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The Effects of *Syzygium Aromaticum* (Clove) Leaf Extracts on Rats Sperm Function

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ABSTRACT

Syzygium aromaticum (Clove) is a small evergreen tree of the myrtle family known for its unopened reddish-brown flower bud with strong aromatic flavor that are used for culinary purpose and are believed to have aphrodisiac properties, including those related to fertility and infertility diseases. The flower buds of *Syzygium aromaticum* (clove) have been used in indigenous medicines for the treatment of male sexual disorders in Indian subcontinent. Objective to evaluate the effect of *Syzygium aromaticum* flower bud on male reproduction, using rats as animal model. The aim of this study was to design a dose-response assessment to assess the effects of aqueous leaf extract of *Syzygium aromaticum*. Healthy adult male rats were treated with 1, 10 and 100 mg/kg/day of *Syzygium aromaticum* leaf extract by treated for 45 days. Then assess the weight, Sperm morphology, DNA fragmentation, sperm viability, sperm morphology and sperm motility of the reproductive organs. Results: There was no effect on body weight, there is a positive effect in rats treated with *Syzygium aromaticum* extract at low doses at 1mg/kg/day, sharp and significant decrease in all vital reproductive functions in rats treated with *Syzygium aromaticum* extract at high concentration at 10 and 100 mg/kg/day. The explanation for the increase in sperm functionality might have been induced by the presence of components within the plant of *Syzygium aromaticum* causing the effect on spermatozoa enabling them to reach optimal motility. Thereby enhancing the negative effects observed within this study. This study indicated that the traditional use of this herb has harmful effects on male reproductive health at high doses. This study underscores the potential of Clove leaf fractions as natural aphrodisiacs, warranting further investigation for clinical applications. Conclusion: Treatment with *Syzygium aromaticum* flower bud causes dose-dependent biphasic effect on male reproductive indices in Rats; lower dose of *Syzygium* appears stimulatory, while the higher doses have adverse effect on male reproduction. The results suggest that the lower dose of *Syzygium* may have androgenic effect, but further studies are needed to support this contention.

1. INTRODUCTION

Today, according to the World Health Organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary health-care needs. Medicinal plants or parts of these plants (leaves, rhizomes, roots, seeds, flowers) can be utilized in different forms such as fresh crude form and preparations as teas, decoctions, powdered plant material, or extracted forms of medicinal agents (juices, water or alcohol extracts, tinctures, essential oils, resins, balsams).

Medicinal plants are generally known and popular for a number of health benefits such as blood pressure decreasing, prevention of cardiovascular diseases, or reducing the risk of cancer also due to their antioxidant activity (Srivastava et al., 1991). The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues (Srivastava et al., 1991). Clove is the dried flower bud of *Syzygium aromaticum* (L.) Merr and Perry. (Family: Myrtaceae) an evergreen tree 10– 20 m in height indigenous to India, Indonesia, Zanzibar, Mauritius and Sri Lanka (Srivastava et al., 1991). *Syzygium aromaticum* commonly known as the deadly carrots (Srivastava et al 1991). It is one of the most important drugs used in indigenous medicine in India, especially reported as aphrodisiac stomachic carminative, antispasmodic (Tajuddin et al., 2003). *Syzygium aromaticum* was considered as an aphrodisiac agent to be useful in conceiving in high doses and act as a contraceptive in low doses however, it may cause some side effects (Brunetti et al., 2020), *Syzygium aromaticum* extract on male reproductive system. Phyto chemical study of the test drug that was carried out according to the methods described by (El-Saber et al., 2020), showed that it contains alkaloids, amino acids, flavonoids, proteins, sterols, reducing sugar, tannins and phenols. However, clove, or its known compound had not been scientifically studied for their effect on sexual function (El-Saber et al., 2020), various efforts have been studied to address sexual dysfunction issues, one of which involves the use of plants with aphrodisiac properties. Aphrodisiacs act as sexual stimulants and can enhance libido (El-Saber et al., 2020). The leaves of the clove plant are among the plants that contain saponins, flavonoids, tannins, sterols, and essential oils, acting as natural antioxidants (Biernasiuk et al., 2022). Clover leaf oil contains phenolic components such as eugenol and eugenol acetate, as well as caryophyllene and sesquiterpenes, suggesting its potential as an aphrodisiac that enhances sexual desire However, thus far, the testing of the aphrodisiac effect of Clove leaves has only extended to its extract and oil (Biernasiuk et al., 2022). The possible adverse effects of various medicinal plants on fertility remain an enigma. In this regard, it is important to note that some plants contain estrogenic substances which may induce alterations to gametogenesis production (Han et al., 2017). Several authors have been investigating a variety of medicinal plants and proved that these plants could potentially cause reductions in sperm density alter androgenic secretion and reduce motility and density of mice spermatozoa Conclusive studies by (Haro-González et al., 2021). Study of hydro alcoholic extract (50%) of clove using only mounting frequency and mating performance as the marker for sexual function in normal male mice, and the results from our study demonstrated the aphrodisiac activity and safety from short term toxicity of the test drug (Hatami et al., 2019). The aim of this study was to examine the effect of different concentrations of extracting *Syzygium aromaticum* on Rats sperm function taking in consideration that defective sperm function has been linked to loss of fertilization in males.

