

***In vivo*, *In vitro* and *In silico* Aphrodisiac Activity of the Hydroalcoholic Fruit Extract of *Fragaria ananassa* in Male Wistar Rats for the Treatment of Erectile Dysfunction Induced by Metronidazole**

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Abstract Erectile dysfunction (ED) is one of the most common sexual disorders in men which occurs due to various biological, environment and rarely genetic factors. The study is conducted due to various side effects of the currently available erectile dysfunction drugs such as PDE 5 inhibitor. The objective of the study is to evaluate the aphrodisiac activity of *Fragaria ananassa* fruit extract in drug-induced erectile dysfunction in rats. The fruit of *Fragaria ananassa* was extracted by the hydroalcoholic extraction method and evaluated for its preliminary phytochemical investigation and further molecular docking studies were carried out. The hydroalcoholic extract of the fruit at different doses (500mg/kg and 1000mg/kg) is used for *in vivo* studies carried out in rats and various physical mating parameters, serum testosterone, FSH, LH, AST, ALT, TC, and antioxidant SOD, CAT and LPO were estimated along with the histopathology studies and compared with standard sildenafil (4.5mg/kg). The phytochemicals screening confirms the presence of the flavonoids, glycosides, phenols, in *Fragaria ananassa* fruit extract (FAFE). In metronidazole induced model, FAFE shows a significant effect against dysfunction by improving the serum hormonal levels and restoration of the

natural antioxidant system (SOD, CAT & LPO) and histology of the testis, which serves as evidence for the aphrodisiac activity of FAFE. From the present study, it can be concluded that the hydroalcoholic extract of *Fragaria ananassa* fruit extract (FAFE) possesses aphrodisiac activity and enhances erection. FAFE is effective in ameliorating the effects of metronidazole induced ED model in rats.

Keywords Erectile Dysfunction, *Fragaria ananassa*, Molecular Docking, Metronidazole, Sildenafil

1. Introduction

Erectile dysfunction (ED) is a common sexual and urologic disorder that affects men of all ages, and it is defined as the inability to maintain and achieve the penile erection which is harder to satisfy the sexual intercourse [1]. Thus, sexual dysfunction leads to loss of erection, diminished libido, premature ejaculation, arousal disorder and insufficiency in detumescence [2]. At present

according to statistical data, there may be 150 million erectile dysfunction men around world. According to predictions, the population may reach 322 million by 2025. Type 2 diabetes affected men are more 3 times likely affected by erectile dysfunction [3]. The aphrodisiac substance is referred to the agents which has the ability to increase and maintain the sexual desire, arousal and performance such as Viagra, levodopa and Amyl nitrite [4,5]. Erectile dysfunction has ageing as a major contributing factor. The patients' age, Cardio-vascular, and Diabetes mellitus conditions are co-morbidities that can disturb the molecular processes that support penile erections, which can hasten the decline of erectile function [6].

The nitric oxide, cyclic guanosine monophosphate (cGMP) and cyclic amino monophosphate (cAMP) have a significant role in contraction and relaxation of the smooth muscles, which is essential for the penile erection. Impotence may be occurred due to imbalance in the penile smooth muscle relaxation. This imbalance can be caused by hormonal, neurogenic, vasculogenic, psychogenic, and other pharmacogenic factors, as well as alteration in nitric oxide/ cGMP, cAMP pathways [7]. The current treatment option for erectile dysfunction includes sildenafil [Phosphodiesterase type 5 inhibitors], having treatment efficacy with numerous adverse effects associated with dizziness, headache, and minor inhibitor in PDE6 which is commonly present in rod and cone photoreceptor of eyes [8,9].

Plants with high levels of sterols, such as sitosterol and campesterol, stimulate the body's receptors and various hormones, including testosterone, to boost performance and libido. The amino acid arginine is required for the production of nitric oxide [10]. Recent studies on the effects of purwoceng extracts on erection performance revealed evidence that these extracts increase testosterone levels in peripheral tissue by converting stigmasterol to testosterone [11]. Since there is now no effective treatment for erectile dysfunction, investigators are always looking for novel pharmacological compounds or technological advances [12].

