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The Activities of Antifertility Ethanol Extract Guava Leaves (*Psidium Guajava* L.) based on The Analysis Of Cement and Display of Immunohistochemistry Cyclooxygenase-2 In Testis Of Mice (*Mus Musculus L.*)

(Aktivitas Antifertilitas Ekstrak Etanol Daun Jambu Biji (*Psidium Guajava* L.) berdasarkan Analisis Semen dan Tampilan Imunohistokimia *Cyclooxygenase-2* pada Testis Mencit (*Mus Musculus L.*))

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Abstract: Population growth increase, without support from good economic conditions, requirer population control. One of the program to control population growth is family planning program (KB). The participation of men in family planning programs is very important. Male contraception can utilize natural materials derived from plants. One of the herb is guava leaves (*Psidium guajava L.*) which contains many active substances that can inhibit the process of spermatogenesis. The purpose of this study was to determine the effect of ethanol extract guava leaves to the quality (concentration, viability, morphology, and motility) of spermatozoa and the expression of Cyclooxygenase-2 (COX-2) in the testes of mice (*Mus musculus* L.). The research used experimental design with completely randomized sampling method. Four treatment groups, namely P0 using aquadest as a control 0,5 mL, P1 using 15 mg/25g body weight extract orally, P2 using 20 mg/25g body weight extract orally, and P3 using 25 mg/25g body weight extract orally. Data were analyzed using One Way ANOVA followed by Bonferroni Post Hoc test 5%. The results showed that ethanol extract guava leaves with a dose of 15mg/25g body weight had significantly decreased sperm quality and increased expression of COX-2 in the testes of mice. This research indicates that guava leaves have a potential to be used as a herbal contraceptive.

Keywords: Guava leaves, contraceptive, sperm quality, COX-2.

Abstrak: Laju pertumbuhan penduduk yang meningkat, tanpa diimbangi dengan keadaan ekonomi yang baik, menyebabkan terjadinya ledakan penduduk. Oleh karena itu, salah satu cara yang dapat dilakukan melalui program keluarga berencana (KB). Keikutsertaan pria dalam program KB sangat penting. Sarana kontrasepsi pria dapat memanfaatkan bahan alami yang berasal dari tumbuhan. Salah satunya adalah daun jambu biji (*Psidium guajava* L.) yang mengandung banyak zat aktif yang dapat menghambat proses spermatogenesis. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian ekstrak etanol daun jambu biji terhadap kualitas (konsentrasi, viabilitas, morfologi, dan motilitas) spermatozoa dan ekspresi *Cyclooxygenase-2* (*COX-2*) pada testis mencit (*Mus musculus L.*). Penelitian ini menggunakan studi eksperimental dengan metode rancangan acak lengkap (RAL) dengan empat kelompok perlakuan, yaitu P0 sebagai kontrol diberikan aquadest 0,5mL, P1 diberikan ekstrak 15mg/25g berat badan, P2 diberikan ekstrak 20mg/25g berat badan, dan P3 diberikan ekstrak 25mg/25g berat badan yang diberikan selama 35 hari. Data yang diperoleh dianalisis menggunakan *One Way Anova* yang dilanjutkan dengan uji *Post Hoc Bonferroni* 5%. Hasil penelitian menunjukkan bahwa pemberian ekstrak etanol daun jambu biji dengan dosis 15mg/25g berat badan sudah berpengaruh terhadap penurunan kualitas spermatozoa dan meningkatkan ekspresi *COX-2* pada testis mencit. Hal ini mengindikasikan bahwa daun jambu biji berpeluang untuk digunakan sebagai bahan kontrasepsi herbal.

Kata kunci: Daun jambu, kontrasepsi, kualitas sperma, *COX-2*.

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PREFACE

INDONESIA'S population growth is increasing every year cannot be resolved properly yet, till raises concerns because both population growth and welfare growth are not balance. Based on BPSRI data, the population from 1980-2010 has increased, so the Family Planning Program increasingly recommended to the public[1]. Family Planning (KB) program as part of national development is one of the ways to plan and adjust the space of birth.

There are various kinds of hormonal contraception available, such as; pills, injections or depots that are implanted under the skin, as well as the IUD (intra uterine device) planted in the uterus and the ovum tube impeding contraception (tubektomi) used for women who have make sure not to get pregnancy anymore. To complement the female contraception condoms are also provided[2].

