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Effect of Javanese Ginseng (*Talinum Paniculatum* (Jacq.) Gaertn) Leaf Extract on Spermatozoa Quality of Mice (*Mus Musculus*)

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ABSTRACT

Java Ginseng (*Talinum paniculatum* (Jacq.) Gaertn) or commonly known as som jawa is one of the local plants in Java. Pharmacological studies have proven its potential as an antidiabetic, antinociceptive, antimicrobial, antioxidant and to treat digestive disorders. Research on Javanese Ginseng leaves proves its benefits on improving mating behavior and increasing libido of male rats, while its roots are proven to improve the quality of mice spermatozoa, but there has been no further research on the effect of Javanese ginseng leaf on the quality of mice spermatozoa. This study aims to determine the effect of Javanese ginseng leaf extract on testicular weight and spermatozoa quality of mice. The design used was a one-factor RBD with 4 treatments, namely the control group (P0) which was only given food and drink, group P1 was given a dose of *T. paniculatum* (Jacq.) Gaertn leaf extract in a number of doses of 0,98mg/20g, 2,8mg/20g, 7,7mg/20g. Each treatment consisted of 5 replicates. The parameters observed were testicular weight, amount, motility, viability, morphology of male mice cauda epididymis spermatozoa. Giving ethanol extract of *T. paniculatum* (Jacq.) Gaertn leaves to mice by gavage for 16 days. The observational data were analyzed with ANOVA (Analysis of Variance) and continued with Duncan's test at a level of 5%. Based on the results of the study that have been analyzed using one way ANOVA, it shows that ginseng leaf extract increases testicular weight, type A motility, viability and amount of spermatozoa, but morphology between control and treatment groups (P1, P2, P3) are not significantly different ($p>0,05$). The most effective dose of *T. paniculatum* leaf extract was 7,7mg/20mg (treatment P3) which showed the best results in the parameters and was significantly different from treatment P0 (control).

1. Introduction

Research from the World Health Organization (WHO) shows that 11-15% of couples who have difficulty getting offspring are due to male infertility factors (WHO, 2021). Infertility in men can be caused by a variety of factors. Biological factors such as genetic abnormalities, antibodies and systemic diseases. Environmental factors such as exposure to toxins or radiation and lifestyle factors such as smoking, actively drinking alcoholic, using illegal drugs also affect infertility. Male infertility can be treated in various ways, such as with hormone therapy, drugs or surgery. Hormone therapy or drugs generally have side effects such as headaches, nausea, seizures, increased blood pressure, blurred vision and resistance (WHO, 2018). The use of traditional medicines can be used as an alternative treatment due to the lack of side effects.

Traditional medicine is closely related to Indonesia. Local people often utilize plants to solve various health problems, both as complementary and substitute drugs. Indonesia has many efficacious plants, one of which is Javanese ginseng (*Talinum paniculatum* (Jacq.) Gaertn) or commonly called som jawa. It is one of the plants that can be used as traditional medicine.
Javanese ginseng is commonly used for cooking by Indonesians, especially the leaves. Ginseng leaves are cooked and used as vegetables for side dishes. Phytochemical screening and standardization of Javanese showed the presence of flavonoids, saponins, tannins and other polyphenolic compounds (Suharsanti and Sulistyanto, 2012). The types of flavonoids known are quersetin, kaempferol, anthocyanins, chlorogenic acid, caffeic acid, and ferulic acid (Andarwulan et al., 2012). The content is similar to Korean ginseng which is believed to help increase stamina and male sexual performance.

Javanese ginseng roots can be used as herbal medicine to overcome body fitness, relieve fatigue, cold sweats, dizziness, aphrodisiac, cough phlegm, lung inflammation, diarrhea, irregular menstruation and vaginal discharge (Ikhtimami, 2012). Ginseng leaves can be used to increase breast milk production, increase appetite, as a medicine for ulcers, and aphrodisiac (Hariana, 2008).

