

## Ethanollic Extracts of Young Papaya Seed (*Carica papaya*, Linn) Decrease Spermatocytes, Leydig Cells, and Androgen Receptor of Leydig Cells of Adult Mice (*Mus musculus*)

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### Abstract

Background: Uncontrolled population growth is one of the problems in Indonesia. Among the various types of wild plants that can be used as anti-fertility drugs are papaya plants. This study aims to determine the effect of extracts on the number of spermatozoa, Leydig cells and androgen receptor (AR) expression on Leydig cells as an anti-fertility mechanism. Materials and Methods: This was an experimental, randomized posttest-only controlled group study. We used a total of 36 Wistar rats as the subjects of this study. The rats were classified equally into two groups by simple random sampling method. Group E rats received 0.5 mL ethanol extract of papaya seeds for 50 days (orally), and the rats in Group C (control) received 0.5 cc aquabidest for 50 days. Results: The treatment group showed a decrease in the number of spermatozoa (201.86 vs. 94.3,  $p < 0.001$ ), a decrease in the number of Leydig cells (7.61 vs. 3.32,  $p < 0.001$ ), and decreased AR expression of Leydig cells (46.19 vs. 18.48,  $p < 0.001$ ) compared to the control group. Conclusion: ethanol extracts of young papaya seeds significantly reduce the number of spermatocytes, Leydig cells, and the AR expression of the Leydig cells.

**Keywords:** *Young papaya seed, Male anti-fertility, Androgen receptor, Leydig cells.*

### Introduction

Indonesia is a country with a very high-dense population. The population in Indonesia is currently around 250 million people and is the fourth most populous country in the world. If projections are made using scientific calculations, this country will have a population of more than 270 million people in 2025, more than 285 million in 2035 and 290 million in 2045 [1].

The welfare of the nation depends on the quality and competence of its human resources to sustain the nation's future. To avoid an explosion in population, the family planning program has become a national program in Indonesia. In Indonesia, the participation in family planning program is still dominated by women, while men have not participated as many since male

contraception is very limited and have some drawbacks [2,3].

It is essential to strive for the development of an ideal male contraceptive drug. An alternative is by finding ingredients from plants that have anti-fertility property. One plant-source ingredient that is known to possess antifertility property is papaya seeds. Papaya seed extract can cause damage to the germinal epithelium, and degeneration of spermatosides and spermatids [4].

In papaya, ethanol extracts reduce the motility of spermatozoa in mice [5]. The n-hexane fraction, chloroform fraction, and methanol fraction of papaya seeds can reduce sperm counts, motility, increase spermatozoa abnormalities, and significantly reduce spermatozoa viability [6].

Young papaya seeds are one of the natural ingredients that have antifertility properties. This research was conducted to find out if the local Balinese papaya ethanol extract inhibits spermatogenesis by decreasing spermatozoa cells, Leydig cells and testosterone receptors.

## Materials and Methods

This was an experimental, randomized posttest-only controlled group study conducted at Udayana University, Bali, Indonesia in 2018-2019. We used a total of 36 Wistar rats (12-weeks old, 150-200 g weight) as the subjects of this study. The study protocol was approved the Committee of Ethical Research of Udayana University. The rats were classified equally into two groups by simple random sampling method. Group E rats received 0.5 mL ethanol extract of papaya seeds for 50 days (orally), and the rats in Group C (control) received 0.5 cc aquabidest for 50 days.

## The Making of Ethanol Extract of Papaya Seeds

The young papaya fruit is peeled-off and the seeds are collected. The seeds were then put in hot water until they are submerged for about one minute in order to stop the process of cell metabolism and enzymatic reactions. Papaya seeds are drained and then dried in an open room with good air circulation, avoiding any direct contact to the sunlight. The drying process was 2-weeks long. The dried papaya was then mashed into a powder. A total of 5 kg of papaya seeds

(dried powder) was macerated with 70% ethanol (in a 1:10 mixture) in a dark extractor bottle at room temperature for 24 hours. After that, 1,000 grams of simplicia and 7.5 liters of solvent were put into the mixture. The mixture was soaked for five days while stirring every 6 hours, filtered, and then we macerated the pulp again with 2.5 liters of solvent, and macerated again with the similar protocol. The filtrate obtained was evaporated with freshdrier to obtain concentrated ethanol extract. Concentrated papaya seed extract is then ready to be used for this study.

## Preparation and Treatment of Experimental Animals

Thirty-six adult male Wistar rats, aged 12 weeks, weighing 150-200 grams were then adapted into a similar environment and food for two weeks. Food provided in the form of rat food and drinks (in ad libitum dose). Papaya ethanol extract was administrated orally using a straw.

