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The Detection of Testicular Protein Expression of Rats (*Rattus norvegicus*) after being treated with Ethanol Extract of Fenugreek Seeds

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Abstract**—***One of the testes' primary functions is to produce the haploid germ cells needed for sexual reproduction. Good vitality can be achieved through a healthy biological pattern and require lasting persistence throughout a productive male's life. This study aims to detect protein expression in rats' testes after being treated with ethanol Fenugreek seeds. As many as 32 male rats (Rattus norvegicus), aged 2-3 months (250-300 g) without anatomical abnormalities were used as experimental animals. They were divided into four groups: P0 as a control group was treated with normal drinking water, while groups of P1 to P3 were given 500, 1000, and 1500 mg kg^{-1} body weight/day of ethanol extract from Fenugreek seeds. The experiment of testicular protein expression was conducted with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The testes data were analyzed by One-way Analysis of Variance (ANOVA) and the Duncan Mean Range Test. The study concluded that Fenugreek's effect is believed to have an inducing impression on protein synthesis in the cells making up the testes. Besides, body weight, body weight gain, testicular weight, and testicular protein showed no significant difference (P> 0.05).*

Keywords**—body weight, Fenugreek, protein expression, rats, testis.**

I. INTRODUCTION

Environmental, health, and lifestyle factors in recent years have contributed to the deterioration of male fertility. Exposure to intense sunray, increasingly toxic pesticides, radiation, and other harmful matters are clear grounds of infertility in men [1]. Approximately 30-50% of infertility occurs in men. Infertility in men affects the immune system (5%), hypogonadism (10%), varicocele (17%), systemic diseases (3%), urogenital infections (9%), general infections (8%), unexplained testicular disease (8%), sexual arousal (6%), and other causes (8%). Infertility is a dysfunction of the reproductive system characterized by the inability to achieve a clinical pregnancy after 12 months of unprotected sexual intercourse. The amount, consistency, motility, and morphology of sperm are primarily dependent on male fertility, and many couples have problems with such complaints, as mentioned above [2].

The haploid gene cells in the testes are needed for the reproductive system, and the process requires lifelong care from a fertile male. Spermatogenesis is a series of events that gradually happens in the seminiferous canal's basal region; spermatogenic stem cells were divided into mitotic spermatogonia. Spermatogonia are divided into primary and secondary spermatocytes, which are turned into spermatids by meiosis after several mitosis cycles. In the lumen zone of the seminiferous conductors, the spermatids are transformed into spermatozoa (sperm) by the process of spermiogenesis. The various spermatogenesis stages can

be distinguished because each morphological cell type is closely correlated with each phase of spermatogenesis [3].

Medicinal herbs are widely used to treat male infertility in many countries through several exercises. Due to high medical costs, some people choose alternative medicine. In many countries, herbs are used to treat male infertility through several therapeutic methods. Herbs with high antioxidant properties are often considered more promising than invasive/pharmaceutical procedures to treat sperm defects, sexual disorders, erectile disorders, and premature ejaculation. Some plants that increase male fertility include celery, fennel, black seed, German chamomile, saffron, Fumaria parviflora, Origanum vulgare, and carrots [4]. Fenugreek is a grassy plant that is considered to have medicinal properties because of its essential nutrient content. Fenugreek (*Trigonella foenum graecum L.*) contains antioxidants and is an old plant deemed to have various properties to treat blood sugar, overfat, antimicrobial, anticancer, and increase sexual capability.

Fenugreek is a perennial herb whose seeds contain protein (25-36% of the plant's dry weight) and various vitamins [5]. The seeds contain several essential nutrients such as iron, calcium, phosphorus, potassium, and other mineral components. Fenugreek seeds consist of a large amount of fiber, phospholipids, glycolipids, oleic acid, linolenic acid, choline, and other practical elements. In *Ayurvedic* medicine, Fenugreek has a long history as a traditional treatment for diabetes, indigestion, elevated lipids, and edema (fluid retention) in the feet. Also, for humans and livestock, Fenugreek is a healthy source of dietary protein. The seeds have a strong and bitter taste.

Fenugreek seeds in cosmetic and medical applications are a source of yellow dye. The application of seed extracts (essential oils, saponins, and flavonoids) has been extensively studied conventionally and pharmacologically. Due to their antioxidant properties and health benefits, many researchers have learned that germinated Fenugreek is much more beneficial than dried seeds [6]. This research aims to detect mouse (*Rattus norvegicus*) protein expression after being treated with ethanol extract from Fenugreek seeds.