Fragaria ananassa, commonly known in English by the name strawberry and is a widely grown hybrid species of the genus *Fragaria*, cultivated globally for their fruit. It is reported for various activities and a special role in cardiovascular disease, inhibiting platelet aggregation, and inflammation, improving endothelial function, and having antioxidant capacity, chemotherapy, and evidence of having the anti-obesity, antidiabetic, antihypertensive, and anti-inflammatory effects. The chemical constituents of the fruit are obtained from the "Dr. Duke phytochemical and ethnobotanical databases (U.S. Department of Agriculture)" database [13-15]. To investigate the plant's aphrodisiac effect, first evaluate the active components of *Fragaria ananassa* for the molecular docking process to determine their binding affinity. The docking studies were confirmed by the *in vivo* experiments.

2. Materials and Methods

2.1. Chemicals and Kits

Metronidazole and sildenafil are procured from Omega Pharma and ZIM Laboratories Limited. Progesterone and Estradiol benzoate were procured from Nuclotec Remedies Pvt. LTD, and Helax healthcare Pvt. LTD. Kits were procured from TransAsia Bio-Medicals Ltd, (ERBA). All other chemicals used were of analytical grade.

2.2. Plant Authentication

Fruits were obtained from the local vendor of Bangalore, Karnataka, India. The fruit was identified and authenticated by Dr. Rama Rao, Research officer (Botany), Central Ayurveda Research Institute, Kanakapura Main Road, Bengaluru, Karnataka, India. Voucher specimens' number SMPU/CARI/BNG/2022-23/208 have been kept in Central Ayurveda Research Institute, Kanakapura Main Road, Bengaluru, Karnataka, India.

2.3. Preparation of Fruit Extract

The extract is prepared using hydroalcoholic extraction method of ethanol and water. Initially, the collected fruits were chopped into small pieces and homogenized in a solution composition of ethanol/water/ formic acid (80:20:0.1, v/v/v) for 50g of fruit material. Extraction is maximized using an IMLH magnetic stirrer in dark for 2 hours and centrifuged at 2400rpm for 2 sequential times for each 15 min to sediment solids and the supernatant was filtered and concentrated using a rotary evaporator and aliquots were diluted in water for further use in animals [16].

2.4. *In vitro* Quantitative Phytochemical Analysis

The Total phenol content was measured using "Folin-Ciocalteu reagent-based assay" [17] and "Aluminum chloride colorimetric method" was used to estimate the flavonoid content [18].

2.5. *In silico* Molecular Docking Studies

To investigate the biomolecular interactions and receptor binding affinities of various compounds, docking studies were performed. Using the Autodock Vina program [19], Autodock vina tools, UCSF Chimera 1.16 [20], Avogadro 1.2.0 [21], Biovia Discovery Studio 2021 client by Dassault Systemes, PyRX [22], and PyMOL, the docking studies were carried out. The crystal structure of the complex of human phosphodiesterase 5 (PDB id:2H42), was docked with 58 selected chemical constituents of *Fragaria ananassa* and standard sildenafil.

2.6. Animals

Thirty swiss strain adult male rats (12 weeks) weighing about 180-220g were used and thirty swiss strain adult females weighing about (160-180g) were used for the present study. The study was approved by the Institutional Animal Ethics Committee (approval number: PESCP/IAEC/145/2023).

2.7. Acute Toxicity Studies

The Acute oral toxicity studies of *Fragaria ananassa* will be carried out according to OECD 425 guidelines. The sample dose up to 5000mg/kg body weight will be administered orally (oral gavage) to Rat using rat oral gavaging needle (not more than 2ml/100g) [23].

2.8. Animal's Groupings and Drug Administration

The swiss male rats were divided randomly into 5 groups (each group of six animals). Group 1 was the normal animal administered with distilled water for 30 days (vehicle); Group 2 was disease control administered with metronidazole 200mg [24] (for 1-14 days) and remained untreated; Group 3 was standard control initially administered with metronidazole 200mg (for 1-14 days) and treated with sildenafil (4.5mg/kg) for 15-30 days; Groups 4 and 5 were treatment groups (FAFE 500mg and FAFE 1000mg), and were standard control initially administered with metronidazole 200mg (for 1-14 days) and treated with *Fragaria ananassa* fruit extract of 500mg/kg and 1000mg/kg. Sexual Mating behavioral parameters were recorded after the experimental period.

By repeatedly injecting the female rats with estradiol benzoate (10 µg/100 g body weight) and progesterone (0.5 mg/100 g body weight) 48 hours and 4 hours before mating, respectively, the female rats were artificially induced into oestrus (heat). The experiment started after confirmation of the female rats' receptivity [25].