Several studies show that Javanese ginseng's root affects the quality of spermatozoa. Javanese ginseng roots have been shown to maintain and increase the number of type A spermatozoa from mice (Dari, 2020). The content possessed by the roots can be used as an assumption that the leaves have the same potential. Research by Septiani (2021) shows that Javanese ginseng leaves affect the mating behavior and libido of male rats. Thus, Javanese ginseng leaves can be used as an aphrodisiac. However, there is no scientific data on the effect of Javanese ginseng leaves on spermatozoa quality. The use of leaves is expected to increase the conservation value of the plant, as there is no need to cut the whole plant. The leaves can also be processed in various ways, such as being made into tea or even side dish.

2. Methodology

2.1 Time and place of study

This research was conducted from February 2023 to April 2023. Plants obtained from Baturiti, Tabanan, Bali. Treatment of the experimental animals is carried out at the experimental animal maintenance room of Biology Study Program, Udayana University. Extracts of *T. paniculatum* were made at the Biochemistry Laboratory and the Laboratory of Genetic Resources and Molecular Biology, Udayana University. The surgery was carried out at the Laboratory of Animal Structure and Development in the Biology Study Program, Udayana University.

2.2 Tools and materials

The materials that have been applied to this research were *T. paniculatum*, mice (*M. musculus*), 96% ethanol, aquadest, 0.9% NaCl, 1% eosin, 2% formalin, filter paper, cotton, tissue, and husks. The tools used were microscopes, optilab, glass bottles, glass slides, surgical instruments (scissors, knives, tweezers, needles), glass objects, trays, sonde, a rectangular cage (plastic tub) with a size of 33x22x15cm, the place for mice to eat and drink.

2.3 Methods

Research design
A one-factor Randomized Block Design (RBD) was used in this study, which consisted of 4 treatments with 5 replications. The treatment of the liquid organic fertilizer application, namely P0 (control, without application of the extract), P1 (0,98mg), P2 (2,8mg), and P3 (7,7mg)

T. paniculatum leaf extract-making process
leaves are sorted wet to separate from the dirt that is attached and then washed thoroughly. After that, it is dried by aerating and not exposed to direct sunlight until it dries, after that it is made into powder by means of a blender. Then 150 grams of extract powder was macerated using 1.5 L 96% ethanol (1:10) for 3 days while stirring. The results are filtered with filter paper to obtain the filtrate and concentrated using a rotary evaporator until the solvent evaporates so that in the end a thick extract is obtained then the results are weighed and several dosages are made which can improve sperm quality in mice.

Measurement of *T. paniculatum* leaf extract dosage
In this study, the dosage of the extract was made refers to research by Septiani (2021):
Average body weight of mice = 20 g = 0,020kg
Dose conversion of rats to mice 0,14

- Dose of 7mg/200g BW Rats

7mg X 0,14
= 0,98mg/20g BW mice
0,98mg will be dissolved with 0,5mL of distilled water

- Dose of 20mg/200g BW Rats

20mg X 0,14
= 2,8mg/20g BW mice
2,8mg will be dissolved with 0,5mL of distilled water

- Dose 55mg/200gBB Rats

55mg X 0,14
= 7,7mg/20g BW mice
7,7mg will be dissolved with 0,5mL of distilled water

T. paniculatum extract application on mice
T. paniculatum extract that has been dissolved with distilled water, then given by gavage with a volume not exceeding the intragastric volume (0.5 ml) of mice. The treatment was carried out on mice once every day, namely at noon at 12.00-14.00 WITA for 16 days with a dose of 0.98mg/20g, 2.8mg/20g, 7.7mg/20g BW mice. On the 17th day, observations were made with the same parameters. Observations included the testicular weight, number, motility, viability, and morphology of the spermatozoa of male mice cauda epididymis

Right testicular weight measurement on mice
After 16 days of treatment, on the 17th day, the male mice were sacrificed and then the right testicle was taken. The organ was taken and washed using 0.9% NaCl, In the next step the testes were placed on filter paper to remove the remaining NaCl, then weighed with digital scales and expressed in grams (g).

