Biosaintifika 14 (1) (2022): 135-146 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

Effect of Neem Leaf Extract on Reproductive Potential of Male Rats Fed High-Fat Diet

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Submitted: 2021-08-08. Revised: 2022-01-23. Accepted: 2022-04-07.

Abstract. Neem leaves contain bioactive compounds, including tannins, saponins, flavonoids, alkaloids, and terpenoids which are thought to have a cholesterol-lowering effect, but are also accompanied by antifertility effects. This study aims to determine the effect of neem leaf ethanol extract on the structure and reproduction of hyper-cholesterol or hyperlipidaemic male rats. The study used a completely randomized design which was divided into 6 groups with 4 repetitions, namely: P0 (negative control), P1 (positive control), P2 (8 mg/gBW simvastatin), P3, P4, and P5 (75, 100, and 125 mg/gBWof neem leaf extract). Parameters observed were testicular structure (epithelial thickness and seminiferous tubule diameter), GSI, ESI, epididymal protein expression and concentration, morphology and number of spermatozoa. Exposure to ethanolic extract of neem leaves had a significant effect on seminiferous tubule diameter, morphology and number of spermatozoa in white rats fed a high-fat diet, but did not differ significantly on the thickness of the seminiferous tubule epithelium and GSI, so it can be concluded that the effect of ethanolic extract of neem leaves in hypercholesterolemic rats decreased the structure and function of male reproduction as indicated by spermatozoa morphology, spermatozoa number, epididymal protein expression, and testicular structure, supported by the results of protein concentration, GSI, and ESI.

Key words: ESI, GSI, seminiferous tubules, simvastatin

How to Cite: Laila, N., Sitasiwi, A. J., & Isdadiyanto, S. (2022). Effect of Neem Leaf Extract on Reproductive Potential of Male Rats Fed High-Fat Diet. *Biosaintifika: Journal of Biology & Biology Education*, *14* (1), 135-146.

DOI: https://doi.org/10.15294/biosaintifika.v14i1.31470

INTRODUCTION

The increased tendency to consume high-cholesterol foods causes disorders of fat metabolism in the blood and triggers several diseases such as cardiovascular, diabetes mellitus, hyperlipidaemia, hypercholes terolemia (Heryani, 2016) to reduce male reproductive function (Widhiantara et al., 2018).

A high-fat diet consisting of 2 g/200 g of duck egg yolk and 100 g of pork oil for 28 days can increase cholesterol levels in rats Dwianita et al (2017). Increased cholesterol levels are positively correlated with triglyceride levels (Ulfa et al., 2018). High triglyceride levels cause low levels of High-Density Lipoprotein (HDL) cholesterol and increased LDL cholesterol formation (Syahrul et al., 2018). Cholesterol is important in the formation of steroids but if it is too high it will give negative feedback in spermatogenesis (Akdogan et al., 2018). High-fat diets can reduce the diameter of the seminiferous tubules which indicates a decrease in spermatogenesis due to reduced testosterone (Samuel et al 2018).

Spermatozoa are a product of spermatogenesis, so by analyzing the quantity and quality of spermatozoa can reflect the processes that occur during their formation, including making a diagnosis if there are abnormalities in the morphology of spermatozoa (aberration). Normal but nonmotile spermatozoa morphology needs to be observed for viability (Fitria et al., 2015).The quality of spermatozoa can be seen in the testes and epididymis, if the cells in the epididymis lack protein, it will result in cell degeneration, namely morphological changes. One of the important proteins found in the tail of spermatozoa is dynein protein. This protein is important because it has ATP-ase activity which functions to maintain internal homeostasis for sodium and potassium ions (Tjiphanata et al., 2017).

People generally use the drug simvastatin to lower cholesterol. Simvastatin works in the body by inhibiting 3-hydroxy-3-methylglutaryl coenzy me-A (HMG-CoA) reductase. Simvastatin is a strong antihyperlipidemic, especially in reducing LDL levels by up to 50% (Dewi et al., 2014). The use of simvastatin for a long time has serious side effects such as stomach ulcers, inflammation of the stomach, liver damage, gallstones and kidney damage (Seo & Wuryandari, 2017), so that people switch to using neem (*Azadirachta indica*) as herbal medicine (Jannah et al, 2020).

Extraction of the test material in this study was carried out using ethanol. Samsudin (2011) states that ethanol is a solvent that produces optimal and non-toxic active ingredients. Phytochemical screening of the ethanolic extract of *Azadirachta indica* leaves which includes flavonoids 0.39%, tannins 0.63%, saponins 0.56%, polyphenols 0.35%, alkaloids 2.84% (Atangwho, 2019), and terpenoids (Supriyanto et al., 2017).

The content of compounds in neem can interfere with cholesterol absorption and inhibit de novo cholesterol synthesis (Duangjai et al., 2019). The content of alkaloids is able to inhibit the performance of lipase enzymes in the gastrointestinal tract so that fat absorption in the body is reduced (Budiarto et al., 2016). Tannins can prevent oxidation of LDL cholesterol, prevent cholesterol reabsorption, stimulate bile salt secretion, and remove cholesterol through faeces (Na'i et al., 2019). Saponins form micelles with bile acids, so the ability of bile acids to form micelles with fatty acids is reduced (Prakoso et al., 2017).

Based on the description above, it is necessary to conduct research on the ethanolic extract of neem leaves in hypercholesterolemic rats to see the effect of improving the structure and function of male reproduction as indicated by spermatozoa morphology, spermatozoa number, epididymal protein expression, and testicular structure.