Meanwhile, contraception for men are still very limited, such as condoms, vasectomy, coitus interruptus that's very difficult to implement, ineffective and inefficient. For the supply of the pill is still very low and the research for it is still underway. Contraceptive drugs work for men based on three locations, namely pretestikuler, testicular, and protestikuler. Infertility in men is obtained by analysis of semen, that is the low percentage of motility and sperm count, discoloration, pH, and sperm morphology[3]. Therefore, contraception for men need to be developed so by that way the involvement of men in family planning programs (KB) increasingly evident.

One way to do is to empower the contraceptive use of natural materials. Plants that are used as contraceptive containing compounds that are antifertilitas, antiesterogenik, and antiimplantasi also good for men, women, or to both. Research about those plants proved that many of those contain alkaloids, flavonoids, isoflavonoids, triterpenoids, xanthon, quinone, steroids, tannins, saponins and essential oil[4].

Society are often consume guava leaves as an antidiarrheal, cure dysentery, colitis, digestive disorders and can be used as an antibacterial. However, they are still lacking of knowledge about the function of the guava leaves can also be used as contraception. Besides containing tannins, guava leaves also contain other substances, such as acid ursolic acid lat, acid guajaverin, essential oils, alkaloids, quercetin that belongs to flavonoids unity, saponins, triterpenoids, and vitamins[5,6].

Flavonoids may inhibit the enzyme aromatase that serves to catalyze the conversion of androgens to estrogen increases the hormone testosterone, that the high concentration of testosterone will be baited back to the pituitary that does not release FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone) [7]. Quercetin, belongs to flavonoid compounds functions to inhibit the hedgehog gametes fusion membrane in the time of fertility. Kuersetin also inhibits the activity of hyaluronidase so that sperm cannot penetrate the cumulus ahead of fertilization[8]

Alkaloids can affect the needed reproductive hormones secretion for the process of spermatogenesis. Atsiri oils do not work on the process of spermatogenesis, but the transport of sperm. Tannins can gather to reduce motility and the viability of sperm[9]. Saponins in the cell cycle can inhibit the the over- high formation of Bcl-2, induces caspase 3 that expressed too low and increase the expression of p53 and trigger the G1 cell cycle arrest. Saponin works on the process of spermatogenesis[10].

Spermatogenesis is a complex process starting from proliferation of germ cell and maturation of spermatogonia into spermatozoa. DNA damage in sperm development phase causes the activity of enzymes cyclooksigenase-2 (COX-2) in the germ cells increases. Cyclooksigenase enzyme (COX) is an enzyme that catalyzes the formation of prostaglandins, a mediator of inflammation, and products of arachidonic acid metabolism. COX enzymes consist of 2 isoenzims, namely COX-1 and COX-2. COX-1 enzyme is constitutive for maintaining normal physiology and homeostasis, whereas COX-2 is an enzyme that is induced in inflammatory cells[11].

Frungieri et al (2007) identified the COX-2 expression in testis showed impaired spermatogenesis. In a recent study has also shown the effects of COX-2 in the regulation of testicular function and male fertility[12]. COX-2 enzyme may have biological relevance in pathogenesis and maintenance of male infertility, and can be used as additional molecular markers for the diagnosis of male infertility disorder[13].

Several studies have reported providing leaves of guava (*Psidium guajava L.*) influences the decrease sperm count, decreased sperm speed, and increased abnormal sperm morphology of male white rat[14]. Research conducted by Khitiyatul Arifah (2006) showed a decrease in the number of sperm cells[15]. The results of research conducted by Ariani, Sri Retno *et al* showed antiimplantasi effect on white mice[16].

Role of *cyclooxygenase-2* in the male reproductive organs are still very poorly researched and there are some reports that showed that COX-2 may have an effect on spermatogenesis. Based on the above, researchers wanted to know whether the ethanol extract of guava leaves provide antifertility effect

based on the examination of semen analysis and examine the role of *cyclooksigenase enzyme-2* (COX-2) in the testes of mice (*Mus musculus L.*)

MATERIAL AND METHOD

MATERIAL. Hemocytometer, microscope, surgical tub, dissecting set, object glass, cover glass, Dako Real Evision Rabbit/Mouse, PT. Link dako (±1 jam) Epitope Retievel: set upmpreheat 65C, Pap pen.