The number of motile cauda epididymal spermatozoa
The left cauda epididymis was placed in a petri dish that already contained 1 ml of physiological saline solution (NaCl 0.9%) at 37oC, then cut into pieces with scissors so that the liquid came out, then stirred until a homogeneous suspension was formed. The spermatozoa suspension was placed on a hemocytometer and observed under a microscope (Wibisono, 2010). Calculations were carried out on motile and non-motile spermatozoa from 100 spermatozoa and the results were in percent form.

Observation of the cauda epididymal spermatozoa viability
Observation and calculation of spermatozoa viability were carried out using sperm smear preparations stained with 1% eosin and observed using a light microscope with 400 x magnification. Viability was determined by not absorbing the eosin dye in spermatozoa cells.

Observation of the cauda epididymal spermatozoa count.
The suspension from observation 3.6.6 was placed into a counting chamber (hemocytometer), then the hemocytometer which already contained a suspension of spermatozoa was then observed under a light microscope with a magnification of 40 X 10 mediated by optical lab software and then photographed. Spermatozoa counted were spermatozoa in 25 large hemocytometer boxes. The spermatozoa head is used as a guide for calculation, the number of spermatozoa is expressed in million/ml.

2.4 Data analysis
The analysis of the data was conducted using one-way ANOVA. If there is a significant effect ($p \leq 0.05$) between treatments, a follow-up test was carried out with the Duncan test (Duncan Multiple Range Test) at the level of 5% using the IBM SPSS Statistics 22 program to determine the difference between treatments.

3. Results and Discussion

3.1 Result

Testicular weight

The results of the statistical test showed that the application of various dose of *T. paniculatum* leaf extract had a significant effect on the average testicular weight of mice at treatment P1, P2 and P3 observations. The lowest average of testicular weight at three observation was found in treatment P1 (extract dose 0,98mg/20g BW) meanwhile the highest average of testicular weight was found in treatment P3 (extract dose 7,7mg/20g BW). Duncan's test showed that treatments P1, P2 and P3 has been significantly different compared to treatment P0, but treatment P2 is insignificantly different compared to treatment P1. Data related to the effect of applying different doses of extract of *T. paniculatum* leaf extract on the testicular weight of sperm of mice are presented in Table 1.

Table 1. The effect of *Talinum paniculatum* leaf extract on testicular weight (gr)

Treatment	Testicular Weight (gr)
P0	0,05 ± 0,013 a
P1	0,07 ± 0,011 b
P2	0,08 ± 0,011 b
P3	0,12 ± 0,016 c

Note: The values in the table above show the average of 20 mice and 4 groups, the numbers accompanied by a different letter notations in the same column show significantly different average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA; P0 = control treatment (without application of extract); P1 = treatment of extract dose 0,98mg/20g BW; P2 = treatment of extract dose 2,8mg/20g BW; P3 = treatment of extract dose 7,7mg/20g BW

Amount of spermatozoa

The results of the statistical test showed that the application of various doses of *T. paniculatum* leaf extract had a significant effect on the number of spermatozoa. The lowest average of testicular weight at three observations was found in treatment P1 (extract dose 0,98mg/20g BW) meanwhile the highest average of testicular weight was found in treatment P3 (extract dose 7,7mg/20g BW). Duncan's test showed that treatments P1,P2 and P3 have been significantly different compared to treatment P0, but treatment P2 is insignificantly different compared to treatment P1. Data related to the effect of applying different doses of *T. paniculatum* leaf extract on the number of spermatozoa are presented in Table 2.

Table 2. The effect of *T. paniculatum* leaf extract on amount of spermatozoa (gr)

Treatment	Amount of spermatozoa (million/ml)
P0	202,20 ± 13,25 a
P1	208,80 ± 11,73 a
P2	218,80 ± 11,41 ab
P3	230,40 ± 12,52 b

Note: The values in the table above show the average of 20 mice and 4 groups, the numbers accompanied by different letter notations in the same column show significantly different average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA; P0 = control treatment (without application of extract); P1 = treatment of extract dose 0,98mg/20g BW; P2 = treatment of extract dose 2,8mg/20g BW; P3 = treatment of extract dose 7,7mg/20g BW

Motility of spermatozoa

The results of the statistical test showed that the application of various doses of *T. paniculatum* leaf extract had a significant effect on the type A motility of spermatozoa in mice, but an insignificant effect on type B, C and D. The lowest average of type A motility at three observation was found in treatment P1 (extract dose 0,98mg/20g BW) meanwhile the highest average of type A motility was found in treatment P3 (extract dose 7,7mg/20g BW). Duncan's test showed that at type A, treatment P3 has been significantly different compared to treatment P0, but treatment P1 and P2 were insignificantly different compared to treatment P0. Data related to the effect of applying different doses of *T. paniculatum* leaf extract on the motility of sperm of mice are presented in Table 3.