II. METHODS

Animal facilities, management, and handling during the experiment were done in compliance with the Guidelines for Care and Use of Biology Laboratory at Diponegoro University and approved by the Research Ethics Committee of the Faculty of Medicine, Diponegoro University number 98/EC/H/FK-RSDK/VII/2018. This research was conducted from March to July 2020 at the Biology Laboratory, Diponegoro University Semarang.

2.1 Preparation of Fenugreek ethanol extract

Fenugreek seeds were obtained from Libya. The seeds were rinsed with tap water. The ethanol extract from the seeds was obtained by mixing with 70% ethanol. The proportion of seeds in ethanol was 100 grams in 1000 mL of 70% ethanol. Dilution was carried out at room temperature for twenty-four hours and stirred regularly. The extract was filtered and stored in a dark-closed bottle in the refrigerator until anti-fertility testing was carried out [7].

2.2 Laboratory animals

A total of 32 rats (*R. norvegicus*) male (250-300 g) aged 2-3 months without anatomical abnormalities were used as experimental animals. The rats were obtained from the Laboratory of Biology Department at Semarang State University. After being acclimated for seven days in the laboratory, the mice were kept in a well-controlled room at ± 26 °C and fed ad libitum for the experiment.

2.3 Experimental design and animal care procedures

This study was conducted in a completely randomized design. All rats used were divided into four groups. Group P0, as a control group, were treated with normal drinking water. The next groups, P1 to P3, were given 500, 1000, and 1500 mg kg^{-1} body weight/day of ethanol extract from Fenugreek seeds. The Fenugreek extract was dissolved in boiling water and stored at room temperature. Treatment was given orally utilizing gavage at 8.00-9.00 am every day for 21 days.

2.4 Bodyweight, feed intake, and drinking water evaluation

Bodyweight was measured every three days during treatment (seven times in total). Drinking water and feed intake were measured every day. At the end of treatment, the mice were starved for 12 hours and then sacrificed with chloroform anesthesia.

2.5 The testes weight and testicular protein concentration

The testes were isolated and washed in saline solution. Later they were weighed and placed on a mortar for grinding. Testicular tissue was dissolved with phosphate-buffered saline (PBS) in a ratio of 1:10 (weight/volume). The testicular solution and PBS were placed in a tube with a volume of 1.5 mL then centrifuged at 3000 rpm for 10 minutes. Measurement of testicular protein levels was carried out by taking a 10μL supernatant and adding up to

1000 µL of PBS in a spectrophotometer tube. The protein content was determined at a wavelength of 450 nm.

2.6 Protein Expression

The testicular weight was weighed in three weeks of the experiment to analyze mouse protein expression. The investigation of testicular protein expression was conducted with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.7 Statistical analysis

Statistical Package of Social Science (SPPS) version 17.0 was used for data management and analyses. The collected data were analyzed by One-way Analysis of Variance (ANOVA) and continued with the Duncan Mean Range Test. The difference in P values at P <0.05 was considered significant.

III. RESULTS AND DISCUSSION

Fig. 1 shows the Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) results for protein expressions.

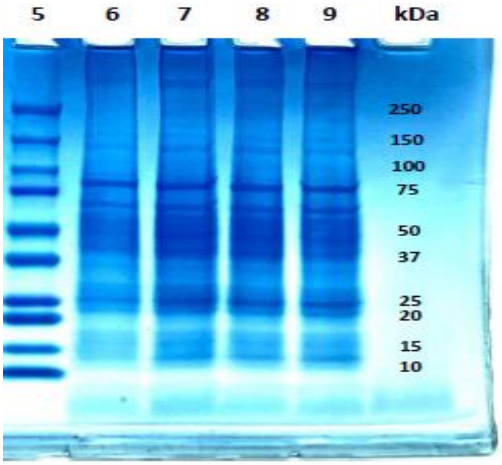

Fig. 1: Results from Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): lane 5: control; lane 6: sample K7; lane 7: sample P1.7; lane 8: sample P3.9; lane 9: sample P3.7

Based on Fig. 1, the protein expression presented in the treatment group is thicker compared to the control group (label 5).