METHODS

This research was conducted at the Research Cage and Laboratory of Animal Structure and Function Biology, BiologyDepartment, Science and MathematicsFaculty, Diponegoro University. Neem leaf was done in Chemical Laboratory, Semarang State University, cholesterol diet analysis at Laboratory of Food Technology and Agricultural Products Testing, Agricultural Technology Faculty, Gadjah Mada University, tracking protein expression in Biochemistry Laboratory and making preparations at Pathology Laboratory, Veterinary MedicineFaculty, Gadjah Mada University.

Materials

The materials used in this study were 24 male white rats (*Rattus norvegicus*) aged two months, ethanol extract of neem leaves (*Azadirachta indica*), simvastatin, duck egg yolk, used cooking oil, commercial feed, distilled water, chloroform, 10% neutral buffered formalin, ethanol, xylol, paraffin solution, Mayer's egg albumin, haematoxylin ehrlich-eosin.

Acclimation of Animal Experiment

The animals used in this study were 24 male white rats (*Rattus norvegicus* L). Acclimation was carried out in collective cages (20x30 cm) for ±1 week. During the acclimation period, the animals were observed for their physical condition and behaviours, given standard feed and drinking water on an ad libitum basis, and will be weighed to determine initial body weight before treatment.

Making High Fat Diet Feed

The high-fat diet consisted of commercial feed and used cooking oil. The used cooking oil in this study was obtained from one litre packaged of cooking oil used to fry 450 g of tofu for 10 minutes at temperature 150-1650 C with a deep fat frying technique

(Muhartono, 2018), nine times of frying (Hanung et al., 2019). High-fat diet feed is made by adding 3 ml of used cooking oil every 30g commercial feed, then stirring. Feeding high-fat diet in this study was necessary to obtain hyperlipidaemia condition in rats in accordance with the specified time.Cholesterol in commercial feeds is 0.49 mg/g, while cholesterol levels in high-fat feeds are 0.76 mg/g.

Neem Leaf Ethanol Extract Production

Neem leaf samples were obtained from campus area of Faculty of Science and Mathematics, Diponegoro University. The leaves are dried in an oven at a temperature of 45-500 C. Leaves are declared dry if theyreach aconstantweight after weighed twice in a row (Hasana et al., 2019), then extracted with maceration method(70% ethanol).The neem leaves weredried and blended to become a powder. One hundred grams of the powder was extracted with a ratio of 1: 3 of the neem leaf powder and the solvent. After that, thesolution was macerated for 48 hours in a closed room by shaking it using a rotary shaker. Then, the sample was filtered using filter paper and then collected in an Erlenmeyer flask. Afterward, the filtrate was evaporated with a rotary evaporator at 40^0C until a concentrated extract was obtained in the form of a powder (Supriyanto et al., 2017).Theresults of extraction were maintained at 4^oC in a closed frosted jar (Isdadiyanto et al., 2021)

Test Animal Treatment

High-fat diet was given every morning for 45 days (±30g), then 75ml water was given along with feeding. Duck egg yolk was given every two days (morning), while simvastatin and neem leaf extract were given orally every afternoon for 44 days.Negative control treatment (P0): commercial feed, positive control treatment (P1): high fat diet and duck egg yolk orally 2.5 ml/gBW, P2: high fat diet and duck egg yolk orally 2.5 ml/200g /BW+8 mg/g/BW simvastatin in 1ml distilled water, P3: P1+75 mg/g/BW neem leaf extract in 1ml distilled water, P4: P1+100 mg/gBW neem leaf extract in 1 ml distilled water, and P5: P1+125 mg/g/BB neem leaf extract in 1ml of distilled water.The treatment was given by means of a cannula syringe attached to the syringe tube.Thecannula syringe was inserted straight and slowly until half of the syringe entered the cavity of the orris to the stomach of the rat, after that the rat was injected and the cannula syringe was slowly pulled out.

Dissection

Rats were weighed and checked for cholesterol before surgery. Dissection begins by preparing a dissection tool, chloroform, and a plastic container. The test animals were put in a plastic container containing 10 drops of cotton that had been moistened with chloroform as a sedative, waited a few minutes until the test animal fainted (Sari et al. 2019). The test animals were placed with the anterior facing up, then dissected by cutting the skin tissue on the anterior-inferior abdomen (Sari et al, 2019). The dissection is carried out until it reaches the thorax, then the thorax is dissected in the mid sagittal way (Alhomaidi et al. 2018) so that the topography of the organ is well visible.

Testicular Preparation

At the end of the treatment, the rats were dissected to collect testicular organs. Histological preparations were made using the paraffin method. Organs were cleaned with 0.9% NaCl solution and dried with tissue paper. Organ fixation was carried out with a neutral buffered formalin solution. Organs that have been fixed are put in 50 to 100% graded alcohol, absolute alcohol: xylol, xylol: paraffin (1:1), then pure paraffin I, II and III. After that, the tissue was immersed in a paraffin block. The organ was cut transversely (thickness 5 µm), then paraffin tape was affixed to the glass object with Meyer's Albumin. After drying, the preparations were put into pure xylol (15 minutes), 95 to 50% alcohol graded, distilled water, then haematoxylin dye. The preparations were washed with running water and distilled water, graded 30 to 70% alcohol and then to 0.5% Eosin dye followed by 70 to 100% alcohol and pure xylol.

Table 1. The diameter and thickness of seminiferous tubular epithelium

Treatments	Variable	
	Diameter (µm) ± SD	Epithelial thickness (µm) ± SD
P0	348,99[b]± 28.79	95.66[a]± 6.36
P1	326.20[ab]± 29.18	85.06[a]± 6.84
P2	305.30[a]± 16.04	84.32[a]± 11.30
P3	306.25[a]± 17.37	89.45[a]± 13.78
P4	353.84[b]± 38.59	98.50[a]± 8.40
P5	342.08[ab]± 7.48	91.94[a]± 6.37

Notes: Results are the mean ± standard deviation (SD). Mean values in the same column with different letters are significantly different ($p < 0.05$). P0= negative control, P1= positive control, P2= 8 mg/200gBB simvastatin, P3, P4, P5 (75, 100, 125 mg/200gBB neem leaf ethanol extract).