Table 3. The effect of *T. paniculatum* leaf extract on spermatozoa motility

Treatment	Motility			
	Type A	Type B	Type C	Type D
P0	53,20 ± 5,49 a	26,00 ± 3,87 a	18,20 ± 4,60 a	13,40 ± 2,07 a
P1	54,80 ± 5,63 a	26,60 ± 1,67 ab	18,00 ± 5,09 a	13,80 ± 1,48 b

P2	59,60 ± 3,64 a	23,00 ± 2,55 ab	16,20 ± 3,96 a	11,00 ± 2,00 bc
P3	66,80 ± 4,14 b	21,80 ± 4,14 b	12,20 ± 4,08 a	7,80 ± 1,94 c

Note: The values in the table above show the average of 20 mice and 4 groups, the numbers accompanied by a different letter notations in the same column show significantly different average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA; P0 = control treatment (without application of extract); P1 = treatment of extract dose 0,98mg/20g BW; P2 = treatment of extract dose 2,8mg/20g BW; P3 = treatment of extract dose 7,7mg/20g BW

Morphology of spermatozoa

The use of various doses of *T. paniculatum* leaf extract to mice had an insignificant effect on the morphology of spermatozoa. Normal morphology with the smallest average is found in mice with treatment P1 (extract dose 0,98mg/20g BW) which is 61,00%. Mice that are not given leaves extract or treatment P0 (control, which was only given food and drink) produce the second smallest normal sperm morphology which is 63,40% while the biggest average is found in mice with treatment P3 (extract dose 7,7mg/20g BW), which is 68,00%. Data related to the effect of applying different doses of *T. paniculatum* leaf extract on the morphology of spermatozoa are presented in Table 4.

Table 4. The effect of *T. paniculatum* leaf extract on the morphology of spermatozoa (%)

Treatment	Normal sperm morphology (%)	Abnormal sperm morphology (%)
P0	63,40 ± 3,78 a	33,20±2,28 a
P1	61,00 ±5,70 a	29,20 ±4,970 a
P2	64,60 ±2,07 ab	30,80 ±3,49 a
P3	68,00 ±2,34 c	30,00 ±3,67 a

Note: The values in the table above show the average of 20 mice and 4 groups, the numbers accompanied by different letter notations in the same column show significantly different average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA; P0 = control treatment (without application of extract); P1 = treatment of extract dose 0,98mg/20g BW; P2 = treatment of extract dose 2,8mg/20g BW; P3 = treatment of extract dose 7,7mg/20g BW

Viability of spermatozoa

The results of the statistical test showed that the application of various doses of *T. paniculatum* leaf extract had a significant effect on the viability of spermatozoa. The lowest average of testicular weight at three observations was found in treatment P1 (extract dose 0,98mg/20g BW) meanwhile the highest average of testicular weight was found in treatment P3 (extract dose 7,7mg/20g BW). Duncan's test showed that treatment P3 has been significantly different compared to treatment P0, but treatments P1 and P2 are insignificantly different compared to treatment P0. Data related to the effect of applying different doses of *T. paniculatum* leaf extract on the amount of viability of spermatozoa are presented in Table 5.