2.9. Mating Behavior Test: [26,27]

According to the experimental protocol, various concentrations of the drugs are administrated to the several groups of animals. The male animals should be exposed to red dim light at the particular time on 30th day. Already the female animals should be induced artificially into the oestrus phase (heat). The investigation should then be conducted on the most receptive females. The experiment should be carried out at 20:00 in the same lab with same lighting. In the ratio of 1 female to 1 male, the female animals have brought into the cages of the male animals.

2.9.1. Physical Parameters

The various physical mating behavior tests such as Mount frequency, Intromission frequency, Mount latency, Intromission latency, Ejaculatory latency, post-ejaculatory

interval, Copulatory rate, and Index of libido were recorded after the treatment for a time period of 1 hour in the respective light conditions.

2.9.2. Determination of Time of Hesitation & Attraction towards Female

A female and a male rats were placed in a cage containing wooden barrier of 15 cm for separating them, where a male rat is motivated to climb over. The period of the male rat's hesitation before attempting to pass the barrier was measured in seconds. In the same, throughout a 15-minute observation period, scoring for attraction to females was recorded using a score between 0 and 5. The scoring was recorded on 0, 14, and 30th days of treatment.

2.9.3. Effect on Sexual Organ Weight

The testes and epididymis were removed from the animals after they were euthanized, weighed after separating from the attached tissue. The epididymis and testes' wet weights were measured in order to determine the gonadosomatic index.

$$\text{GSI} = (\text{Organ weight}) / (\text{Total body weight}) \times 100$$

2.9.4. *In-vivo* Sperm Count, Sperm Motility

Separate samples of epididymal fluid have been collected and diluted with Sorenson's buffer (pH 7.2). It was normal to make 1 in 20 dilutions. The Neubauer's haemocytometer was used to count sperm. High-powered sperm counting was performed in the RBC area (R1, R2, R3, R4 and R5). The formula for calculating the sperm count is as follows, (Number of sperm counted x dilution factor)/Volume x 1000 = sperm/ml.

2.10. Biochemical Studies

2.10.1. Serum Parameters

Animals were anesthetized on the 30th day and blood was collected by retro orbital plexus/sinus in a tube. The blood was allowed for clotting by incubating in room temperature for 30-40 min, and these tubes were subjected to be centrifuged at 3000 rpm for 10-15 min. Then the supernatant serum was collected using a micropipette and transferred into another centrifuge tube.

The various serum biochemical parameters such as serum testosterone, follicle stimulating hormone (FSH), Luteinizing Hormone (LH) using ELISA kit and total cholesterol (TC) are performed based on Modified Roe Schlaw's method [28].

2.10.2. Tissue Antioxidant Parameters

The isolated testis was gently blotted between the folds of filter paper and weighed on an analytical balance. 10%w/v tissue homogenate is prepared and centrifuged at 10,000 rpm for 20min for removing the cell debris. The supernatant is used for the estimation of superoxide dismutase by the method of "Mc Cord and Fridovich

(1969)" [29], Tissue catalase by the method of "Beer and Sizer (1952)" [30], lipid peroxidation by the method of "ohkawa and ohishi (1979)" [31].

2.11. Histopathology Studies

After sacrificing the rats, testes were removed and immediately preserved with 10% formalin. Then, the specimens were fixed in paraffin, sectioned at 5 μ m and stained with haematoxyline and eosin [32].

2.12. Statistical Analysis

The statistical analysis was analyzed using "One Way ANOVA by Dunnett's multiple comparisons test" using graph pad prism 9 and all the values were expressed in mean \pm SEM. The results are considered statistically significant if 'the p-value <0.5 or less.

3. Results

3.1. Extraction Yield of Fruit Material

The hydroalcoholic extracts of *Fragaria ananassa* fruit of about 2000g were extracted using the required solvent of hydroalcoholic mixture of Ethanol/water/concentrated

formic acid in (80:20:0.1, v/v/v) and percentage yield of the 4% is obtained.

3.2. In vitro Quantitative Phytochemical Analysis

The *In vitro* quantitative phytochemical analysis of FAFE is performed and their results are given in Table 1.

3.3. In silico Molecular Docking Studies

The molecular docking studies were performed with all 58 chemical constituents of the *Fragaria ananassa* with human phosphodiesterase 5 [2H42], and their binding affinity was expressed in kcal/mol. Among 58 constituents the highest binding affinity ligands and their binding affinity were shown in Table 2. The 2D interactions of the highest binding affinity ligands and standard sildenafil were presented in Figure 1.