The preparations were covered with a cover glass with the help of Canadian balsam (according to the method of Setyawati et al., 2017). Histopathological examination of the testes used a light microscope with a magnification of 100 times (10x10) and 400 times (10x40). Testicular histology preparations were then photographed with a digital microscopic camera (Optilab®) with Optilab Viewer (Micronos®) software. Measurement of the epithelial thickness of the testicular seminiferous tubules was performed on 20 intact and round seminiferous tubules randomly using Image Raster software (Micronos®) (Setyawati et al., 2017).

Sperm Quality Observation

On the 46th day, all rats were dissected and their testes were taken. Subsequently, the tip of the cauda epididymis was cut and the ampulla of the ductus deferens was dissolved, collected in a watch glass containing two drops of 0.9% NaCl solution, then stirred homogeneously to observe the quality of spermatozoa (Hasanah & Sukarjati, 2016).

Calculation of Spermatozoa Motility

Spermatozoa motility calculations were carried out based on the 1999 WHO method. One drop of mouse sperm was taken immediately on a glass object and covered with a cover slip. Counting was carried out under a microscope (M=400x) magnification with a count of 100 spermatozoa.

Calculation of Spermatozoa Morphology

Observation of spermatozoa morphology was determined by making smear preparations from one drop of suspension on a glass spermatozoa object and fixed in the air until dry, then dipped in methanol, then dripped with safranin, then dipped rapidly with phosphate buffer 3 times then stained with crystal violet for 10 minutes, washed in running water and dried. Furthermore, normal and abnormal spermatozoa were counted in 100 spermatozoa for each replication. The calculations were carried out under a microscope (M=400x) (method of Elia, 2015).

Epididymal Protein Expression Tracking

The epididymis and the tissue attached to the outside of the testes are cleaned. The distilled water was homogenized with PBS in a ratio of 1:1 (w/v). The epididymis was weighed, then the right epididymis was used for protein expression detection. The epididymis was crushed by chopping and grinding by adding 1:1 PBS solution.

The epididymis that had been destroyed with the addition of PBS solution were then centrifuged twice at a speed of 10000 rpm, for 5 minutes at -40 C. The supernatant was taken and the protein concentration was immediately measured using a spectrophotometer (450 nm). The isolated protein was then run by electrophoresis to determine the expressed protein using the Chemiluminescent Western Blot kit(method of Sitasiwi et al., 2016).

Data Analysis

Histology of seminiferous tubules and spermatozoamorphology were carried out

descriptively, while detection of protein expression was carried out by comparing marker proteins, proteins from the control and the treatment group. The data of testicular weight, epididymal weight and the number of spermatozoa were analysed by analysis of variance (ANOVA). Data that had significant differences were then further tested with Duncan's test with a 95% confidence degree ($p < 0.05$) (Harsojuwono et al., 2011).

Ehical Clearance

The research has been conducted an ethical feasibility test by the Ethical Committee for Experimental Animal Use of Medicine Faculty, Diponegoro University with certificate ethic No.: 101/EC/H/FK-UNDIP/X/2020.

RESULTS AND DISCUSSION

Testicular Structure

The structure of the testes includes the diameter and thickness of the seminiferous tubular epithelium (**Table 1**).

The thickness of the seminiferous tubular epithelium in white rats showed no significant difference in each treatment (Table 1).