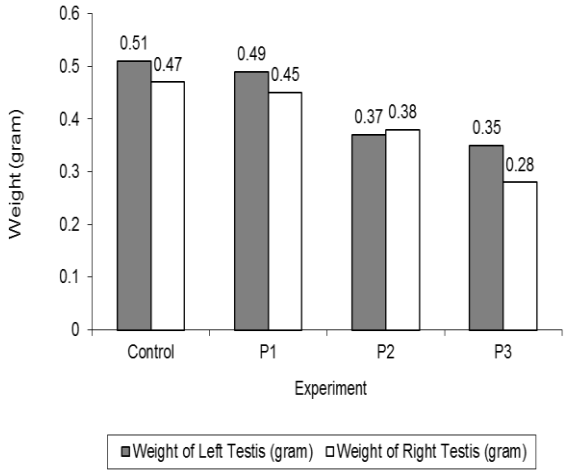

Fig. 2: Testicular weights in the first week.

From Fig. 2, the average weight of the left testes was slightly more massive than the right one after the ethanol extract of Fenugreek seeds was given in the first week for P1-P3. However, Fenugreek extract treatments did not show a significant difference (P> 0.05) on testicular weights in the first week of observation.

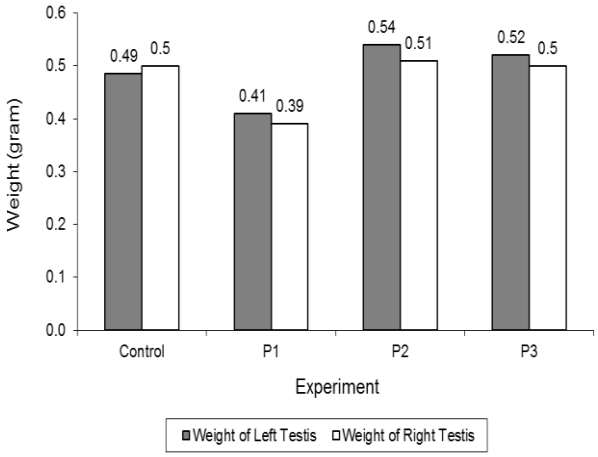

Fig. 3: Testicular weights in the second week.

From Fig. 3, it can be seen that the average weight of the left testes was moderately more massive than the right one after the ethanol extract from Fenugreek seeds was given in the second week for P1-P3. However, Fenugreek extract treatments did not show a significant difference (P> 0.05) on testicular weights in the second week of observation.

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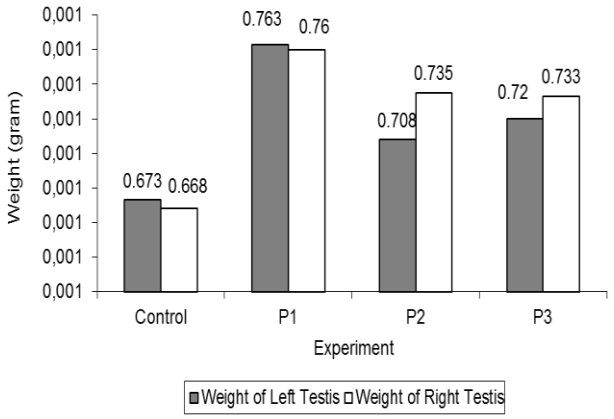

Fig. 4: Testicular weights in the third week.

Based on Fig. 4, the average weight of the right testes was a bit more massive than the left one after the ethanol extract from Fenugreek seeds was given in the third week for P1-P3. However, Fenugreek extract treatments did not show a significant difference (P> 0.05) on testicular weights in the third week of observation.

The analysis results of the body weight, body weight gain, testicular weight, and testicular protein of white rats after being treated with fenugreek extract are shown in Table 1 below:

Table 1. Analysis Results of the Body Weight, Body Weight Gain, Testicular Weight, and Testicular Protein of White Rats after being treated with Fenugreek extract.

Parameters	Weeks	Treatment group			
		P0	P1	P2	P3
Body weight (g)	1st	63.29±14.42	62.34±13.40	61.45±13.55	66.02±17.13
	2nd	84.73±14.30	82.07±14.39	74.89±20.52	81.24±17.09
	3rd	99.11±15.57	99.74±20.64	102.99±9.07	96.95±20.39
	4th	115.08±15.78	129.74±23.10	113.89±6.39	118.79±26.42
Weight gain (g)	1st	35.67±12.03	32.79±11.92	31.87,11.05	30.93±35.32
	2nd	67.72±19.63	63.85±9.90	58.62±19.62	44.39±19.92
	3rd	89.77±27.38	88.61±26.88	90.07±5.05	74.98±23.10
Testicular weight (g)	1st	0.49±0.21	0.47±0.22	0.38±0.24	0.32±0.13
	2nd	0.49±0.21	0.39±0.14	0.52±0.04	0.51±0.07
	3rd	0.67±0.11	0.76±0.07	0.72±0.02	0.73±0.12

No significant difference (P>0.05): P0/Control = (0 mg kg^{-1} Bodyweight; P1 = 500 mg kg^{-1} Bodyweight; P2 = 1000 mg kg^{-1} Bodyweight; P3 = 1500 mg kg^{-1} Bodyweight.