3.4. Acute Toxicity Studies

The acute oral toxicity studies were performed according to acute toxicity OECD 425 guidelines. There is no mortality rate and no toxic signs were observed of rats after administration with *Fragaria ananassa* fruit extract (FAFE) up to 5000mg/kg for acute oral toxicity studies.

Table 1. Quantitative phytochemical analysis

<i>Fragaria ananassa</i>	Total phenolic content in mg GAE (Gallic Acid Equivalent)	Total flavonoid content in mg QE (Quercetin Equivalent)
Fruit extract	62.736 \pm 0.229	3.364 \pm 0.003

Table 2. Docking studies of *Fragaria ananassa* with 2H42

Ligand	Binding Affinity (kcal/mol)	Binding Residues
Beta-Carotene	-11.4	Ala A:289, Ile A:290, Ile A:131, Phe A:236, Val A:248, Ala A:233, Met A:282, Leu A:270, Phe A:252, Leu A:191, Tyr A:78, His A:83, His A:79
Campesterol	-10.3	Leu A:270, Met A:282, Phe A:252, Phe A:286, Val A:248, Leu A: 231, Ile A:131, Ile A:290
Lutein	-11.3	Ser A: 129, Leu A:270, Met A:282, Phe A:252, Val A:248, Phe A:286, Ile A:131, Ile A:290, Leu A: 191, Tyr A:78, His A:79, His A:123, Ile A:146, Val A:126, Met A: 147
Stigmasterol	-11	Leu A:191, Met A:282, leu A:270, Ala A:249, Ile A:279, Phe A:286, Phe A:252, Val A:248
Sildenafil	-9.9	Asn A:128, Asn A:127, His A:79, Met A:282, Phe A:286, Phe A:252, Ile A:234, Val A:248, Ala A:249, Ile A:279, leu A:270