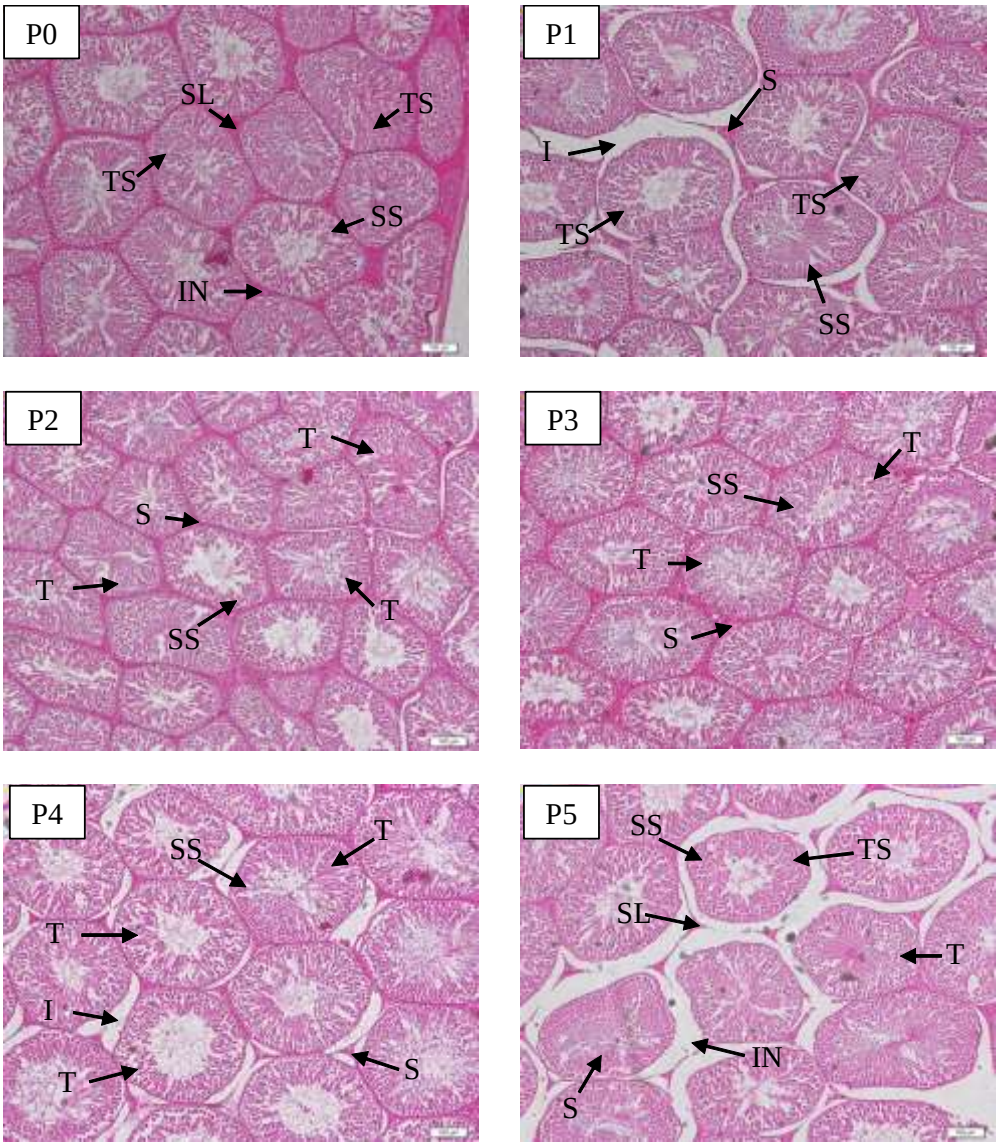


Figure 1. Testicular histology. P0= negative control, P1= positive control, P2= 8 mg/200gBB simvastatin, P3, P4, P5 (75, 100, 125 mg/200gBB neem leaf ethanol extract).TS = seminiferous tubules,SL= Leydig cells, IN= interstitial space, SS= Sertoli cells. (H&E, 10x).

Golalipour et al. (2011) also stated that thickening of the seminiferous tubular epithelium indicates that Sertoli and spermatogenic cells are in good condition and can carry out the spermatogenesis process well.

The testes contain seminiferous tubules where spermatozoa are produced as well as the hormones inhibin, ABP (Androgen Binding Protein) and estrogen (Ilacqua et al, 2017). The seminiferous tubules, especially in epithelial tissue, are composed of two cell populations; spermatogenic cells and Sertoli cells. This tissue determines the diameter of seminiferous tubules.

The histology of seminiferous tubule structure **(Figure 1)** of the negative control group (P0) indicated a normal histology where the histology shows that seminiferous tubule walls of the testis are composed of layers of spermatogenic cells. The seminiferous tubules consist of three main components: basement membrane, Sertoli cells, and germinal epithelial cells consisting of: spermatogonia, spermatocytes, and spermatids (Wahyuni et al, 2012).

The P1 treatment found that the germinal epithelial cells of seminiferous tubules were released into the lumen so that the arrangement of the epithelial cells looked looser.

The loose arrangement of the germinal epithelium causes the number of germ cells to decrease, so that the spermatozoa produced are reduced (Sugiantari et al., 2020), according to the research conducted, the number of spermatozoa decreased in treatment P4 and was not significantly different from P5 treatment. Decreased testosterone causes a decrease in libido, spermatogenesis, and the diameter of the seminiferous tubules. High and low levels of testosterone will result in negative feedback to the hypothalamus which results in disrupted spermatogenesis processes (Setyawati et al, 2017).

Sertoli cells are stimulated by FSH to synthesize LHRH-like substance and steroidogenic stimulating factor. Both of these proteins increase the activity of testosterone biosynthesis by Leydig cells, so it can be said that the increase in testosterone can indirectly be due to the influence of FSH. The indirect effect of FSH on Leydig cells is an increase in the number of LH receptors, hyperplasia and hypertrophy of Leydig cells and an increase in the activity of steroidogenic enzymes (Miyarso et al, 2017).

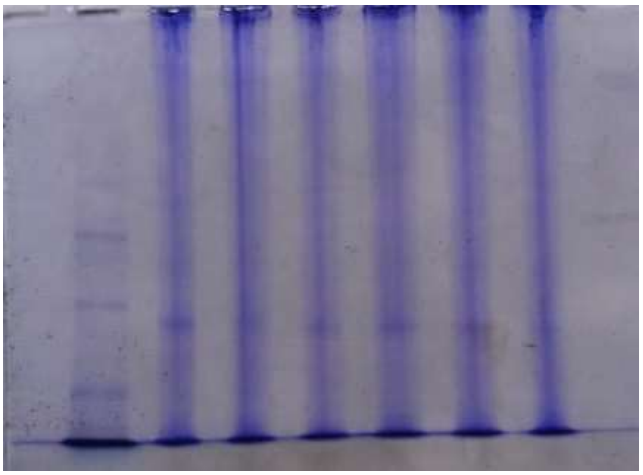
Table 2. GSI and Body Weight of Male White Rats

Treatments	*Gonadosomatic Index* (GSI) ± SD	Bodyweight ± SD
P0	$0.5525^{a} \pm 0.074$	$206.07^{a} \pm 12.63$
P1	$0.5175^{a} \pm 0.025$	$224.64^{a} \pm 9.05$
P2	$0.5825^{a} \pm 0.199$	$191.60^{a} \pm 28.36$
P3	$0.6200^{a} \pm 0.104$	$197.14^{a} \pm 20.43$
P4	$0.6100^{a} \pm 0.072$	$216.43^{a} \pm 9.92$
P5	$0.5125^{a} \pm 0.095$	$200^{a} \pm 26.42$

Results are the mean ± standard deviation (SD) of four replications. Mean values in the same column with different letters are significantly different ($p < 0.05$).

