrane permeability of dead spermatozoa which heads showed pink or red coloration due to the breakage of the membrane.

*Phaleria macrocarpa* showed significant increase in percentage of viable sperm as compared to negative control group. This may be due to *Phaleria macrocarpa* potential in increasing secretion of testosterone hormone through the presence of saponin. This result is in agreement with Koumanov *et al.*,[26] who found that saponin has the potency to increase

testosterone hormone level which is the principal male reproductive hormone and this hormone play a huge role that affects sperm quality.

The increase in percentage of viable sperm in rats given *Phaleria macrocarpa* was also parallel with the result from Che Zairieha[16] that found significantly highest testosterone concentration in *Phaleria macrocarpa* treated rats as compared to rats given distilled water and Andriol® Testocaps™. In addition, a study conducted by Nakayama *et al.*,[27] found that sperm ATP concentration in testosterone-injected group is higher than control group. Generally, the function of mitochondria is the production of energy in the form of ATP. According to Gloria *et al.*,[19] viability and mitochondrial activity were higher in epididymal sperm as compared to ejaculated sperm. Therefore, measurement of sperm ATP concentration may become a possible biochemical method to measure actual fertilizing potential of sperm.[28]

Morphology can be defined as the structure and form of organisms to include the anatomy, histology and cytology at any stage of its life history.[29] Several studies in the literature have reported that percentage of normal sperm morphology is an essential characteristic for in vivo fecundity and in vitro fertilization.[30] Most of these studies indicate that morphology is the best predictor among all of the sperm characteristics. Based on the result obtained, the percentage of normal sperm morphology was not affected across all treatment groups including the rats given *Phaleria macrocarpa*. This indicate that *Phaleria macrocarpa* maintain sperm morphology and does not cause any defect to the morphology of the sperm produced.

## Conclusion

The study showed an increment in sperm characteristics in response to *Phaleria macrocarpa* without causing any defect to the morphology of the sperm produced. This indicated that *Phaleria macrocarpa* was at the safe level and did not have toxic effects on the sperm. Thus, it can be concluded that *Phaleria macrocarpa* offers an alternative way to improve male fertility by improving the sperm quality.

## Conflict of Interest

The authors report no conflicts of interest.

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