Table 5. The effect of *T. paniculatum* leaf extract on viability of spermatozoa

Treatment	Viable	Non Viable
P0	67,40±4,50 a	31,00 ± 2,73 a
P1	62,20±4,76 ab	31,80 ± 3,11 a
P2	67,80 ±4,20 ab	30,20 ± 4,43 a
P3	71,00 ± 2,90 b	28,00 ± 1,58 a

Note: The values in the table above show the average of 20 mice and 4 groups, the numbers accompanied by different letter notations in the same column show significantly different average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA; P0 = control treatment (without application of extract); P1 = treatment of extract dose 0,98mg/20g BW; P2 = treatment of extract dose 2,8mg/20g BW; P3 = treatment of extract dose 7,7mg/20g BW

3.2 Discussion

Mice Testicular weight

Treatment of *T. paniculatum* leaf extract to mice had significant effect on the average testicular weight. This happens because according to Thanamool et al. (2013) *T. paniculatum* leaf extract contains phytoestrogens. Phytoestrogens are secondary metabolites of plants that have a structure similar to endogenous estradiol and are able to bind to estrogen receptors. Phytoestrogens can be found in grains, fruits, and vegetables. Research by Gunnarsson et al. (2009) proved that phytoestrogens can stimulate testosterone synthesis in male goats by increasing the secretion of T3; a hormone known to stimulate Leydig cells. Plasma T3 concentrations were significantly higher in animals fed phytoestrogens, suggesting that the underlying cause of increased testosterone production is the stimulation of T3 secretion. The study confirmed that T3 has an important role in Leydig cell differentiation and function. Leydig cells will later produce testosterone. Another study by Wyse et al. (2022) said that phytoestrogens were shown to increase body weight, growth, and testicular circumference and higher sperm count in bulls. Testicular size is very important to predict the level of testosterone hormone. This was also proven by Akbar et al. (2014) that there is a relationship between testosterone levels and testicular size (circumference and volume) in Timor deer.

Spermatozoa quality

Treatment of *T. paniculatum* leaf extract to mice had a significant effect on increasing the number of spermatozoa.
This increase can occur because according to Rusli and Towaha (2010) the presence of bioactive compounds that can increase the activity of the immune system is very helpful to overcome the decline in the immune system and Javanese ginseng leaves are empirically used to strengthen the immune system. Javanese ginseng leaves by de Araújo Borges Menezes et al. (2021) are a source of nutrients and the leaf extract has antioxidant and antibacterial potential that can be used as a supplement in the diet to improve health. Javanese ginseng leaves also contain phytoestrogens where phytoestrogens by Wyse et al. (2022) proved to increase the number of spermatozoa in bulls. Treatments P1, P2, and P3 have higher mean than P0, but only P3 is significant to P0. The higher average value and significant against P0 can be said that the P3 dose is effective in increasing the number of spermatozoa.

The results showed that treatment of *T. paniculatum* leaf extract in mice had a significant effect on increasing motility type A of spermatozoa. Based on research by Kao et al. (1998) the movement of spermatozoa requires a certain amount of ATP energy used to move the flagellar apparatus, disruption of mitochondrial respiration function can lead to decreased motility and fertility. Ruiz-Pesini et al. (2000) proved that there is a correlation between mitochondrial DNA haplogroup variation and sperm motility. Spiropoulus et al. (2002) also found mtDNA mutations strongly correlated with reduced sperm motility. Point mutations or deletions that occur in mitochondrial DNA can interfere with spermatozoa motility. The function of mitochondria in cells is to produce energy in the form of ATP (adenosine triphosphate). Most of the ATP is produced through the process of oxidative phosphorylation. The energy produced is used by cells for homeostasis, regulation, division, motility, and death (St. John et al., 2005). Java ginseng leaves by de Araújo Borges Menezes et al. (2021) is a source of nutrients and the leaf extract has antioxidant potential. Based on research by Nurjannah and Asadatun (2011), antioxidant intake can prevent cardiovascular disease by reducing the rate of fat oxidation due to its role as an antioxidant. According to Andarina and Jauhari (2017) to neutralize the free radicals produced, antioxidants can eliminate the harmful effects of these free radicals. Under normal conditions, the balance between free radicals and antioxidants is maintained. If the balance of the two factors is disturbed, so that the number of antioxidant enzymes decreases and the number of free radicals increases, infertility disorders will occur. The unimpaired mitochondrial function indicates the absence of substances or compounds that cause mitochondrial mutations. The treatment P3 has a slightly higher standard deviation than P2, but P3 is significant to P0 compared to P2 so it can be concluded that the P3 dose is the most effective for increasing spermatozoa motility.