2. METHOD

Collection and preparation of plant material

Plant materials.

The leaf of *Syzygium aromaticum* (clover leaf) was collected from medicinal plant markets in Benghazi, Libya. The plant species was identified and authenticated by Department of Botany at university of Benghazi.

Processing of plant material.

The leaves were chopped into small pieces that were shade dried over a period of 3 weeks. The dried leaves were milled into fine powder by means of a mixer (IKA WERKE MILL M20). The aqueous extract was prepared by infusing the plant in boiled distilled water for 4 hours. Subsequently, the infusion was filtered using a water filtration pump to ensure thorough separation of herbal material from distilled water. The remaining crude herbal material was discarded and the filtered infusion was freeze-dried into a fine powder using the Alpha HED 10 freeze-dryer (Christ Freeze dryers, Osterode am Harz, Germany). The freeze-drying process took between two to three weeks depending on the volume of *Thapsia garganica* infusion being freeze-dried. The powder was stored in clean polythene bags in a cool at 4°C, dry place until further use.

Experimental Animals

Eighteen Sprague Dawley male rats and with body weight 250-300 g, and two months old were used. They were kept in the animal house of Faculty Medicine and Health Sciences under room temperature (22-25 °C), with 70-80% humidity, and automatic 12 hours light-dark cycle. The rats evaluated to be free from diseases and deformities. The rats were acclimatized for one week before starting treatments. They were group fed with pellet and drinking water was given ad libitum.

Force feeding and sacrificing of rats.

Rats were segregated into control and experimental groups: (n=10 per group) (group of rats force fed with normal tap water for 52 days) (group of rats that were force fed with 10mg/kg *Syzygium aromaticum* extract for 52 days) (group of rats that were force fed with 100mg/kg *Syzygium aromaticum* extract for 52 days),

Body weights of rats were recorded daily and the overall wellbeing of the animals were monitored throughout the feeding process. At the end of the feeding period (52 days), animals were sacrificed by means of rapid cervical dislocation. Immediately after sacrificing, blood was aspirated directly from the heart for the determination of serum testosterone concentration which was performed at a later stage. Blood was incubated for one hour at 37°C and then centrifuged until the serum portion was separated from the pellet. Serum was collected in Eppendorf tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and kept in the -20°C freezer until further use. Afterwards the testes, epididymis, seminal/coagulating glands, kidneys and the liver were removed and the respective organ weights were recorded. Spermatozoa were isolated from the caput- and cauda epididymides for functional analysis of sperm parameters.

Determination of Sperm Morphology

Smears were made after pipetting 20µl of sperm sample onto slides. After allowing these smears to air dry, they were fixed by immersing them in methanol (Sigma Aldrich, Steinheim, Germany) at 4°C for 25 minutes. In order to determine sperm morphology a modified Papanicolaou staining technique was employed. Staining procedure followed by submerging slides (for at least 10 times per solution) in the following; 80% ethanol, 70% ethanol, 50% ethanol and then in distilled water. Following this, slides were then put in Harris Haematoxylin, Papanicolaou solution 1a (Merck 9253) (Merck and Co., Inc. Whitehouse Station, USA) for 15 minutes and then rinsed with running water for 3-5 minutes. Next, slides were submerged in 0.5% HCl; this particular submerging step was repeated after which slides were rinsed with running water for about 5 minutes. Subsequent to the rinsing step, slides were then placed in Scott's water (20g MgSO₄·7H₂O and 2g NaHCO₃ dissolved in 1000ml distilled water).