Mice, guava leaves, NaCl 0,9%, aquadest, Tris Buffered Saline (TBS), xylol, alkohol, normal horse serum (NHS), DAB + Substrat chromogen solutiom dengan pengenceran 20 µL, Litium karbonat (5% dalam aqua), ethanol, COX-2 abcam (ab15191).

METHOD.This study was an experimental study with a completely randomized design that uses experimental animals (*Mus musculus L.*). DDW Strain (Double Distch Webster) is male, healthy, active, ±12 weeks old, having 25-30 g body weight and having 24 tails. Grouped into four groups, namely P0 (control) were given distilled water 0,5 mL, P1, P2, P3 successively given ethanol extract of guava leaves 15 mg/25 g bb, 20 mg/25 g bb, 25 mg/25 g bb) for 35 successive days respectively. This study will be conducted at the Laboratory of Pharmacy, University of North Sumatra, Medan and Anatomical Pathology Laboratory of Murni Teguh Hospital Medan.

Guava leaves obtained directly from the tree in the yard in Stabat district of Langkat. Guava leaves have been chosen in good condition, not too old and too young, healthy (not moldy, perforated), not dry. Guava leaf (*Psidium guajava L.*) previously cleaned and weighed in fresh state, then dried and after dry cut into small pieces and then crushed (blended) until tenuous. Extraction of guava leaves done by maceration method. Maceration method is used, for it's the simplest way of seeking so as avoiding the damage to the active substance. Solvent used in maceration is polar ethanol that it can draw analytes that have the same characteristics as ethanol[16]. Maceration process begins by dissolving guava leaves with solvents; ethanol at a ratio of 1:10 (w/v). Guava leaves as many as 10 parts dissolved, then pour with 75 parts ethanol. Close and for 5 days and protected from light, stirring occasionally. After 5 days the solution was filtered, the pulp is squeezed and washed with liquid ethanol sufficient to obtain 100 parts. The extracts transferred into a closed vessel, allowed to cool place that is protected from sunlight for 2 days. Extracts were then evaporated by evaporator at a temperature of 5.000 ^{o}C and then dried and weighed[17], then assessed the moisture content, total ash and acid insoluble ash content, as well as phytochemical screening. The assessed observation parameters are concentration, viability, morphology and motility, as well as the expression of COX-2 enzyme.

Calculation of The Concentration of Spermatozoa. Mice were dissected and cut the part of cauda epididymis and distal vas deferens, then placed into a glass containing NaCl 0.9% and sliced with a razor blade. Sperm that have a homogeneous suspension is taken with a pipette (50µL) and put into a hemocytometer box Improved Neubaur and examined under a light microscope with a magnification of 400 times[18].

Spermatozoa Viability Calculation was done by giving giemsa colours *Improved Neubauer* hemocytometer, the live spermatozoa do not absorb either the live giemsa or dead observed under a light microscope at 400 times magnification and expressed in percent (%)[18].

Spermatozoa Morphology Calculation. Sperm from the cauda epididymis made preparations to remove the glass object, then dried and giemsa staining for 15 minutes. After that observed with a light microscope and determined the percentage of the normal and the abnormal sperm[18].

Calculation Spermatozoa Motility. Sperm suspension obtained beforehand and left for 5 minutes at room temperature, then this suspension is subsequently dropped into the counting chamber of Improved Neubauer, observed under a microscope to see and count the number of spermatozoa fast moving, not moving, and moving slowly[18].

Making The Net Block Paraffin. Testis tissue was washed with PBS 3-5 x to rid of contaminants. Then fixed with 10% formalin. After that dehydration is done using graded alcohol (30%, 50%, 70%, 80%, 96% and absolute) respectively 60 minutes. Cleaning with xilol 2 times each 60 minutes, then conducted with soft paraffin infiltration for 60 minutes at a temperature of 48 ^{o}C. Then do block in hard paraffin on the mold and allowed to stand for a day. The next day affixed to the holder and the cutting of 4-6 µm thick with a rotary microtome. Do mounting to object glass with gelatin 5%.

Deparafinisasi Process. Object glass immersed in a block of paraffin results xilol 2 times for each during 5 minutes and then, rehydration was done using radiant alcohol (absolute, 96%, 80%, 70%, 50% and 30%) respectively for 4 minutes, then rinsed with H_2O for 5 minutes.