These results have shown that treatment of *T. paniculatum* leaf extract to mice had a significant effect on the viability of spermatozoa. According to Salmah (2014) the percentage of live spermatozoa is determined by the intact plasma membrane. The role of the spermatozoa plasma membrane is to protect spermatozoa organelles and transport electrolytes for spermatozoa metabolism. Damaged plasma membranes will affect the physiological and metabolic functions of spermatozoa and even cause spermatozoa death. According to Azzahra et al. (2016), the intact plasma membrane correlates with spermatozoa motility, the more intact spermatozoa plasma membrane, the more motile spermatozoa. This is directly proportional to the results of the study where there was an increase in motility.

Based on research by Sutanto et al. (2017) free radicals or ROS are molecules that are formed when oxygen molecules combine with other molecules and odd electrons will be formed. Oxygen molecules have stable paired electrons, if there are unpaired electrons in the outer orbit the result is that oxygen will be reactive and unstable. The unpaired oxygen molecule will seek and seize electrons from nearby vital components to release extra energy and return to a stable state. If the free radicals do not bind to antioxidants, the oxidation reaction continues or a cascade leading to cell damage is established. The number of free radicals or oxidants that exceed the healing ability of antioxidants in the cell will cause oxidative stress. Oxidative stress experienced by cells causes cellular damage through the breakdown of important molecules such as DNA, proteins, and lipids. Oxidative stress will

cause excessive lipid peroxidation of the plasma membrane. The plasma membrane of spermatozoa is sensitive to ROS because the majority of the sperm plasma membrane consists of PUFA (Polyunsaturated Fatty Acid). The resulting lipid peroxidation causes leakage in membrane structures (cellular and organelle) and causes cell function disorders, causing rapid loss of intracellular ATP, DNA damage, damage to axons, and apoptosis of spermatozoa cells which ultimately causes morphological abnormalities and decreased spermatozoa motility.

Research by de Araújo Borges Menezes et al. (2021), Septiani et al. (2021), and Suharsanti and Sulistyanto (2012) prove that Javanese ginseng leaves have antioxidant activity. Antioxidants function to add or remove one electron to neutralize ROS, so that free radicals become stable and inhibit the oxidation process. Antioxidants protect cells from free radical damage by donating one free electron to free radicals or accepting one unstable electron so that it becomes stable and stops chain reactions and prevents damage to lipids, proteins, and DNA. Lachman et al. (2003) said polyphenolic compounds, especially flavonoids are effective antioxidants because of their ability to bind free radicals from fatty acids and oxygen. Treatment P3 is significant against P0. Treatment P3 has a smaller standard deviation value than P1 and P2. The average of treatment P3 is also greater than P1 and P2, so it can be concluded that the P3 dose is effective in increasing the level of viability.

The application of *T. paniculatum* leaf extract to mice in various doses produces an insignificant influence on morphology. The effect that is not statistically significant is thought to be due to research conducted in a very short time. According to Hess et al, (2008), the length of spermatogenesis in mice is 35.5 days with 4 seminiferous epithelial cycles, where 1 epithelial cycle takes 207 ± 6 hours or 8.65 days. This study was conducted for 16 days, meaning that Javanese ginseng leaf extract only affects 2 out of 4 epithelial cycles. To be able to observe the overall quality of spermatozoa appropriately, the study should be conducted for at least 36 days.

4. Conclusion

The testicle's weight and quality of spermatozoa can be increased by the use of *T. paniculatum* leaf extract in the concentration range of 0,98-7,7mg extract dose on observation parameters and had a significant effect on the parameters of amount, motility also the viability of mice spermatozoa, while it has no significant effect on morphological parameters. The most effective concentration in increasing the testicle's weight and quality of mice spermatozoa is treatment P3 (application of extract dose 7,7mg/20g BW) with results that differ significantly from treatment P0 (control) in the parameters of amount, motility and viability of spermatozoa.

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