## Making Testicular Histology Preparations

The rats were put down by intracardial injection of Pentothal. The testes were then mutilated, then were put in a 10% formalin buffer for 24 hours. A dehydration procedure using alcohol followed, and then we put in toluene and blocked with paraffin. Histology incision was made with microtomes, 3-5 microns thick, and stained with Hematoxylin Eosin (Figure 1).



Figure 1: Examples of FOVs for the sperm counts from out study: Group E (left) and Group C (right)

## Counting the Number of Androgen Receptors of Leydig Cells

The number of androgen receptors on leydig cells is measured by immunohistochemical method using the Rabbit Anti-phospho-

Androgen Receptor Polyclonal Antibody (Ser 578) with Bioss-0943 R (Figure 2). We read them in five fields of view (FOV), and then count the cells divided by all cells, then multiplied by 100%.

## Data Analysis

Descriptive analysis was used to present data from each group such as age, sex, number of spermatozoa cells, number of Leydig cells, and testosterone receptors in Leydig cells. Data distribution of rats from each experimental group was tested with Shapiro-Wilks test. The data variants were then tested for homogeneity.

If the data is homogeneous and has a normal distribution then a t-test is performed. The Mann-Whitney test is performed otherwise. We used SPSS 24.0 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) For all statistical analysis. A p-value of <0.05 was considered statistically significant.

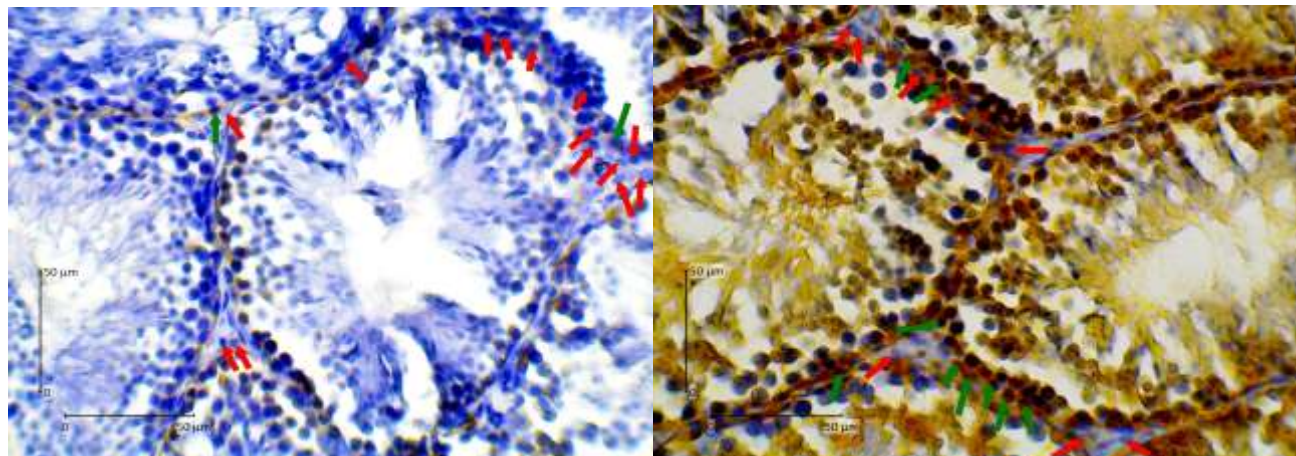


Figure 2: AR expression examples in our study: E group (left) and C group (right)

## Results

Thirty-six Wistar rats were used in this study according to the study protocol. The Shapiro-Wilk test in this study resulted in a normally distributed data.

Therefore, we continued to the parametric test, using the unpaired t-test (Table 1). We found significant differences between the two groups in terms of sperm counts, Leydig cell counts, and AR expressions on the Leydig cells.

Table 1: The comparison of sperm counts, Leydig cell counts, and AR expression on the Leydig cells between the two groups

Variables	Groups		Mean difference	CI95%	p
	E	C			
Sperm counts (cells), mean±SD	94.30±12.06	201.86±16.54	107.59	97.76-117.37	<0.001
Leydig cells, mean±SD	3.22±1.18	7.61±1.35	4.28	3.42-5.14	<0.001
AR expression of Leydig cells, mean±SD	18.48±2.58	46.19±2.7	27.71	25.91-29.51	<0.001

AR: androgen expression; CI: confidence interval; SD: standard deviation

## Discussion

The results showed the average number of spermatozoa in the treatment group was  $42.72 \pm 3.33$  cells/FOV, compared to control's  $75.89 \pm 4.71$  cells/FOV. The low count of spermatozoa cells in this study are probably caused by active substances contained in ethanol extracts of young papaya seeds (steroid, triterpenoid, and alkaloid). These substances are thought to be antifertility agent that possess cytotoxic, anti androgen/estrogenic effects [7]. The decrease in the number of spermatozoa is also thought to be due to the estradiol and progesterone hormones contained in papaya seed extract

[8]. The hormone progesterone or estradiol will cause disruption of FSH and LH secretion.