Imunohistokimia staining method (IHC) COX-2 abcam (ab15191). Slides were washed by using TBS pH 7.4 for 5 minutes, then blocking with *Dako Flex* performed for 15 minutes, washed in TBS pH

7.4 for 5 minutes. After that, it was incubated using (anti-cyclooxygenase-2 antibody (ab15191), Rabbit polyclonal to cyclooxygenase-2) each for 1 hour. Wash again in TBS pH 7.4 for 5 minutes, drop with secondary antibody HRP Flex brand Dako for 30 minutes. After that, rewash the slide in a solution of TBS pH 7.4 or *Tween* for 5-10 minutes. Then, the slide is ready to be dropped with DAB+dako flex by diluting 20µL substract (lasting for 5 days at a temperature of 2-80 ^{o}C after being mixed) for 5 minutes, washed again with running water and performed counterstain with hematoxylin for 10 minutes for each. Wash it under running water for 5 minutes, then dip preparat slide into lithium carbonate (5% in aqua) for 2 minutes, wash with running water and the liquids drying process done again previously with dehydration process using alcohol 80%, 96%, absolutely 5 minutes per each, then clearing with xylol solution 1, 2, 3 for 5 minutes, then mounting and closed the slide with a cover glass and preparat is ready to be viewed. The slides were observed under a light microscope with a magnification of 400x, the expression of COX-2 is marked with brown color in the seminiferous tubules. The average number of positive germ cells COX-2 expression than - average number of germ cells positive COX-2 per 200 seminiferous tubules (modified[19].).

The data were obtained and analyzed using One-Way ANOVA followed by Bonferroni Post Hoc Test 5% (significant if $p < 0.05$).

RESULT and DISCUSSION

Ethanol extract of guava leaves (*Psidium guajava L.*) that was used in the study had a yield of 14.2%, water content of 7.48%, total ash content of 4.62%, and acid insoluble ash content of 0.32%. Phytochemical content obtained from the phytochemical screening of guava leaves can be seen in Table 1.

Table 1. Screening result of fitochemical of guava leaves.

Class of compounds	Ethanol extract of guava leaves
Alkaloid	+
Flavonoid	+
Tanin	+
Saponin	+
Triterpenoid	+
Atsiri Oil	+

Based on observations of concentration, viablitias, morphology, and motility, as well as COX-2 IHC appearance of the testes of mice is shown in the following tables 2 until 6 and picture 1 until 7.

Tabel 2. Results of the calculation of the concentra-tion of spermatozoa were treated for 35 days.

Treatment group	n (Deuteronomy)	Concentration of sperm ($\bar{x}$±SD)
P0	6	101.06±6.33
P1	6	21.81±5.61
P2	6	18.25±1.91
P3	6	14.89±2.21

Tabel 3. The calculation result viability of spermatozoa that treated for 35 days.

Treatment group	n (Deuteronomy)	Viability of sperm ($\bar{x}$±SD)
P0	6	94.72±0.43
P1	6	35.97±3.74
P2	6	32.08±1.15
P3	6	30.83±1.49

Tabel 4. The calculation result morphology of spermatozoa that treated for 35 days.

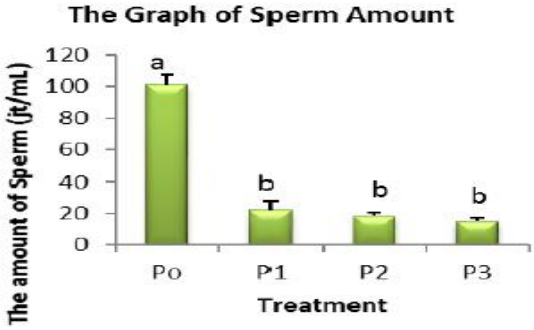
Treatment group	n (Deuteronomy)	Morfology of sperm ($\bar{x}$±SD)
P0	6	94.28±1.04
P1	6	42.22±4.07
P2	6	34.67±2.21
P3	6	29.72±1.36

Tabel 5. The calculation result motility of spermatozoa that treated for 35 days.