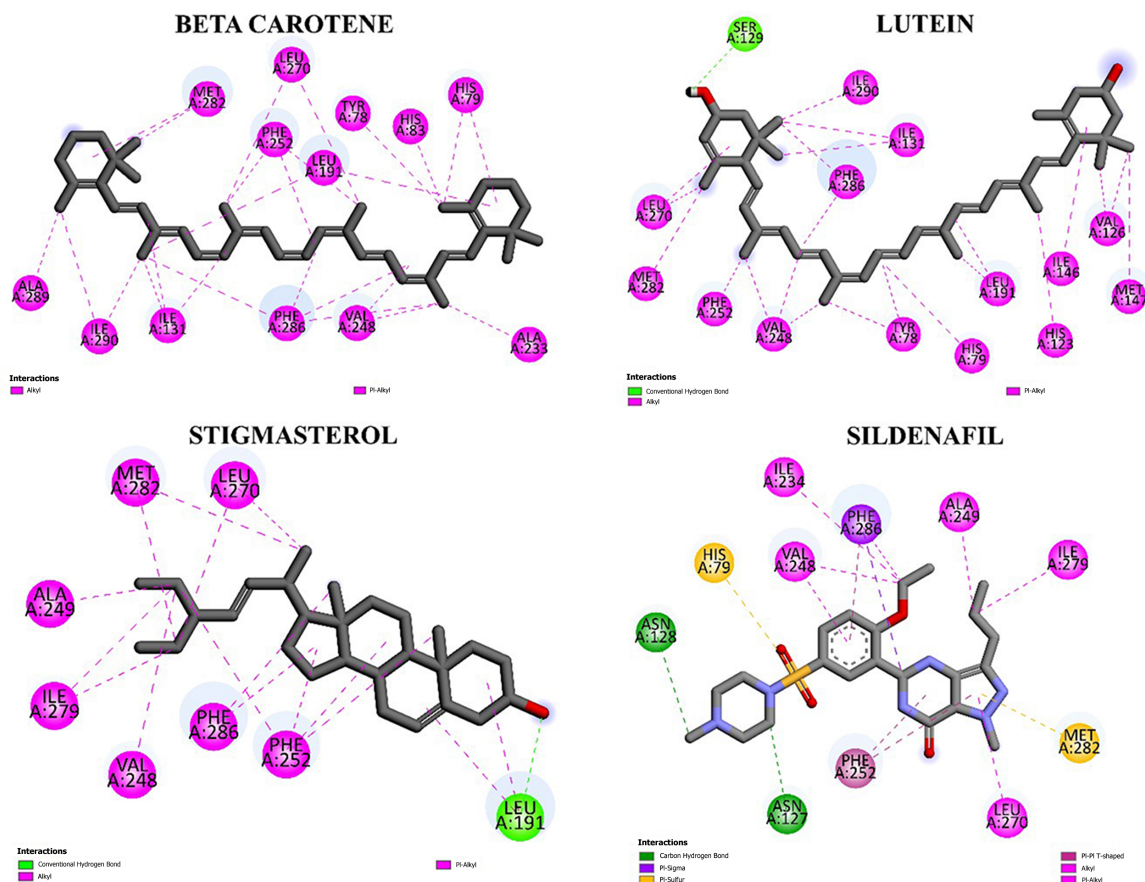


Figure 1. The 2D interactions of beta-carotene, Lutein, Stigmasterol and Sildenafil with 2H42

3.5. Effect of Drug in Physical Parameters

The test for the physical parameters as indicated in Table 3 shows that FAFE has significantly increased ($p < 0.0001$) in the mating parameters. The effects were compared with the disease control group, similarly standard group of sildenafil also had a positive effect where the physical parameters are effective by a dose dependent manner as higher dose of FAFE shows similar effect of standard group.

3.6. Effect on Hesitation Time & Attraction towards Female

The data for the hesitation time & attraction towards female are indicated in Table 4. A significant difference is observed in day 14 after inducing of disease when compared to normal control and after treatment thus FAFE shows a significant reduction in hesitation time and improved attraction towards female when compared with disease control and as similar in standard group also has improved attraction.

3.7. Effect on Sexual Organ and Sperm Count

In terms of the sexual organ weight like testis, epididymis and sperm count, a significant increase in their

weight of these organs and improved sperm count after treatment are observed in significance of $p < 0.0001$. Thus, the data are visualized in Figure 2.

3.8. Effect on Biochemical Analysis

The effects of the biochemical analysis are indicated in Figure 3, thus the FAFE shows a significant difference in the biochemical serum analysis such as serum testosterone, total cholesterol, LH and FSH, when compared to disease control and similarly sildenafil shows a significant difference. Then, in antioxidant parameters such as SOD and catalase, significant increase and significant reduction are observed in LPO levels after treatment as compared to untreated disease control.

3.9. Effect on Histology of Testis

The effects of the drug and FAFE on the histology of the testis are indicated in Figure 4. The administration of the sildenafil and FAFE effective in the treatment against metronidazole induced damage of testis (Shrunken seminiferous tubules with disorganization of spermatogenic germ cells) is conducted, and there is recovery in Shrinking and disorganization of the cells. In FAFE 1000mg it has better and viable seminiferous tubules and spermatocytes.

Table 3. Effect of physical parameters recorded on day 30 after the treatment

TREATMENT	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5
Mount frequency (sec)	883.3 ± 30.7	316.67 ± 30.73	750 ± 34.1 ****	700 ± 36.5 ****	816 ± 30.7 ****
Intromission frequency (% Intromitted)	533.3 ± 42.16	150 ± 22.3	500 ± 25.18 ****	383.3 ± 30.73 ****	516.6 ± 30.7 ****
Mount latency (min)	5.833 ± 0.307	14.67 ± 0.421	7.17 ± 0.6 ****	11.17 ± 0.307 ****	8.17 ± 0.477 ****
Intromission latency (min)	3.33 ± 0.21	9.83 ± 0.401	4 ± 0.36 ****	7.5 ± 0.22 ***	5.17 ± 0.401 ****
Ejaculatory latency (%)	450 ± 22.36	66.6 ± 21.08	433.3 ± 21.08 ****	283.3 ± 30.73 ****	400 ± 25.81 ****
Post-ejaculatory interval (min)	8.16 ± 0.307	23 ± 0.96	10.16 ± 0.65 ****	14.66 ± 0.55 ****	11.33 ± 0.55 ****
Copulatory rate (% Copulatory rate)	23.6 ± 0.51	8.88 ± 0.7	20.83 ± 0.83 ****	18.05 ± 0.79 ****	22.2 ± 0.82 ****
Index of libido (%)	883.3 ± 30.7	386.33 ± 30.73	750 ± 34.1 ****	700 ± 36.5 ****	816 ± 30.7 ****

Data are expressed in mean ± sem (n=6). *** p<0.001, **** p<0.0001 as compared to disease control (one-way ANOVA followed by Dunnett's test).