Estradiol will cause suppression of the hypothalamus and anterior pituitary, causing GnRH and gonadotropin hormones to be inhibited [9]. The estrogen hormone will inhibit the process of spermatogenesis because it results in degeneration of the seminiferous tubular epithelium. While progesterone will inhibit FSH secretion which results in disruption of spermatogenesis [10]. If FSH secretion is disturbed, spermatogonia maturity will be



disrupted. Disruption of the spermatogonia cells will cause the process of spermatogenesis to be disrupted as well. FSH and testosterone act as survival factors for the survival of spermatocytes and spermatids through the regulation of intrinsic and extrinsic apoptotic pathways, where spermatid cells will become spermatozoa [11]. The number of spermatozoa is one of the parameters that is widely used to test fertility. It is also used to test the antifertility effect of a compound. Previously, papaya seeds have been widely studied in their effect as one of the natural ingredients which has antifertility properties.

Papaya seed extract can inhibit the population of spermatogonia and primary spermatocytes in male *Rattus norvegicus* rats. Another research shows that papaya seed's methanol extract can inhibit spermatogenesis causing necrosis of the epithelial germinal testes [12]. The number of Leydig cells in the treatment group was lower than in the control group. The decrease in the number of Leydig cells is probably caused by the active substances contained in papaya seed extract (steroid and triterpenoids, alkaloids) which are antifertility.

The active substances contained in papaya seeds can have cytotoxic, anti-androgenic or estrogenic effects. The decrease in the number of Leydig cells is also thought to be due to the estradiol and progesterone contained in the extract. The estradiol and progesterone will suppress the secretion of FSH and LH. If LH is inhibited, the function of Leydig cells to produce testosterone will be disrupted because on the surface of Leydig cells there are many LH receptors [13]. Progesterone will also inhibit FSH secretion which results in disruption of spermatogenesis [14].

A decrease in Leydig cells will cause a decrease in the production of the hormone testosterone, because these Leydig cells produce about 95% of the hormone testosterone in the body. In the process of spermatogenesis in addition to testosterone, FSH is also needed. FSH stimulates Sertoli cells to form androgen binding protein (ABP) [15]. The testosterone hormone produced by Leydig cells will be transported by ABP with very high concentrations to the site of spermatogenesis.

In this study, the average expression of androgen receptors in the Leydig cells in the treatment group was  $28.11 \pm 3.06\%$ , compared to  $55.07 \pm 2.49\%$  that we found in the control group. The mechanism that may be involved in the low expression of androgen receptors (AR) is the active alkaloids contained in the extract of young papaya seeds. Previous research has shown that the alkaloids contained in pepper (*Piper longum*) have been reported to reduce the amount of AR in prostate cancer cell culture (Pca).

The study shows that alkaloids can reduce the full-length expression of AR in the ligand-binding domain (LBD) [16]. The low number of AR expression due to alkaloids is likely due to the increased degradation of AR through the ubiquitin-proteasome pathway [17]. ARs are a type of nuclear receptor that is activated by testosterone or dihydrotestosterone in the cytoplasm and translocated into the nucleus [18].

Androgen receptors (AR) are involved primarily in the development of male-specific phenotypes during embryogenesis, in spermatogenesis, sexual behavior, and fertility during adult life. Activation of autocrine AR by testosterone is important for the maturation and function of Leydig cells and regulation of steroidogenic enzymes [19]. Furthermore, AR autocrine signaling in Leydig cells protects the seminiferous tubular epithelium and protects Leydig cell apoptosis in rats.

The number of receptors in the cell largely determines its reaction to hormones. If enough hormones and receptors are in the cell, the ligand (testosterone) binds and activates the receptor [18]. As a result of the reduction in AR in Leydig cells, steroidogenesis was inhibited so that spermatogenesis stops at the spermatid stage.

## Conclusion

The ethanol extract of young papaya seeds inhibits spermatogenesis of male rats, where the number of spermatozoa cells in the treatment group is lower than in the control group, the number of Leydig cells in the treatment group is lower than in the control group, and testosterone receptor expression in the treatment group was lower than in the control group.

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