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Effect of Papaya Seeds *(Carica papaya)* and Gandarusa Leaves (*Justicia gendarussa)* on the Spermatozoa Quality of Male Mice

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Abstract

Papaya seeds (*Carica papaya* L.) and gandarusa leaves (*Justicia gendarussa* Burm.f.) are considered medicinal plants that can be used as natural male contraceptives. Papaya seeds contain alkaloids, triterpenoids, saponins, and papain. Meanwhile, the gandarusa leaves contain flavonoids. These compounds have the function of antifertility for men. The purpose of this study was to determine the effect of papaya seeds (*Carica papaya* L.) ethanol extracts, gandarusa leaves (*Justicia gendarussa* Burm.f.), and its combination on motility, viability, and morphology of male mice spermatozoa (*Mus musculus)* most effective decrease concentration on motility, viability, and morphology of male mice spermatozoa (*Mus musculus*). This research was an experimental study. The samples of this study were 18 male mice with a body weight of 20-30 grams, aged 3-5 months, and were divided into six groups. The treatments given were control, papaya seed ethanol extract, gandarusa leaves ethanol extract, and its combinations to concentrations of P1 Na CMC1%, P2 (papaya seed 100mg/kgW), P3 (gandarusa leaf 50mg/kgW), P4 (papaya seed : gandarusa leaf 50:50 mg/kgW), P5 (papaya seed : gandarusa leaf 50:100 mg/ kgW), P6 (papaya seed : gandarusa leaf 100:50 mg/kgW). The extract was given for 35 days. On the 36th day, the mice were dissected to take their epididymis to observe the spermatozoa's quality (motility, viability, morphology). Those observations used a light microscope. Data were analyzed using one-way ANOVA and LSD post hoc tests. This study resulted differences in P1, P2, P3, P4, P5, and P6 towards motility, viability, and morphology of spermatozoa ($p< 0.05$). The conclusion of this study showed that both single and combination ethanol extracts of papaya seeds and gandarusa leaves could reduce the motility, viability, and morphology of spermatozoa of male mice, and the effective dosage in reducing motility, viability, and morphology of spermatozoa is P3 gandarusa leaves 50mg/kgW.

Keywords: Ethanol extract; Gandarusa leaves; Papaya seeds; Spermatozoa

1. INTRODUCTION

The high urban population growth rate in developing countries has caused many problems. The most obvious problems are the uncontrolled growth rate and the increase in poverty rates in many cities. Therefore, the government has planned family planning programs (*KB*) to overcome this problem (Margono, 2013).

Family planning (*KB*) is an effort to control childbirth, ideal pregnancy interval and age pregnancy, natality, and control pregnancy through protection and assistance promotion according to reproductive rights to create a good quality family. Family planning allows couples of childbearing age to prevent pregnancy, plan the number of their children, and measure the interval of births (Dewi and Rahmawati, 2019)

Indonesia is implementing the family planning program (KB). It is used to achieve a thriving and healthy family. Data from the National Population and Family Planning Agency (*BKKBN*) shows that female contraceptive methods used (93.66%) are much higher than male contraceptive methods (6.43%). It shows that men's participation in contraceptive methods is minimal, and women still dominate contraception use (Walansendow *et al*., 2016).

Moreover, it has been found that 18 types of medicinal plants in Indonesia have the potential to be male antifertility (Febrianti and Sukarjati, 2015). It is a plant that contains alkaloids, which can affect spermatogenesis and suppress the secretion of reproductive hormones. Flavonoids can reduce the motility and viability of spermatozoa (Laili, 2018). Indian people use gossypol and papaya (Carica papaya) for male contraception (Lohiya et al. 2015). Giving papaya seed extract can cause infertility with reduced fertility, decreased sperm count, and motility (Pathak et al. 2018).

One medicinal plant with alkaloids and flavonoids that can affect sperm quality is papaya (*Carica papaya* L.). People in Indonesia have long used this plant as medicine. Based on prior research, one of the effective herbal contraceptives is papaya seeds (Satyarsa *et al*., 2017). Young papaya (*Carica papaya* L.) seeds of the California variety have a higher active compound content than old ones. Young papaya fruit can be seen from the fruit's skin, which is still green, and the seeds are yellowish white (Udoh et al., 2019). Therefore, young papaya seeds have a more significant effect on decreasing the quality of spermatozoa (Raji, 2015).