Table 3. The mean value of ESI

Treatments	*Epididymis SomaticIndex* (ESI)
P0	0.24
P1	0.23
P2	0.26
P3	0.38
P4	0.25
P5	0.24



Marker P0 P1 P2 P3 P4 P5

Figure 2. Epididymal protein expression. P0= negative control, P1= positive control, P2= 8 mg/200gBB simvastatin, P3, P4, P5 (75, 100, 125 mg/200gBB neem leaf ethanol extract).

Sertoli cells play an important role in controlling germ cell development. Androgens are also a major factor in supporting germ cell development and Sertoli cells are the main target for the action of androgen hormones (Verhoeven et al. 2010). Androgens are actively involved in the initiation of spermatogenesis by acting as germ cell survival factors and cooperating with FSH in supporting spermiogenesis (Hasbi & Gustina 2018). Spermatogenesis in the seminiferous tubules is a complex process involving mitotic cell division, meiosis and the process of spermiogenesis (Cordeiro et al., 2018).

GSI (Gonadosomatic Index) and ESI (Epididymis Somatic Index)

Table 2 shows that the lowest GSI value was in treatment P5 and the highest GSI value was in treatment P3, so it can be assumed that the higher the dose of neem leaf extract, the lower the GSI value in male white rats. Muslichah & Wiratmo (2013) also stated that the gonadal maturity level (GSI) in male rats was inversely proportional to the dose of neem leaf extract given. A decrease in the gonadosomatic index (GSI) indicates a reduction in reproductive capacity (Wang et al, 2021).

The highest ESI value was in the P3 treatment and there was an increase in the ESI value from the control treatment (**Table 3**). Both GSI and ESI are often used as indicators of biological disorders in reproduction. The results of the research by Abdulkareem & Nanakali (2019) showed a decrease in GSI and ESI in rats treated with tetrachlorodibenzo-p-dioxin (TCDD), which is a toxic chemical compound that can affect male fertility.

Epididymal Protein Expression and Concentration

The results of detection of epididymal protein expression in male white rats fed a high-fat diet after exposure to neem leaf ethanol extract can be seen in **Figure 2**.

Disturbances in protein biosynthesis, either due to low hormone levels or impaired function of the cell membranes that make up the reproductive organs, cause changes in protein expression (Sitasiwi et al, 2019)

The results of measuring the concentration of epididymal protein with a wavelength of 595 in male white rats fed a high-fat diet after exposure to the ethanolic extract of neem leaves can be seen in **Table 4**.

The epididymis is an organ rich in a number of proteins or molecules (Akmal et al, 2015). Spermatozoa maturation in the epididymis depends on the molecules secreted by the epithelium (Dube et al., 2008). In addition, the epididymis contains a family of

Table 4. Epididymal Protein Concentration

Treatments	Epididymis
P0	0.8026
P1	0.6739
P2	0.6729
P3	0.7478
P4	0.7377
P5	0.6343

Notes: P0= negative control, P1= positive control, P2= 8 mg/200gBB simvastatin, P3, P4, P5 (75, 100, 125 mg/200gBB neem leaf ethanol extract).

Table 5. Morphology and Number of Spermatozoa

Morphology ± SD	Number of Spermatozoa ± SD
$0.23^{a} \pm 0.07$	$215.07^{c} \pm 19.73$
$0.42^{b} \pm 0.04$	$138.49^{ab} \pm 33.04$
$0.37^{b} \pm 0.11$	$148.49^{b} \pm 49.67$
$0.3b^{a} \pm 0.06$	$147.91^{b} \pm 31.82$
$0.60^{c} \pm 0.09$	$94.99^{a} \pm 13.23$
$0.28^{ab} \pm 0.13$	$110.41^{ab} \pm 15.78$

Notes: Results are the mean ± standard deviation (SD) of four replications. Mean values in the same column with different letters are significantly different ($p < 0.05$ P0= negative control, P1= positive control, P2= 8 mg/200gBB simvastatin, P3, P4, P5 (75, 100, 125 mg/200gBB neem leaf ethanol extract).

proteinase and protease inhibitor genes (Sipillä et al., 2009). The P1 - P5 treatment showed lower protein levels than the control. This can be interpreted that the administration of neem extract in hyperlipidaemic rats can cause protein biosynthesis disorders so that protein concentrations decrease.

Morphology and Number of Spermatozoa

Table 5 shows a decrease in the number of spermatozoa in the P4 treatment, where the morphology was most normal but the number was the least, so it can be said that at this dose there was a disturbance in both the process of spermatocytogenesis and the process of spermiogenesis.

Ofoego et al (2017) stated that ethanolic extract of neem leaves at a dose of 500 mg/kg given to male white rats had an effect on decreasing sperm count, sperm motility and sperm morphology, as well as lowering serum testosterone levels when compared to controls. Neem leaf extract contains alkaloids, flavonoids (Ayini et al, 2014), coumarins, saponins, tannins, and terpenoids (Irais et al, 2020). Flavonoids inhibit the aromatase enzyme (Hargrove et al, 2011). The inhibition of the enzyme, which functions to catalyze the conversion of androgens to estrogen (El-Kersh et al, 2021), will increase the number of testosterones. The high concentration of testosterone will have a negative effect on the pituitary not to release FSH (Follicle Stimulating Hormone) and/or LH (Luteinizing Hormone) thereby inhibiting the spermatogenesis (Wuwungan et al, 2017).

Syamsuddin (2021) stated that the decrease in the number of spermatozoa caused by alkaloid compounds can cause degeneration of sperm cells and reduce the number of sperm cells. This alkaloid compound also provides a cytotoxic effect that can interfere with germ cells and spermatogenic cells. The decrease in the number of spermatogenic cells is also caused by decreased levels of the hormone testosterone as a result of chemical compounds that are cytotoxic and cause disruption of spermatogenic cell metabolism such as alkaloids, triterpenoids and steroids. These compounds are also owned by the ethanol extract of neem leaves.