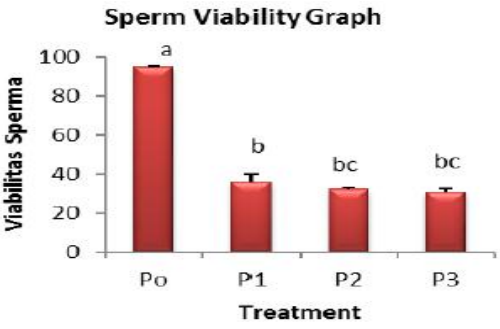
Treatment group	n (Deuteronomy)	Motility of sperm grade a ($\bar{x}$±SD)	Motility of sperm grade b ($\bar{x}$±SD)	Motility of sperm grade c ($\bar{x}$±SD)	Motility of sperm grade d ($\bar{x}$±SD)
P0	6	83.47±3.22	8.06±2.28	4.17±1.05	4.03±1.70
P1	6	10.28±1.64	15.83±1.29	29.31±2.44	44.58±2.57
P2	6	9.03±0.82	14.94±2.26	29.36±3.24	46.67±2.30
P3	6	6.14±0.92	10.69±2.66	31.08±1.59	52.08±2.16

Tabel 6. IHC staining assessment of COX-2 in the testes of male mice that treated for 35 days.

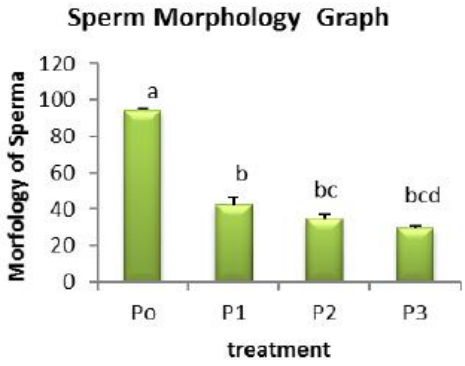
Treatment group	The Amount of multiplication results	Percentage (%)
P0	4	2.06
P1	45	23.20
P2	64	32.99
P3	81	41.75
amount	**194**	**100.00**



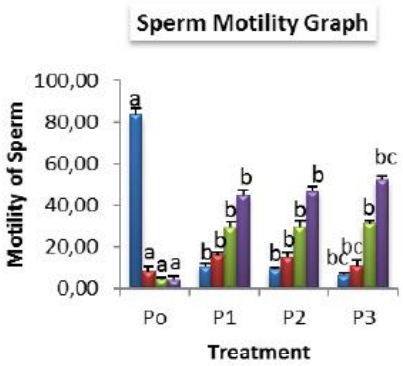
Picture 1. Graph of spermatozoa concentration of adult male mice (juta/mL).



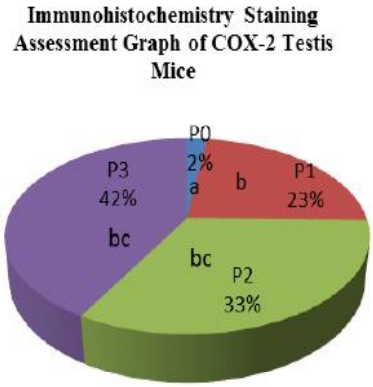
Picture 2. Graph of viability of adult male mice.



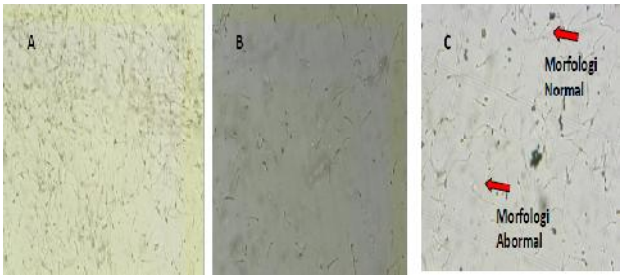
Picture 3. The morphology graph of spermatozoa of mature male mice.



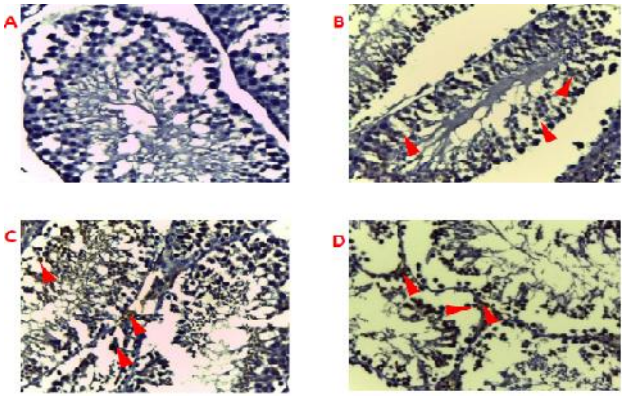
Picture 4. Graph of motility of adult male mice
grade a grade b grade c grade d.