In addition to papaya seeds, one plant with an antifertility effect is the gandarusa plant (*Justicia gendarussa* Burm.f.). Some Papuan have used this plant as male antifertility, which is empirically used in Indonesia. Prior research shows that gandarusa leaves contain 12 flavonoid components (Bagia et al., 2011).

There are several mechanisms in the use of male contraception, namely as anti-spermatogenesis agents that suppress the production of spermatozoa, preventing sperm maturation and blocking the transport of sperm through the vas deferens (Sharma et al., 2019). The researchers also found that the use of male contraception containing hormones has a weakness. Namely, it can cause side effects when used for a long time even though there are conditions that must be met for ideal male contraception, including being safe, effective, reversible, and has no effect on libido (Kamal et al. 2020).

Based on the description above regarding the low participation of men in family planning programs, the researchers are interested in researching the effect of papaya seeds (*Carica Papaya L.*) ethanol extract, gandarusa leaves (*Justicia Gendarussa* Burm.F.) and its combinations on spermatozoa quality of male mouse (*Mus Musculus*)

2. MATERIALS AND METHODS

2.1. Materials

The materials to be used in this study were papaya seed extract (*Carica papaya* L.), gandarusa leaf extract (*Justicia gendarussa* Burm.f.), aquadest (Smart-Lab; Tangerang, Indonesia), ethanol 70% (Brataco; Tangerang, Indonesia), Cloroform (Merck; Darmstadt, Jerman), eosin-y solution (Merck; Darmstadt, Jerman), methanol (Brataco; Tangerang, Indonesia), safranin, buffer phosphate, gentian violet (Onemed; Tangerang, Indonesia), Na CMC 1% (Merck; Darmstadt, Jerman).

2.2. Methods

2.2.1. Preparation of papaya seeds and gandarusa leaf extract.

Samples of papaya seeds (*Carica papaya* L.) were obtained by separating the white, young papaya seeds and cleaning them from the dirt that sticks by washing them with running water (wet sorting). Then, it is dried in an oven at a temperature of 45-50^{o}C; after drying, the papaya seeds are powdered until they become like flour using a blender. Papaya seed powder (*Carica papaya* L.) was put into a jar and extracted by maceration method and 70% ethanol soluble was added. Ethanol is polar, so it is expected to be able to extract compounds that have polar properties. The solvent or filter will penetrate the cell wall of the simplicia. Maceration was carried out for three days, stirring three times a day. The maceration results were filtered to separate the filtrate and residue. Then, the filtrate is concentrated using a rotary evaporator and a water bath until the solvent evaporates and the extract becomes thicker but can still be poured. The thick extract was weighed according to the treatment dose.

The samples of gandarusa leaves (*Justicia gendarussa* Burm.f.) were cleaned of adhering dirt to separate impurities or other foreign materials that were not used after being cleaned of impurities and then dried by aerating to dryness. After that, the smooth leaves of gandarusa (*Justicia gandarusa* Burm.f.) were put into a jar and soaked using 70% ethanol solvent until all simplicia were submerged and soaked for three days. After three days, the extracted water was separated from the extract, and the liquid obtained was filtered with the help of a vacuum pump to produce filtrate, then evaporated using a rotary evaporator to form a thick extract. The thick extract was weighed according to the treatment dose.

2.2.2. Extract dosage

The dosage of the papaya seed ethanol extract P2 100mg/kgW is calculated so that the dose is weighed so that the extract is 0.3 grams, the ethanol extract of gandarusa leaves P3 50mg/kgW is weighed as much as 0.15 grams, the combined dose of P4 is 50;50mg/kgW each from the seed extract. Papaya and gandarusa leaves weighed 0.15 grams, P5 50:100mg/kgW papaya seed extract weighed 0.15 grams. Gandarusa leaves weighed 0.3 grams, P6 100:50mg/kgW seed extract Weighed 0.3 grams of papaya and 0.15 grams of gandarusa leaves, each dissolved with 1% Na CMC and then made up to 100 ml in volume, then administered orally using a probe/cannula (blunt tip needle) according to the weight of the mice.