Sajuthi et al (2017) reported that neem seed extract 0.25 and 0.5 g/kgBW caused a decrease in testicular function and interfered

with the developmental stage of spermatogenesis, namely at the spermatid stage. The recovery period of 36 days after administration of the extract was not sufficient to restore the physiological conditions of the mice's reproduction to their original state. The existence of this study confirmed that neem leaf extract had an effect on morphology and spermatozoa, where a dose of 0.5 g/kgBW was also equivalent to 100 mg/200 gBW used in this study. Future studies should be found the concentration of each phytochemical in neem leaves so that compounds that have an active role in preventing infertility can be identified.

CONCLUSION

Effect of ethanolic extract of neem leaves in hypercholesterolemic rats decreased the structure and function of male reproduction as indicated by spermatozoa morphology, spermatozoa number, epididymal protein expression, and testicular structure, supported by the results of protein protein concentration, GSI, and ESI.

REFERENCES

Abdulkareem, S. M., & Nanakali, N. M. (2019). Quercetin reduces oxidative stress damage to reproductive profile induced by 2,3,7,8-tetrachlorodibenzop-dioxin in male albino rats (*Rattus norvegicus* L.). *Applied Ecology and Environmental Research* 17 (6):13185-13197.

Akdogan, M., Y. Nasir, N. Cengiz, A. Bilgili. (2018). The effects of milled Tribulus terrestris, Avena sativa, and white ginseng powder on total cholesterol, free testosterone levels and testicular tissue in rats fed a high-cholesterol diet. Ankara Universitesi Veteriner Fakultesi Dergisi, 65(3), 267-272.

Akmal, M., D. Masyitah, Hafizuddin, & Fitriani. (2015). Epididimis dan Perannya pada Pematangan Spermatozoa. *JESBIO* 4(2): 1-9.

Alhomaidi, J., Jatmiko, S.W., Triliana, R. (2019). Pengaruh frekuensistress fisik terhadapkadar malondialdehide(mda) dan superoxide dismutase(sod) jantung tikusbetina dengandiet atherogenik. *Jurnal Bio Komplementer Medicine*, 6(1), 86-95.

Atangwho, I.J., Ebong, P.E., Eyong, E.U., Williams, I.O., Eten, M.U., Egbung, G.E. (2009). Comparative Chemical Composition of Leaves of some Antidiabetic Medicinal Plants: *Azadirachta indica*, Vernonia Amygdalina and Gongronema Latifolium. *African Journal of Biotechnology*, 8 (18).

Ayini, U., S. Harnina B., & T.C. Dewi. (2014). Efek Antibakteri Ekstrak Daun Mimba (Azadirachta indica A. Juss) terhadap Bakteri Vibrio algynoliticus Secara In Vitro. Biosaintifika 6(1), 67-75.

Budiarto, M.A., Yuniwarti, E.Y.W. (2016). Pengaruh Pemberian Tepung Daun Jati Belanda (Guazuma ulmifolia L.) dalam Pakan terhadap Kadar Trigliserida Darah dan Lemak Abdominal AyamBroiler. *Buletin Anatomi dan Fisiologi (Bulletin of Anatomy and Physiology)*, 1(1), 43-47.

Cordeiro, F., Gonçalves Jr, V., Moreira, N., Slobodticov, J.I., de Andrade Galvão, N., de Souza Spinosa, H., ... & Bernardi, M.M. (2018). Ivermectin acute administration impaired the spermatogenesis and spermiogenesis of adult rats. *Research in veterinary science*, *117*, 178-186.

Dewi, R., Santhi, D.D., Sukrama. & Karsana R. (2014). Simvastatin Generik. *Indonesian journal of Clinical Pathology and Medical Laboratory*. 20(2), 106-110.

Duangjai, A., A. Ontawong, C. Srimaroeng. (2019).Siamese neem flower extract suppresses cholesterol absorption by interfering NPC1L1 and micellar property in vitro and in intestinal Caco-2 cells. Res Pharm Sci., 14(3),190-200.

Dube, E., L. Hermo., P.T. Chan and D.G. Cyr. (2008). Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men. *Biol Reprod*,78,342–351.

Dwianita, C., Tandi, J., Dermiati, T. (2017). PengaruhPemberian Ekstrak Etanol DaunTalas (Colocasia esculenta (L.) Schott) terhadap Penurunan Kadar Kolesterol Total Darah Tikus Putih Jantan

(Rattus norvegicus) yang Diinduksi Pakan Tinggi Lemak dan Streptozotocin. *Farmakologika: Jurnal Farmasi*. 14(2), 83-90.

El-Kersh, D.M., Ezzat, S. M., Salama, M. M., E.A. Mahrous, Y.M. Attia, M.S. Ahmed &M.M. Elmazar. (2021). Anti-estrogenic and anti-aromatase activities of citrus peels major compounds in breast cancer. *Scientific Reports* 11(7121).

Elia, L. S., Janette. M. Rumbajan. (2015). Kualitas Spermatozoa Tikus Wistar (*Rattus norvegicus*) setelah Pemaparan Obat Nyamuk Elektrik Berbahan Aktif *Transflutrin*. *Jurnal E-Biomedi*. 3 (1): 274-279.

Fitria, L.M., Cut, M.T. Andreas, S.B. (2015). Profil Reproduksi Jantan Tikus (*Rattus norvegicus* Berkenhout, 1769) Galur Wistar Stadia Muda, Pradewasa, dan Dewasa. *Jurnal Biologi Papua*. 7(1), 29–36

Golalipour, M.J., Balajadeh, B.K., Ghafari, S., Azarhosh, R.,&Khori, V. (2011). Protective Effect of *Urtica dioica* L. (Urticaceae) on Morphometric and Morphologic Alterations of Seminiferous Tubules in STZ Diabetic Rats.