Table 4. Effect on hesitation time and attraction towards female rats using barrier method

TREATMENT	Hesitation time in sec			Cumulative scores for the attraction towards females		
	Day 1	Day 14	Day 30	Day 1	Day 14	Day 30
GROUP 1	159.5 ± 4.5	151 ± 2.35	148.5 ± 3.78	30	30	30
GROUP 2	157.8 ± 3.4	770.6 ± 44.97	849.3 ± 32.08	30	2	12
GROUP 3	152.3 ± 10.1 #	811.16 ± 56.32 #	451.1 ± 7.08 ****	30 #	4 #	21 **
GROUP 4	160 ± 9.4 #	814.5 ± 54.5 #	536.6 ± 26.4 ****	30 #	6 #	24 **
GROUP 5	153.83 ± 9.04 #	854.83 ± 45.16 #	201.6 ± 8.2 ****	30 #	6 #	27 ***

Data are expressed in mean ± sem (n=6). # indicates no significance, ** p<0.01, *** p<0.001, **** p<0.0001 as compared to disease control (one-way ANOVA followed by Dunnett's test).

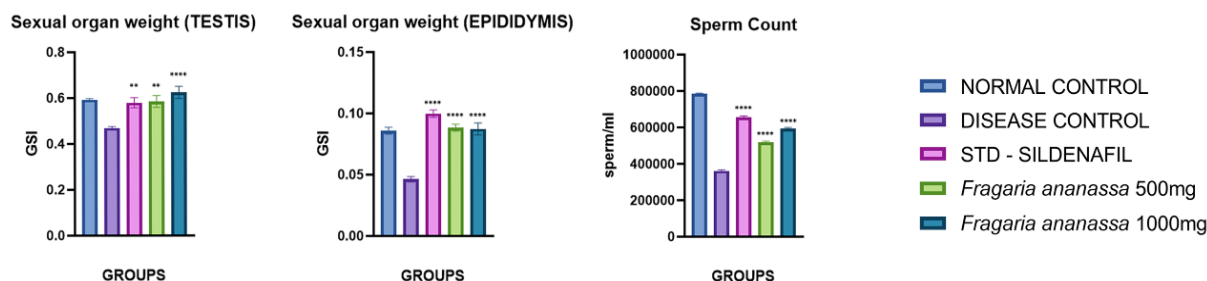


Figure 2. The effects of FAFE in Sexual organ weight and sperm count

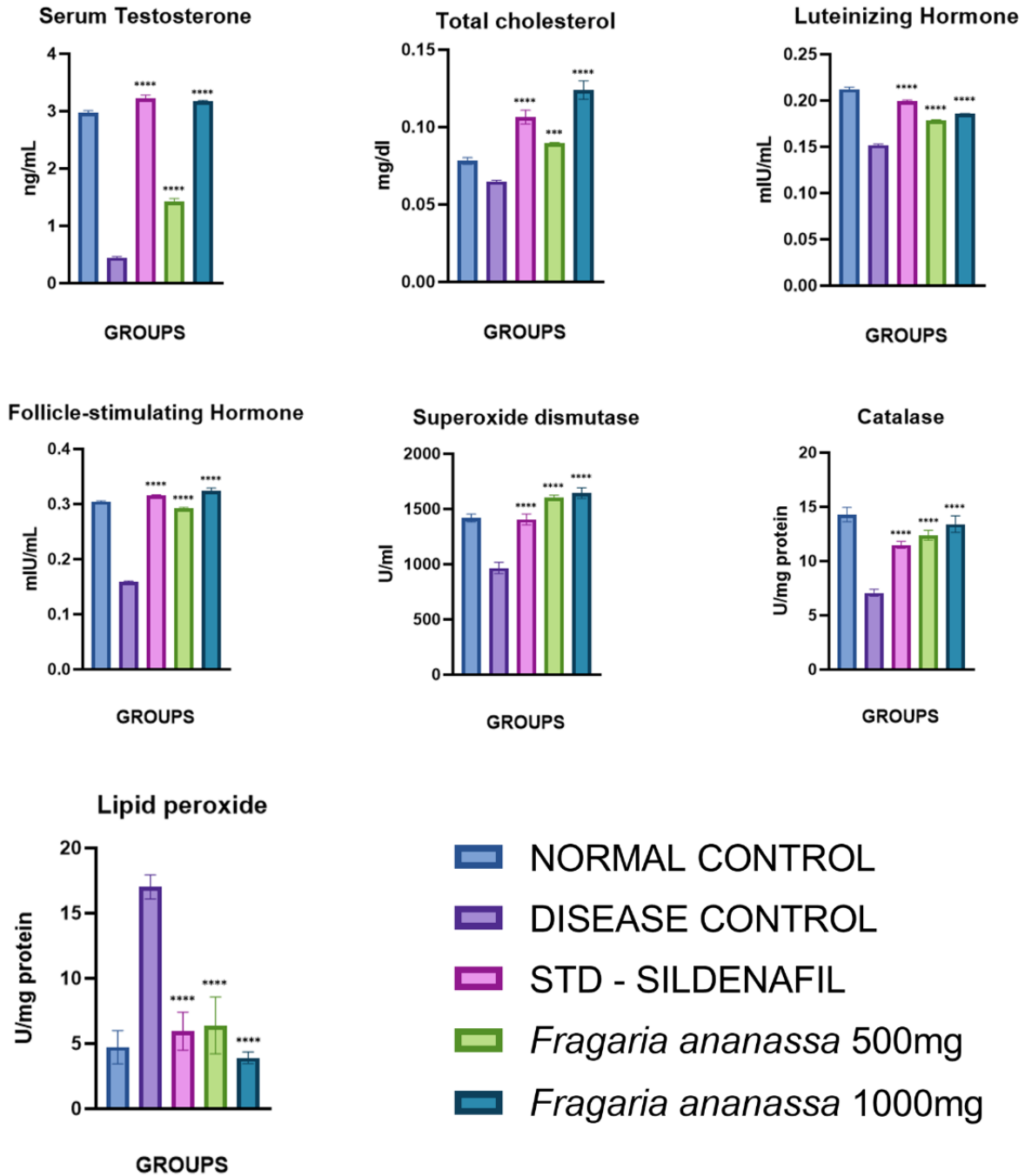


Figure 3. The effects of FAFE in biochemical analysis

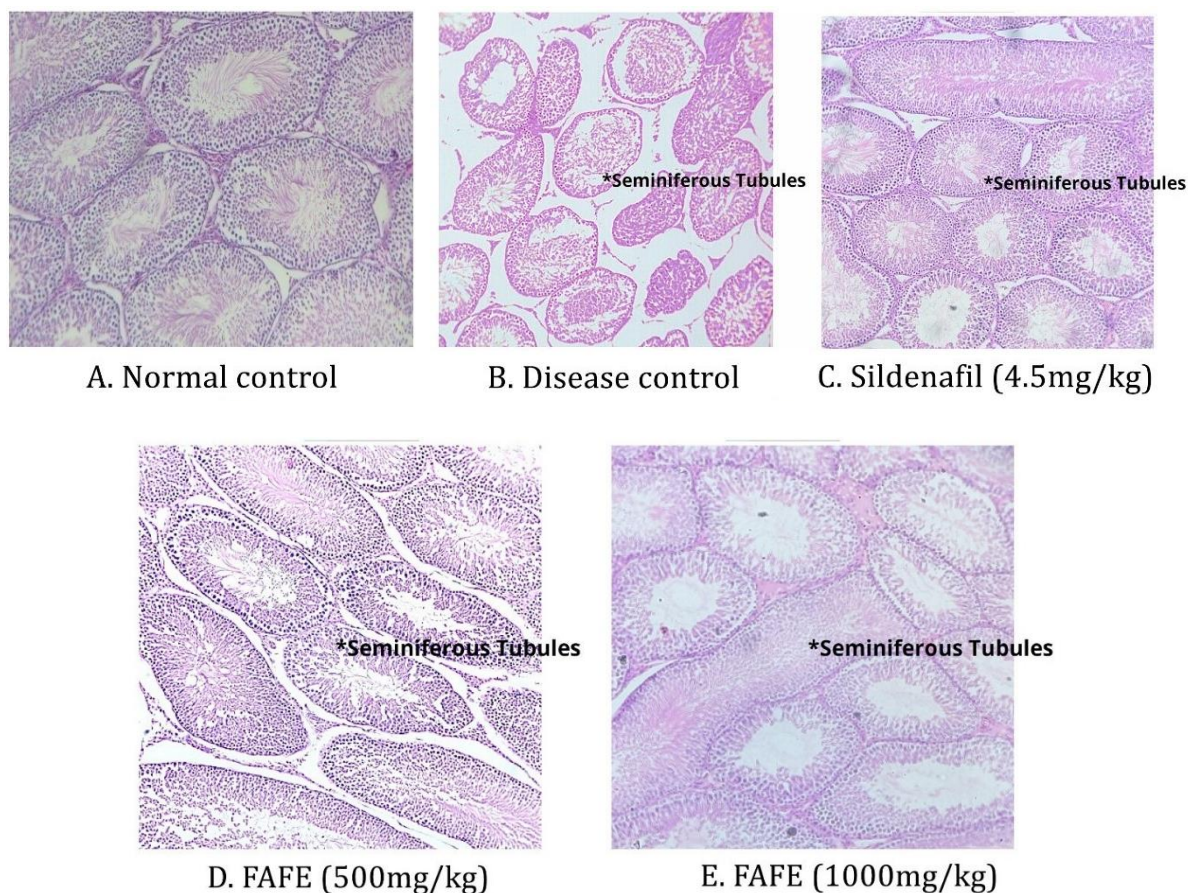


Figure 4. The histopathology studies of testis

4. Discussion

Erectile dysfunction (ED) significantly impacts men's quality of life and overall well-being. Several factors contribute to the development of ED, such as age, chronic diseases, lifestyle choices, and psychological issues [33,34]. Numerous epidemiological studies have revealed a substantial prevalence of ED in developed countries, underscoring its significance as a critical health issue [24].