2.2.3. The spermatozoa quality effect of male mice with papaya seeds and gandarusa leaves extract

Experimental animals first prepare a place for rearing experimental animals, which includes a drum, a place to eat and drink for mice, and feed for mice; then, the mice are acclimatized at laboratory temperature for seven days to adapt to new environmental conditions. General observations were made while adapting to standard food environmental conditions, and the body weight was weighed before the dose was determined. Types of extracts include Na CMC control (A), papaya seed extract (B), gendarusa leaf extract (C), papaya seed extract, and gendarussa leaf extract (D). The doses were 1% Na CMC, 100 mg/kgW, 50mg/kg W, and 50:50 mg/kgW. 100:50 mg/kgW, 50:100 mg/kgW. The extract was given to mice once a day for 35 days with a calculated dose according to the dose of mg/kg BW of mice; on day 36, all mice were anesthetized with ether or chloroform, dissected, and both testes were taken. Next, cut the tip of the cauda epididymis, accommodated in a watch glass containing two doses of 0.9% NaCl solution, and then stirred homogeneously to observe sperm quality. The parameters observed in this study were the calculation of motility, viability, and morphology of spermatozoa.

2.2.4. Data analysis

Data analysis used the one-way ANOVA test. If the test results showed a significant difference, the test would be followed by the LSD (Last Significant Different) test. The first step of using LSD is sorting the average treatment from the largest to the smallest. The second compares the average difference of a pair of treatments with the calculated value. The last, if the difference value is smaller than before, the average treatment is in one line.

3. RESULT AND DISCUSSION

In this study, samples of young papaya seeds (*Carica papaya L*) of California type were taken from Mangki Village, Cempa Regency, Pinrang City and Gandarusa Leaves (*Justicia gendarussa Burm.f.*) were taken from Duriasi Village, Konawe Regency, Kendari City. The experimental animals used in this study were mice (*Mus musculus*).

From the results of sperm motility observations in male mice (*Mus musculus*), the average values produced are P1 88.67%, P2 14.67%, P3 54.67%, P4 84.00%, P5 84.33% and P6 62,67%. The effective dose influencing the decrease in sperm motility of male mice (*Mus musculus*) is at a P2 of 14.67% (Table 1). At this dose, the progressive spermatozoa are less than the progressive spermatozoa in P1 (Control). It is in line with research (Wijayanti, 2016), which stated that spermatozoa's motility is abnormal if the calculation result is < 32% of progressively moving spermatozoa (Figure 1). This decrease is due to the presence of compounds in papaya seeds, namely saponins, and triterpenoids, which provide hormonal effects by affecting cell membranes so that they can interfere with the function of cell membranes in transporting food or nutrients.

Table 1. The average percentage value of spermatozoa motility in male mice**.**

No	Treatment Group	Percentage of result % ± SD
1	Na CMC 1% control	86.66 ± 9.45
2	Papaya seed extract 100 mg/kgW	14.67 ± 4.16
3	Gandarusa leaves extract 50 mg/kg	72.33 ± 4.04
4	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 50 mg/kgW	84.00±12.12
5	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 100 mg/kgW	84.33±11.01
6	Combination of papaya seeds 100 mg/kgW and gandarusa leaves 50 mg/kgW	62.67±15.66

The prior research by Hasanah and Sukarjati (2016) showed that 100 mg/kg BW papaya seed extract effectively reduced spermatozoa motility, and a mixture of papaya seed extract and neem leaves with a concentration of 100:100 mg/kg BW has the most effect on decreasing motility. It also can reduce the concentration of spermatozoa due to the presence of compounds from both extracts that can interfere with the transport process of spermatozoa, namely agglutinating sperm, and affect reducing motility and vitality. Therefore, the spermatozoa cannot reach the ovarium. (Hasanah and Sukarjati, 2016).