Hanung, A. (2019). Pengaruh frekuensi penggorengan minyak jelantah terhadap diameter dan gambaran histopatologi lumen aorta tikus wistar (*Rattus novergicus*). *Jurnal Kedokteran Diponegoro*, 8(1), 26-37.

Hargrove, J.L., Greenspan, P., Hartle, D.K., & Dowd, C. (2011). Inhibition of Aromatase and α-Amylase by Flavonoids and Proanthocyanidins from Sorghum bicolor Bran Extracts. *Journal of Medicinal Food* 14(7-8).

Harsojuwono, B.A., Arnata, I.W., Puspawati, G.A.K.D. (2011). *Rancangan percobaan: teori, aplikasi spps dan excel*. 1 ed. Lintaskata publishing. ISBN 978-602-99853-1-3

Hasanah, W., Sukarjati. (2016). Pengaruh pemberian ekstrak biji pepaya (*Carica papaya* L.) dan ekstrak daun mimba (*Azadirachta indica* A. Juss) dan campuran ekstrak biji pepaya dan ekstrak daun mimba terhadap kualitas spermatzoa mencit (*Mus musculus*). *Wahana*, 67(2), 59-69.

Hasana, A. N., Sitasiwi, A. J., & Isdadiyanto, S. (2019). Hepatosomatik Indeks dan Diameter Hepatosit Mencit (*Mus musculus* L.) Betina setelah Paparan Ekstrak Etanol Daun Mimba (*Azadirachta indica* Juss.). Jurnal Pro-Life, 6(1), 1-12.

Hasbi, H., & Gustina, S. (2018). Androgen Regulation in Spermatogenesis to Increase Male Fertility. *WARTAZOA. Indonesian Bulletin of Animal and Veterinary Sciences*, *28*(1), 13-22.

Heryani, R. (2016). Pengaruh Ekstrak Buah Naga Merah terhadap Profil Lipid Darah Tikus Putih Hiperlipidemia.*Jurnal Ipteks Terapan*. 10 (1): 8-17.

Ilacqua, A., Francomano, D., & Aversa, A. 2017. *The Physiology of the Testis*. Springer International Publishing.

Irais, C.M., Claudia, B.R., David, P.E., Ashutosh, S., Rubén, G.G., Agustina, R.M., ... & Luis-Benjamín, S.G. (2020). Leaf and Fruit Methanolic Extracts of *Azadirachta indica* Exhibit Antifertility Activity on Rats' Sperm Quality and Testicular Histology. *Current Pharmaceutical Biotechnology*.

Isdadiyanto, S., S.M. Mardiati, & A.J. Sitasiwi. (2021). Blood-Glucose Levels of Rats Given High-Fat Diets after Administration of Neem Leaf Ethanolic Extract. *Biosaintifika*,13(2), 142-148.

Jannah, B.M., S. Isdadiyanto, & A.J. Sitasiwi. (2020). Histopathological of White Rats Aorta Induced by High-Fat Feed After Administered by Neem Leaf Ethanolic Extract.*Biosaintifika*, 12(3), 370-378.

Miyarso, C.S., Widiastuti, T.C., & Kiromah, N.Z.W. (2017). Pengaruh Pemberian Ekstrak Akar Pasak Bumi (*Eurycoma longifora* Jack) Terstandar Terhadap Gambaran Histopatologik Testis Tikus Wistar. *Jurnal Ilmiah Kesehatan Keperawatan*, *13*(3).

Muhartono. (2018). Minyak jelantah menyebabkan kerusakan pada arteri koronaria, miokardium, dan hepar tikus putih (*Rattus norvegicus*) jantan galur

sprague dawley. *Jurnal Kedokteran Unila*, 2(2), 129-135.

Muslichah S & Wiratmo. (2013). Efek antifertilitas fraksi n-heksana, fraksi kloroform danfraksi metanol biji saga (*Abrus precatorius* L.) dan bijipepaya (*Carica papaya* L.) terhadap tikus jantan galur wistar. *Boptn/Dipa Unej* 2(1): 1-13.

Na'i, A., Yasinta, R. & Mulyani, S. (2019).Uji Efek Kombinasi Eeds dan Dpw terhadap Penurunan Kadar Kolesterol Total Tikus Putih Jantan Hiperkolesterolemia-Diabetes. *Farmakologika Jurnal Farmasi* 16(1), 1-12.

Ofoego, U.C., Nwachukwu, O., Ekwujuru, E.U., Mbagwu, S., Nwakanma, A., Nweke, E.O., & Ofoego, A.N. (2017). Ethanolic Seed Extract of *Cucurbita pepo* (Pumpkin) Protects the Testis from *Azadirachta Indica* (Neem) Induced Damage. *Journal of Natural Sciences Research, 7*(24), 120-131.

Prakoso, L.O., Yusmaini, H., Thadeus, M.S., Wiyono, S. (2018). Perbedaan efek Ekstrak Buah Naga Merah (*Hylocereus polyrhizus*) dan Ekstrak Buah Naga Putih (*Hylocereus undatus*) terhadap Kadar Kolesterol Total Tikus Putih (*Rattus norvegicus*). *Jurnal Gizi dan Pangan*, 12(3), 195-202.

Sajuthi, D., Agil, M., Arifiantini, R. I., Winarto, A., & Lisanti, E. (2017). Kualitas Spermatozoa Mencit (*Mus musculus* albinus) Jantan Pasca Pemberian Ekstrak Air Biji dan Ekstrak Air Daun Mimba (*Azadirachta indica* A. Juss). *Abstrak*. IPB Repository, Bogor.