Determination of Motility

The epididymis was placed on a warmed-up Petri dish in 3ml of HTFM-HSA medium. Human Tubular Fluid Medium (HTFM) was made up according to Quinn et al. (1985). The ionic composition of the medium was as follows; 2.9655g/ml NaCl (Merck, Modderfontein, South Africa), 0.175g/ml KCl (Merck), 0.1505g/ml CaCl₂·2H₂O (Merck), 0.025g/ml MgSO₄·7H₂O (KIMIX, Eppindust, South Africa), 0.025g/ml KH₂PO₄ (Merck), 0.00025g/ml Phenolred, 1.05g/ml NaHCO₃, 0.25g/ml Glucose (anhydrous), 0.018g/ml Na-Pyruvate (VWR International Ltd. Biochemical, Pool, England), 1.9991ml Na-Lactate (60% Syrup), 2.603g/ml HEPES (Sigma Aldrich, Steinheim, Germany). Before use, the osmolarity of the medium was adjusted to 280mOsmol/kg and 1% of Human Serum Albumin (HSA) was added to the medium.

Determination of DNA fragmentation by means of the TUNEL assay

The TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling) assay is used for the detection of DNA strand breaks [8]. Adhering to the protocol provided within the kit (Detection System Fluorescein, Promega, Mannheim, Germany) and as described previously by (Hatami et al 2019). In this study, the percentage of green fluorescing sperm (TUNEL-positive sperm) was determined. In addition, slides without TdT enzyme were prepared which served as negative controls. After samples were centrifuged for 10 minutes at 300xg, the supernatants were discarded and the pellets were resuspended in PBS (Oxoid Ltd, Hampshire, England). Fifty µl of sperm suspensions were then pipetted onto Superfrost slides (Knittel Gläser, Braunschweig, Germany) and subsequent smears were made. Smears were allowed to air dry after which they were fixed by immersion in freshly prepared 4% formaldehyde (KIMIX Chemicals, Eppindust, South Africa) dissolved in PBS; this was done for 25 minutes at 4°C.

Determination of sperm viability

One of the most basic and essential methods used to identify dead from live sperm is the viability stain. The eosin-nigrosin staining technique was used to determine viability according to (Chandra et al., 2022). The staining solution was prepared by dissolving 0.67 g of Eosin Y and 0.9 g of NaCl in 100 ml of distilled water with gentle heating. Thereafter, 10 g of nigrosin was added to the eosin solution and brought to a boil. To remove coarse and gelatinous precipitates the solution was filtered through filter paper and subsequently, the filtrate was stored in a dark glass bottle at room temperature until use. To stain, a ratio of 1:1 was used where 50 µl of the sperm suspension were added to 50 µl of the Eosin-Nigrosin in an Eppendorf vial. A smear was then made on a glass slide and left to air dry. Slides were then viewed with a 100X oil immersion objective in the bright field using a light microscope. A total of 200 spermatozoa were counted and the percentage of live sperm was then calculated. Dead sperm appear pink/purple and live sperm white.

3. ETHIC APPROVAL

Studies involving animals

Protocols for animal experiments were approved by the animal experimental ethics committee of the Benghazi university; all animal housing and experiments were conducted in strict accordance with the institutional Guidelines for care and use of Laboratory Animals at Benghazi university Faculty of Science Zoology department

4. RESULT

1. Effect of *Syzygium aromaticum* on sperm viability

After exposing to different concentrations of *Syzygium aromaticum*, washed sperm samples showed a significant dose-dependent trend (ANOVA trend analysis: $P=0.026$) towards lower viability rates were observed at higher concentrations of *Tapisa gercanica* (Figure 1). Nevertheless, only the highest concentration (100 $\mu\text{g/ml}$) used differed significantly ($P=0.012$) from the control, while the difference at 1 $\mu\text{g/ml}$ *Tapisa gercanica* was marginally significant decreased ($P=0.026$). In contrast, results for the washed spermatozoa neither revealed decrease difference to the control, with trend (ANOVA trend analysis: $P=0.0028$) (Figure 1).