Metronidazole exhibits reproductive toxicity by inhibiting spermatogenesis in rats. According to the statement, in rats administered with 200mg/kg and 400mg/kg [35] of metronidazole, there were reductions observed in testis weights and spermatogenesis. The administration of higher doses of metronidazole resulted in degenerative changes in the testicular seminiferous epithelium and reduced epididymal sperm count [36]. This impairment in the testicular affects the erection property by causing cellular and hormonal changes such as a reduction in luteinizing hormone, testosterone, and follicle-stimulating hormone levels [37].

In the present docking studies, the Phosphodiesterase type 5 [2H42] is inhibited and increases the concentration of cGMP causing dilation of corpus cavernosum tissues and penile erection. The chemical constituents of the *Fragaria ananassa* have the best binding affinity towards

PDE 5 receptor which are responsible for the erection of the penis.

In the present study of metronidazole induced model, it was observed that there was a decrease in the level's hormones LH, FSH and Testosterone, and this leads to the reduction physical mating as of the hormonal imbalance in the disease group than the normal control and this reduced mating, Intromission frequency and increased in mating, Intromission, Ejaculatory latency and hesitation time. Therefore, *Fragaria ananassa* at the dose of 500mg/kg and 1000mg/kg shows a significant increase in the serum hormonal levels and enhanced the mating, Intromission frequency and reduced in ejaculation latency and hence the attraction towards female by reducing the hesitation time is similar to that of the standard treatment with sildenafil.