Samsudin. (2011). *Biosintesa Dan Cara Kerja Azadirachtin Sebagai Bahan Aktif Insektisida Nabati*. Balai Penelitian Tanaman Rempah dan Aneka Tanaman Industri. Sukabumi.

Samuel, R.J., O. Joy, P.K. Murthy, S. Iyer, S.G. Ramachandra and K. Thamaraiselvi. (2018). Effect of feeding high fat diet on reproductive parameters in male mice. *Indian J. Anim. Res*., B-3528, 1-5.

Sari, R.A., Ariani R.D. & Rahma T. (2019). Pengaruh frekuensi stres fisik (forced swimming) terhadap kadar leptin serum tikus betina. *Jurnal Bio Komplementer Medicine* 6(1), 79-85.

Seo, A.L. Wuryandari, W. (2017). Seduhan Daun Mimba *(Azadirachta indica A. Juss)* sebagai Penurun Kadar Kolesterol Tikus *(Rattus norvegicus)*. *Artikel Ilmiah Disertasi*. Akademi Farmasi Putra Indonesia Malang.

Setyawati I, Putra I, & Roni N. (2017). Histologi tubulus seminiferus dan kadar testosteron tikus yang diberi pakan imbuhan tepung daun kaliandra dan kulit nanas. *J Vet*, 18, 369-377.

Sipilä P, Jalkanen J, Huhtaniemi IT, Poutanen M. Novel epididymal proteins as targets for the development of post-testicular male contraception. Reproduction. 2009 Mar;137(3):379-89. doi: 10.1530/REP-08-0132. Epub 2009 Jan 8. PMID: 19131560

Sitasiwi, AJ., Artama, W.T., Budiyanto, A. Dharmana, E. (2016). Pelacakan protein *wingless-type mmtv integration site family member*-4 pada uterus mencit. *J Vet*, 17(1), 71-77.

Sitasiwi, A.J., Isdadiyanto, S., Mardiati, S.M. (2019). Pelacakan Eskpresi Protein pada Testis Mencit (*Mus musculus*) setelah Paparan Esktrak Etanol Daun Mimba (*Azadirachta indica*). *Jurnal Sain Veteriner*. 37(1), 90-96.

Sugiantari, I.A.P., Suaskara, I.B.M., & Suarni, N.M.R. (2020). Konsentrasi Spermatozoa Dan Ketebalan Tubulus Seminiferus Tikus Putih Jantan Setelah Pemberian Seduhan Daun Kelor (*Moringa oleifera* L.). *Metamorfosa: Journal of Biological Sciences, 7*(2), 97-104.

Supriyanto, S., Rifa'i, B.W., Yunianta, M. (2017). Uji Fitokimia dan Aktivitas Antioksidan Ekstrak Daun Mimba (*Azaradiracta indica* A.Juss). Prosiding Snatif Ke-4. 523-529.

Syahrul, K., Kairufan, C.F., Durry, M.F. (2018). Efek Pemberian Madu terhadap Gambaran Histopatologik Aorta Tikus Wistar *(Rattus norvegicus)* yang diinduksiMargarin. *Jurnal e-Biomedik (eBm).* 6(1), 45-50.

Syamsuddin, M. I, Turalaki, G. L. A. & Tendean, L. E. N. (2021). Pengaruh Pemberian Ekstrak Biji Pepaya (Carica papaya L.) terhadap Kualitas Sperma. eBiomedik. 9(1):26-37

Tjiphanata, S., Queljoe E.D., Sudewi S. (2017). Pengaruh Pemberian Ekstrak Etanol Daun Dadap Ayam (*Erythrina variegata* L.) Terhadap Kualitas Spermatozoa Tikus Putih Jantan Galur Wistar (*Rattus norvegicus*). *Pharmacon*, 6 (3): 91-98.

Ulfa, P.M., Alioes, Y., Putri, B.O. (2018). Pengaruh Pemberian EkstrakBiji Melinjo(*Gnetum gnemon*) Terhadap Kadar Trigliserida padaTikus dengan Diet Tinggi Lemak. *Jurnal Kesehatan Andalas*, 7(2), 192-197.

Verhoeven, G., Willems, A., Denolet, E., Swinnen, J.V., & De Gendt, K. (2010). Androgens and spermatogenesis: lessons from transgenic mouse models. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1546), 1537-1556.

Wahyuni, S., Srihadi, A., Muhammad, A., Tuty, L.Y. (2012). Histologi dan Histomorfometri Testis dan Epididimis Muncak *(Muntiacus muntjak muntjak)* pada Periode Ranggah Keras. *Jurnal Veteriner*. 13(3), 211-219.

Wang, T., Kong, H., Shang, Y., Dupont, S., Peng, J., Wang, X., …, & Wang, Y. (2021). Ocean acidification but not hypoxia alters the gonad performance in the thick shell mussel Mytilus coruscus. *Marine Pollution Bulletin* 167: 112282.

Widhiantara,I.G., Permatasari, A.A., Ayu, P., Ferbian, M.S., Ni, P.E.S.D. (2018). Ekstrak Daun Sembung (*Blumea Balsamifera*) Memperbaiki Histologi Testis Tikus Wistar yang Diinduksi Pakan Tinggi Lemak. *Jurnal Bioteknologi & Biosains Indonesia*. 5(2), 111-118.

Wuwungan, C., Edwin de Q., Defny S. W. (2017). Kualitas spermatozoa tikus putih jantan galur wistar (*Rattus norvegicus* L.) setelah pemberian ekstrak etanol daun sirih (*Piper betle* L.). *Pharmacon*, *6*(3).