Picture 5. Assessment graph of IHC staining of COX-2 adult male mice.



Picture 6. The test results analysis picture of adult male mice sperm; A. the control group; B. the treatment group; C. morphology of adult male mice sperm.



Picture 7. The results COX-2 IHC outward appearance picture on mice testis; A. the control group B. the treatment group 1, C. the treatment group 2, D. treatment groups 3.

Ethanol extract of guava leaves were used in this study met the criteria that have been determined in accordance with the Decree of the Minister of Health in 2009 that the condensed extract yield >12.3%, water content <10%, total ash content <9%, and the ash content is not soluble acid <0.8%[20]. Results of phytochemical screening guava leaf extract contains active ingredients, namely alkaloids, flavonoids, tannins, saponins, triterpenoids, and atsiri oils that will influence the process of spermatogenesis.

From research conducted showed that the ethanol extract of guava leaves with a dose of 15mg, 20mg, and 25 mg / 25g bb may decrease the concentration, viability (live), normal morphology, and motility, as well as increasing the expression of COX-2 in the testes of mice (*Mus musculus L.*). COX-2 expression in the testes of mice found in Leydig cells. A decline in the quantity and quality as well as increased expression of COX-2 in mouse Leydig cells caused by active substances contained in the ethanol extract of guava leaves[4].

Flavonoids are compounds that function as antiandrogenik by inhibiting the enzyme aromatase. The aromatase enzyme is an enzyme that works to catalyze the conversion of androgens to estrogens that increases testosterone. The high concentration of testosterone will be feedback negatively baited to the pituitary, namely to release the follicle stimulating hormone (FSH) or luteinizing hormone (LH) that will inhibit spermatogenesis (number of spermatozoa produced decreased, an increase in abnormal sperm morphology). Alkaloids also work to press FSH and LH leading to impaired spermatogenesis. The disruption of the hormone testosterone causes the viability of spermatozoa in the epididymis decreased[21]. The reduced viability of spermatozoa can also be due to reduced spermatozoa causes the fluid for sperm in epididymis disrupted due to the decreased of testosterone[22].

Alkaloids also can interfere with the activity of the enzyme ATPase in the membrane of the sperm cell was disrupted, so that the movement of sperm becomes impaired. ATPase enzyme serves to maintain internal homeostasis of sodium and potassium ions, if the enzyme is impaired concentration increased intracellular Na^+ and Na^+ across the membrane decreases, thereby expending Ca^{2+} decreased as well[23]. With the disruption of the sperm membrane permeability will cause the transport of nutrients necessary for the spermatozoa adversely durability and impaired movement[23].

Tannins also one of the active substances contained in the ethanol extract of guava leaves. Tannins shown to have antioxidant activity, inhibiting tumor growth and inhibit enzymes such "reverse" transkiptase and DNA of topoisomerase[24]. Tannins contained in the bitter melon leaves can be used as anti-fertility in experimental animals because it can inhibit protein synthesis. Tannins on the Curcuma domestica can crumple spermatozoa[25]. By its agglomerate spermatozoa causes a decrease in vitality and movement of spermatozoa. While atsiri oils work to disturb the transport of sperm to fertilize ovum[26].

The disruption in the testicles causes COX-2 expression increased in the seminiferous tubules tissue. COX-2 expression was found in the Leydig cells showed impaired Leydig cells. Leydig cells function to produce testosterone. This further supports that the active substances contained in the ethanol extract of guava leaves disturb the testosterone production, so the quantity and quality of sperm decreases. In accordance with the results of research of Fungieri *et al*[12]. states that the presence of COX-2 in the testis showed the existence of spermatogenesis disorder[12]. Likewise the research of Perrotta showed the impact of COX-2 in the regulation of testi function and male fertility[13]. So it can be seen that the ethanol extract of guava leaves disturb the spermatogenesis of mice with the presence of COX-2 in the testes of mice. COX-2 has a function to protect the testes from any disruption, so when the process of spermatogenesis is disturbed COX-2 present trying to protect the testes[27].

Ethanol extract of guava leaves can be used anti-fertility, because the value of the quantity and quality are far below the normal range of research conducted by Montoto *et al*[28].

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