2. Effect of *Syzygium aromaticum* on sperm motility

Although a marked decrease in total motility at the highest concentration (100 $\mu\text{g/ml}$) was found for washed spermatozoa when compared to the control after incubation with *Syzygium aromaticum* only a marginal significance ($P=0.036$) was found for washed spermatozoa. For samples, which were just washed, a significant trend (ANOVA trend analysis: $P=0.0041$) to lower total motility rates could be observed towards higher concentrations of *Syzygium aromaticum*. For progressively motile spermatozoa no trend was observed for low concentration (ANOVA trend analysis: $P=0.146$). Moreover, although the rates were lower concentration ($P=0.01820$) and highest concentration ($P=0.00670$) at the highest concentration used, significant decline in progressive motility was found (Figure 2).

3. Effect of *Syzygium aromaticum* on Sperm morphology.

The effect of an aqueous extraction of *Syzygium aromaticum* on sperm morphology is illustrated in figure 3. Treatment with the low dose (1 $\mu\text{g/ml}$) *Syzygium aromaticum* extract showed to have had an increase on the percentage of sperm with normal morphology ($96.60 \pm 1.05\%$). Although the mean was lower to the control ($69.05 \pm 1.13\%$), the level of significance ($P=0.0035$) was not reached. However, a significant decrease ($P<0.001$) in the percentage of sperm with normal morphology was observed after treatment with the high dose ($85.8 \pm 1.14\%$) (100 $\mu\text{g/ml}$) of *Syzygium aromaticum* extract when compared to the control ($84.05 \pm 1.13\%$) (Figure 3).

4. Effect of *Syzygium aromaticum* on DNA fragmentation

With regard to DNA fragmentation, the treatment of washed spermatozoa with increasing concentrations of *Syzygium aromaticum* resulted decrease in high dose (100 $\mu\text{g/ml}$). In addition, no detrimental effect of the *Syzygium aromaticum* extract on sperm DNA fragmentation was found for low dose (1 $\mu\text{g/ml}$) washed spermatozoa (ANOVA trend analysis: $P=0.031$) (Figure 4), nor for (10 $\mu\text{g/ml}$) (ANOVA trend analysis: $P=0.009$). Nevertheless, a marked increase at a concentration of 100 $\mu\text{g/ml}$ in the washed sperm set up was found ($P=0.005$). However, this appeared to be only within the statistical variation.

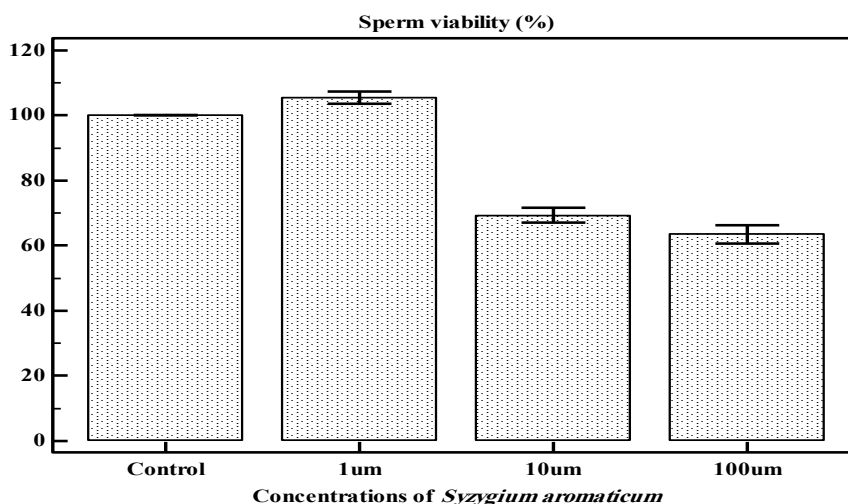


Fig. 1 The effect of different concentrations of crude *Syzygium aromaticum* extract

Fig. 1 The effect of different concentrations of crude *Syzygium aromaticum* extract (1,10, and 100 µg/ml) on viability as determined by means of the trypan blue incorporation in the triple stain *in vitro*. Values shown as mean±SEM (n=18). Significant difference was found when compared concentrations with control, as well as the result obtained after ANOVA in significant (P=0.016). However, a trend analysis after repeated measures analysis revealed a significant trend (P=0.0042).