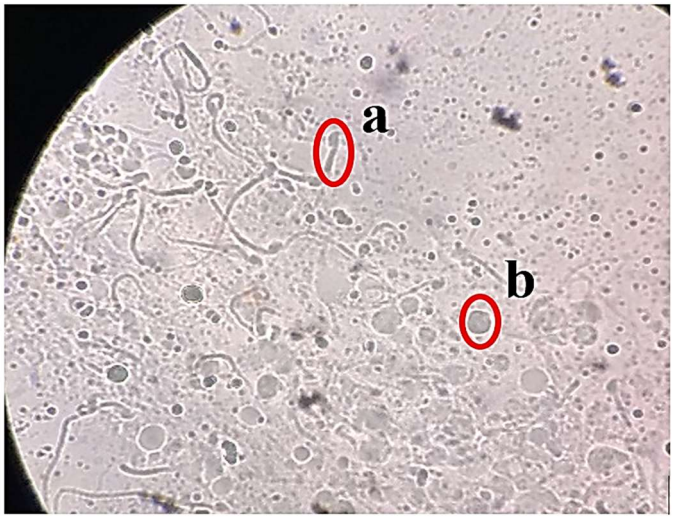

Figure 1. Representative microscopic appearance of spermatozoa motility in male mice. Description: Moving sperm(a) and Immobile sperm (b).

The percentage of spermatozoa motility differs in value (Table 1). It is because the weight and volume of spermatozoa fluid produced from each experimental animal significantly affect sperm quality. Meanwhile, in the treatment group, the value was changed considerably. It was because, apart from the compound content in the plant, the length of observation also greatly affected the quality of spermatozoa. In this observation, the various times were used according to the accuracy of observing sperm cells under a microscope, so the difference in values was significant.

From the percentage of the average value of spermatozoa motility in male mice, the data normality test was carried out using Shapiro Wilk, which showed the values of P1, P2, P3, P4, P5, and P6 where p>0.05. It means the data was normally distributed. Then, the homogeneity test was followed, which obtained p>0,05, meaning the information was homogeneously distributed. Next, a one-way ANOVA test is carried out, and the data obtained is 0.001 p<0.05, which means a significant (significant) difference in each treatment group. Last, in the post hoc test using the LSD (Least Significant Difference) method, there was a significant difference p<0.05.

Table 2. The average value of spermatozoa viability in male mice.

No	Treatment Group	Percentage of result % ± SD
1	Na CMC 1% control	75,67±30.92
2	Papaya seed extract 100 mg/kgW	35,67±21.00
3	Gandarusa leaves extract 50 mg/kgW	4,67±3.20
4	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 50 mg/kgW	69,33±2.08
5	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 100 mg/kgW	88,33±8.32
6	Combination of papaya seeds 100 mg/kgW and gandarusa leaves 50 mg/kgW	53,33±12.22

In this observation, the diluted spermatozoa were dripped with 2% Eosin-y solution to determine the difference between dead spermatozoa (sucking many colors/having a pink color) and live spermatozoa (slightly sucking color/transparent). So, in observing the viability of the spermatozoa of male mice (Mus musculus), the average values produced are P1 75.67%, P2 35.67%, P3 4.67%, P4 69.33%, P5 88.33%, and P6 53.33%. It can be seen in the graph of 2 effective doses influencing the decrease in viability, namely at P3 of 4.67%, where at this dose, the viability of spermatozoa was less than the viability of spermatozoa in P1 (control). It follows the theory (Wijayanti, 2016), which states that viability is abnormal if the value is < 58% and the ability of spermatozoa to survive is tiny. The decrease in spermatozoa viability is thought to be affected by the decreasing percentage of motile/moving spermatozoa (Bagia et al., 2011). In addition, the active compounds in gandarusa leaves, namely flavonoids, which act as antifertility, are estrogenic compounds capable of stimulating estrogen formation in the body, increasing estrogen levels. This increase provides negative feedback to the anterior pituitary that does not release FSH and LH, disrupting testosterone hormone secretion. The presence of disturbance in these hormones will affect the quality of spermatozoa, such as the movement of spermatozoa (Laili, 2018).

The percentage of spermatozoa viability had significantly different values, especially in the control group or without extract (Table 2). In that group, there was a decrease in viability in the mice 1 group. It is because after staining, the spermatozoa were left for approximately 2 minutes to see the different colors in the head (*corpus*) of the spermatozoa. It turns out that the

stagnation causes the viability of the spermatozoa to decrease so that the following observation after staining is no longer resisted (Figure 2).