IV. DISCUSSIONS

In previous studies, Fenugreek has been understood as a possible source of anti-fertility, abortifacient, emmenagogue, and uterine stimulant agents [8, 9, 10, 11]. However, some studies assessed Fenugreek extract as a complement supporting testosterone [12, 13, 14, 15] through animal and human experiments as it possessed many active elements such as flavonoids, alkaloids, amino acids, coumarins, vitamins, saponins, and other antioxidants. Yet, the effects covering those investigations were inconsistent.

Saponins, a family of glycosylated triterpenes, found 4.8% in Fenugreek seeds with two major steroidal compounds: diosgenin and yamogenin. They acted as anti-fertility and caused teratogenicity through estrogenic and

androgenic activities [16, 17, 18]. Distinct characters of Fenugreek extract have shown an androgenic and anabolic effect in a male's reproductive system.

A histological study of testes from rats after being treated with Anabolic-androgenic steroids (AAS) [19] showed different effects in shape on seminiferous tubules, resulting in abnormality of normal testicular functions and mass. Their study also exhibited evidence that direct exposure to AAS had some interruption consequences in average testosterone production, causing spermatogenesis regulation disorders and eventually decreased sperm count and mortality.

Besides, many elements affected the volume and dimensions of the testes: all variability in compactness, the elasticity of scrotal surface, compression of scrotal content, and other compositions around the testes such as the epididymis, tunica vaginalis, hydrocele, spermatic fascias, and scrotal skin, up to the experience of analysis by the researchers [20].

However, from the ANOVA test results: no significant difference between the effects of Fenugreek seeds' ethanol extract on the testes weight in the first week of observation; there was also no significant difference between the effects of Fenugreek seeds' ethanol extract on the testes' average weight; there was no significant difference between the effect of Fenugreek seeds' ethanol extract on testes weight in the third week.

Thus, the contained proteins belonging to Fenugreek seeds, including diosgenin and yamogenin, identified as most steroidal sapogenins [21], did not significantly influence the weight gain of left and right testes in the present study. It is also believed that the effects of prohormone components found in Fenugreek seed extract are likely to have a low impression in Leydig cells that are supposed to affect further the normal secretion of testicular testosterone and the entire testicular functions.

From the present experiment, Fenugreek seeds' effect was not significant in affecting the increase in testicular weight, indicated by the absence of a significant difference between animals' testicular weight in the first week, second week, and third week. Thus, there was no significant difference in testicular weight towards the P0, P1, P2, and P3.

The testes are large olive-sized oval organs located in the scrotum, held together at both ends by a spermatic cord structure. The testes are responsible for producing testosterone and sperm. Inside the testes is a mass of coiled tubes called seminiferous tubules accountable for making sperm cells through spermatogenesis [22].

The entire male reproductive system relies on hormones that stimulate or control the function of cells or organs. Male replication hormone (FSH), luteinizing hormone (LH), and testosterone are the chief hormones involved in the male reproductive system. The pituitary at the base of the brain develops FSH and LH. For sperm development (spermatogenesis), FSH is required, and LH stimulates testosterone production, which is needed for continued spermatogenesis. Muscle mass and strength, fat distribution, bone mass, and sex drive are also closely associated with testosterone [21].

Sperms were transferred to the epididymis to complete its development [23]. The sperm then travel to the vas deferens or sperm ducts. The seminal vesicles and prostate gland produce a white fluid called seminal fluid; when stimulated sexually, they combine with sperm to form semen. When a man is sexually aroused, blood floods the erectile tissue, and the penis becomes stiff and erected, resulting in the muscles around the body contract and push semen into the tubes and urethra due to the stimulation. This process is called ejaculation, in which semen leaves the man's body by the urethra. A man can accommodate up to 500 million sperm each time they ejaculate [23].

The sperm viability test is used to assess whether non-motivated/dallying sperm are alive or dead and when the sperm motility is below 5-10%. This test is useful in primary ciliary tardive, where ultra-structural defects of sperm flagella cause low motility. No widely accepted morphological evaluation of the mouse sperm classification scheme is available; spermatozoa are measured in this system as (a) ordinary, (b) abnormally shaped head separate from flagella, (c) deformed head separate from flagella, (d) abnormally-flagellated deformed head, (e) irregular head, (f) head defects, (g) other flagella defects with normal heads, and (h) normal heads.