Thus, the sexual organ weight such as testis and epididymis and sperm count is reduced by the effect of metronidazole in the disease control due to cellular damage. Therefore, *Fragaria ananassa* at the dose of 500mg/kg and 1000mg/kg shows a significant recovery in the and sexual organ weight such as testis and epididymis and a significant increase in the sperm count similar to that of sildenafil as standard treatment.

The present findings indicate that FAFE possesses a powerful antioxidant activity as evident from its ability to

prevent or to reduce the LPO, and increase in the level of SOD, CAT as there was an elevated lipid peroxidation and increase in antioxidants in impotency rats. These results are supported by the histopathological results of disease control that has the disorganization and shrunken of seminiferous tubules, and in the standard treatment sildenafil recovers the seminiferous tubules and appearance of normal appearing Leydig cells. Thus, the treatment group of FAFE 1000mg has the viable cells and is composed of spermatogonia, spermatocytes and spermatids.

5. Conclusions

The results obtained in the present study indicate the aphrodisiac activity of the *Fragaria ananassa* for the treatment of the erectile dysfunction. These effects may be due to the possible mechanism, by enhancing the hormonal levels and by inhibiting the PDE5 and increase the cGMP level and NO level and enhance the relaxation of the penile smooth muscle as it is confirmed by the molecular docking studies. It should be proved by molecular and cellular level investigation clinically to identify the active constituent for treating erectile dysfunction.

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