From the percentage of the average value of spermatozoa viability in male mice, the data normality test was carried out using Shapiro Wilk, which showed the values of P1, P2, P3, P4, P5, and P6 where $p>0.05$. It means that the data was normally distributed. Then, the homogeneity test was followed, and a $p>0,05$ was obtained. The data is homogeneously distributed, a one-way ANOVA test is carried out, and the data obtained is 0.001 $p<0.05$. It means that there is a significant difference between the treatment groups. The post hoc test will continue using the LSD (Least Significant Difference) method. Therefore, it resulted in a significant difference of $p<0.05$.

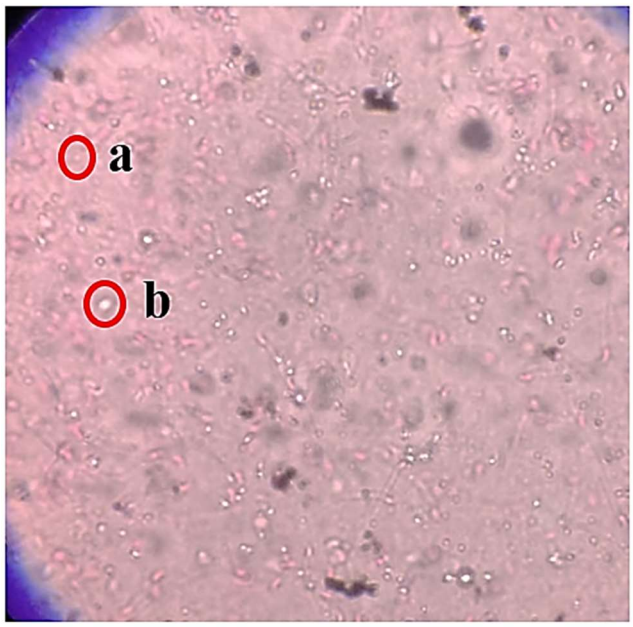

Figure 2. Representative microscopic appearance of spermatozoa viability in male mice. Description: Pink sperm/ dead sperm (a) and Colorless sperm/live sperm (b).

In this morphological observation, the spermatozoa taken are first fixed in the air, and the cells can be attached to the slides. If the cells are not fixed on the slide, the cell layers to be stained can be washed off during the staining procedure, then dipped in methanol to maintain the cell structure remains the same as the original, then washed with safranin and dyed with phosphate buffer. It is a substance needed to maintain pH when a small amount of acid or base is added to the solution. Then, it is stained with crystal violet, the main stain that will give color to microorganisms that will be observed, which can ease this process. Then, it was washed under running water to reduce the excessive color during observation, followed by observation under a microscope. It obtained the result of P1 51.00%, P2 87.00%, P3 6.67%, P4 39.00%, P5 42.67%, and P6 68.33%. It can be seen in the graph that three effective doses most affect the decrease in normal morphology of spermatozoa, namely at P3 6.67%. In that dose, there are fewer normal spermatozoa than normal spermatozoa in the control group. These results follow the study (Wijayanti, 2016), which states that spermatozoa morphology is abnormal if the total

percentage is <40%. The increased abnormality of spermatozoa was due to the presence of Gandarusa leaf extract, which can reduce the weight of the testes, epididymis, and vas deferens by interfering with the function of these organs in the production and maturation of spermatozoa in the epididymis. It causes the mature spermatozoa to decrease in quality due to a spermatozoa defect, which causes the sperm to be abnormal and unable to move forward. The greater the number of abnormal spermatozoa, the ability to move (motility) of the spermatozoa will decrease so that abnormal spermatozoa will have difficulty in movement and cannot move forward quickly towards the ovarium.

Based on the observations, the most common abnormalities found were primary ones. Those are abnormalities in the neck, such as without a head and a tail. Meanwhile, secondary abnormalities include entangled tails and fractures in the tail. It also found that the primary abnormalities are due to disruption of spermatogenesis in the phase of spermatogenesis. It occurs when spermatozoa are formed from spermatids, while secondary abnormalities are thought to occur during spermatozoa maturation disorders in the epididymis. Then, it results in the discovery of abnormal spermatozoa (Figure 3). In prior research, ethanol extract of gandarusa leaves at 0.195 mg/kg W can reduce motility and viability and increase spermatozoa abnormalities in mice (Bagia et al., 2011). The previous researchers stated that the extract of gandarusa leaves LD_{50} was 180 mg/kg BW of mice, which was included in the practical and non-toxic category (Bagia et al., 2011).