Other researchers classified several anomalies on the sperm head and tail, including bare hooks, heads of banana, amorphous, pinheads, two-heads twisted heads, and bent tails; effectiveness of such sperm is difficult in their function of fertilization. Although the classifications are very highly subjective, sperm head size is a continuous variable with widely accepted definitions, primarily when they are categorized as small heads. In many classification schemes, an individual sperm can be classified into more than one category [24].

Generally, epididymitis or testicular sperm issues have been reported in several studies on reproduction system studies. A sperm head (caput), body (corpus), tail (*cauda*)

of the epididymitis may have sperm epididymitis. Sperm tally is affected by sexual activity. This sperm count can help necropsy tests regarding sperm-filled in cauda for at least a week [23].

Testicular sperm number usually means a homogeneous and resistant sperm headcount. The nucleus of the spermatozoa becomes highly condensed during spermiogenesis, and the nuclear material becomes widely interlinked. When the spermatid nucleus matures, it becomes relatively immune to trauma as homogenization. All other cells and immature spermatozoa are killed through this treatment, and a reasonably accurate estimate of the number of spermatozoa can be obtained during the final spermatogenesis maturation process. In a necropsy test, the number of spermatic heads of the testis can be estimated, where the tissue is frozen and examined further.

The testicular tissues as samples for testing protein expressions were resolved in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS–PAGE) gels. For analysis, labeled samples were run parallel on a preparative gel from stacking gel to separating gel. The protein expressions presented in the treatment groups (lane 6, 7, 8, and 9) showed several thicker bands compared to the control group (lane 5). Relative flow for a given polypeptide varied with gel density, and different polypeptides can hold identical molecular masses.

After electrophoresis and staining of the gel, as shown in Fig. 1, SDS-Page results showed that proteins from Fenugreek are believed to have an inducing impression on protein synthesis in the cells making up the testes; most of them are somewhat heavy, shown by bold colors mostly found in the mid-lane. Most of the bands were the same, but some exciting and apparent bands could be leading to the difference in protein concentration values. One bar (strip) on a gel can therefore consist of one or more polypeptides. Bands of similar mass and concentration that were moving together indicate that the polypeptides are related in structure. They were consistently higher and showed positively contributed expression compared to the control group (lane 5) as Fenugreek seeds' protein content was discovered to be 235-246 g kg^{-1}, and lipid content in the range of 40-100 g kg^{-1} [25].

Legume crops, including Fenugreek (*Trigonella foenum graecum L.*), are high-quality foodstuffs that provide nutritional and functional benefits at a low price. Fenugreek is widely planted in China, India, Turkey, Canada, Australia, North Africa, South Africa, and Southern Europe [26, 27]. Fenugreek is one of India's top export products and has long been recognized as a powerful herb in traditional medicine. Fenugreek seeds contain proteins with the desired configuration of amino acids, lipids, and biogenic elements. Fenugreek seeds contain large amounts of Fe, P, Ca, Zn and Mn, and are plentiful in vitamins A, B1, C, and nicotinic acid. This study's examination proved that a factor like amounts of Fenugreek seeds extracts affect the protein expressions.

Although there were differences in values between body weight, body weight gain, testicular weight, and testicular protein, the difference was not much contrasting. Through this study, it was discovered that Fenugreek extract treatments did not show a significant difference ($P > 0.05$) on the parameters from the first week to the third week of observation (Table 1). This study showed that Fenugreek extract's impression ranging from 500, 1000, and 1500 mg kg^{-1} body weight/day was not significantly altering the male rats' body weight gain in 21 days of observations. The increase in body weight observed in this study ranged from 30-40 grams per week, and the significant difference between the control and treatment groups was non-existent.

The average body weight of rats ranged from 275-375 grams at 56 days of age [28], and the bodyweight of the mice observed in this study was smaller since the animals used had a smaller body weight from the start. Compared with the results of a previous study in histology [29], it can be inferred that the body weight gain in this study nevertheless displayed normal body weight.

V. CONCLUSION

This study concluded that Fenugreek seeds had no significant effect on the increase in mice's testis weight from the first until the third week of observation. A factor like amounts of ethanol extract from Fenugreek seeds affected the protein expressions, shown by an inducing impression on protein synthesis in the cells making up the testes. This research is anticipated to grow as a supplementary directory for research related to Fenugreek seed extract in health and general science domains. It is expected that further research covering more diverse extracted concentrations can be carried out in the future so that broader recommendations for finding the best extract configuration could be attained.

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