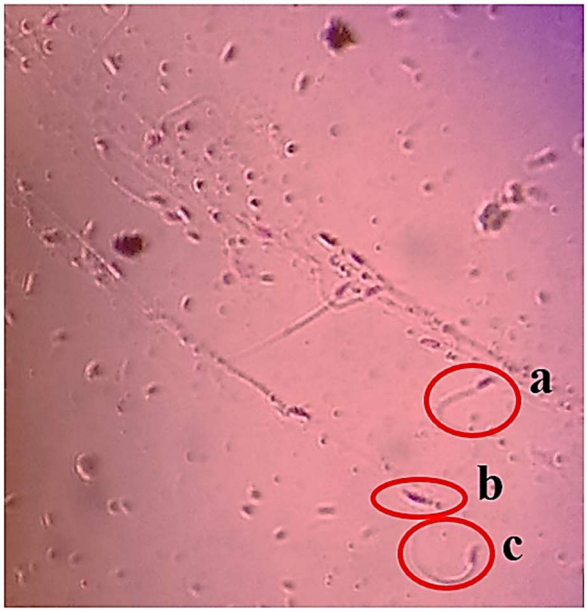

Figure 3. Representative microscopic appearance spermatozoa morphology of male mice. Description: Entangled sperm tail (a), sperm without tail (b) and abnormal movement (c).

Male mice's most decreased spermatozoa morphology (Table 3) shows that the most significant decrease in normal spermatozoa occurred at P3. It was due to the compound content in the Gandarusa leaves extract, which was more potent than the state after being combined. In addition, it was caused by one of the error factors during administering the liquid extract that

was administered orally, not entirely drunk by the experimental animals according to the specified volume. The volume of spermatozoa fluid obtained during the observation process differed, so the decrease was more significant than after combined.

From the percentage of the average value of spermatozoa morphology in male mice, the normality test of the data was carried out using Shapiro-Wilk. It showed the values of P1, P2, P3, P4, P5, and P6 where $p>0.05$, which means the data was normally distributed. Then, the homogeneity test obtained the $p>0,05$ value. It means the information is homogeneously distributed, then a one-way ANOVA test is performed, and the data obtained is 0.003 $p<0.05$, meaning there is a significant difference in each treatment group. Then, the post hoc test using the LSD (Least Significant Difference) method revealed a significant difference $p<0$.

Table 3. The average value of spermatozoa morphology in male mice.

No	Treatment Group	Percentage of result % ± SD
1	Na CMC 1% control	51,00±36.71
2	Papaya seed extract 100 mg/kgW	87,00±4.35
3	Gandarusa leaves extract 50 mg/kgW	6,67±1.52
4	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 50 mg/kgW	39,00±15.00
5	Combination of papaya seeds 50 mg/kgW and gandarusa leaves 100 mg/kgW	42,67±7.50
6	Combination of papaya seeds 100 mg/kgW and gandarusa leaves 50 mg/kgW	68,33±16.50

Previous studies have shown that abnormal spermatozoa morphology increases because papaya seed extract causes abnormal cell organelles in the neck of spermatozoa, namely vacuolization in mitochondria. It causes the function of mitochondria in producing energy to be not optimal and thus affects the motility of spermatozoa (Lohiya *et al*., 2017). The content of papain enzymes in papaya seed extract can also reduce total lipids in testicular and epididymal tissue. Papaya seed extract reduces the activity of the lipoprotein lipase enzyme. It inhibits the absorption of nutrients from the gastrointestinal system. In contrast, the energy produced from these nutrients is needed for spermatogenesis in the testes and the maturation of spermatozoa in the epididymis (Basha and Cangamma, 2018).

4. CONCLUSION

Based on the research that has been done, it can be concluded that the administration of extracts of papaya seeds (*Carica papaya L.*) and gandarusa leaves (*Justicia gandarusa Burm.f.*) and their combination toward the quality of spermatozoa of male mice (*Mus musculus*) can reduce motility, viability, the morphology of good spermatozoa both in single or combination. The test results p value <0.005. The effective dose in reducing spermatozoa's motility, viability, and morphology is P3 ethanol extract of gandarusa leaves 50 mg/kgW.

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