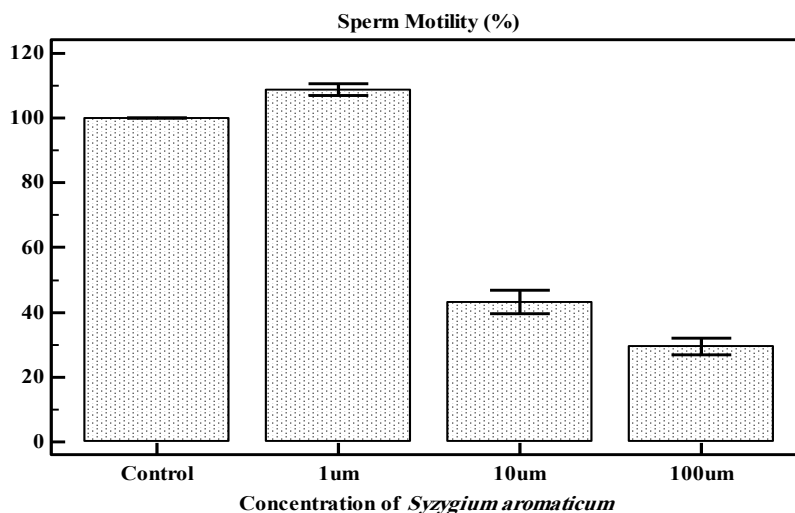


Fig. 2 Comparison of the percentage of sperm motility within the control

Fig. 2 Comparison of the percentage of sperm motility within the control (group of rats not treated with *Syzygium aromaticum* extract low dose (group of rat's force fed with 1µg/ml *Syzygium aromaticum* and high dose (group of rats force fed with 100 µg/ml *Syzygium aromaticum*. Motility was significantly decreased (P=0.0036) after treatment with the low dose of *Syzygium aromaticum* whereas the high dose compared to the control showed a decreased that reach high level of significance (P=0.0024).

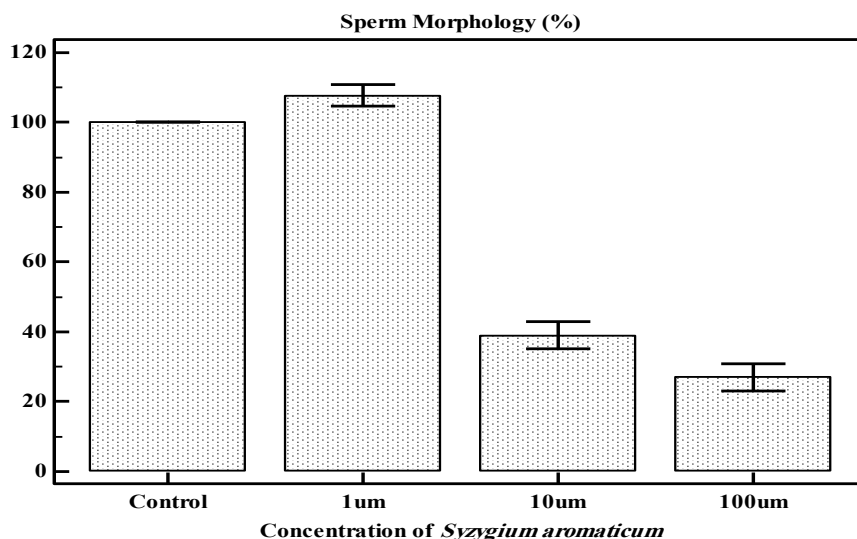


Fig. 3 Effect of treatment with *Syzygium aromaticum* extract on normal sperm morphology within the control

Fig. 3 Effect of treatment with *Syzygium aromaticum* extract on normal sperm morphology within the control (group of rats not treated with *Tapisa gercanica*), lowdose (group of rats force-fed with 10mg/kg *Syzygium aromaticum* extract) and high dose (group of rats force-fed with 1µg/ml *Syzygium aromaticum*). The percentage of sperm with normal morphology in the low dose group showed a decrease when compared to the control. However, this decrease was significant (P=0.0022). An extremely high significant decrease (P<0.001) in the percentage of sperm with normal morphology was detected in the high dose group compared to the control.

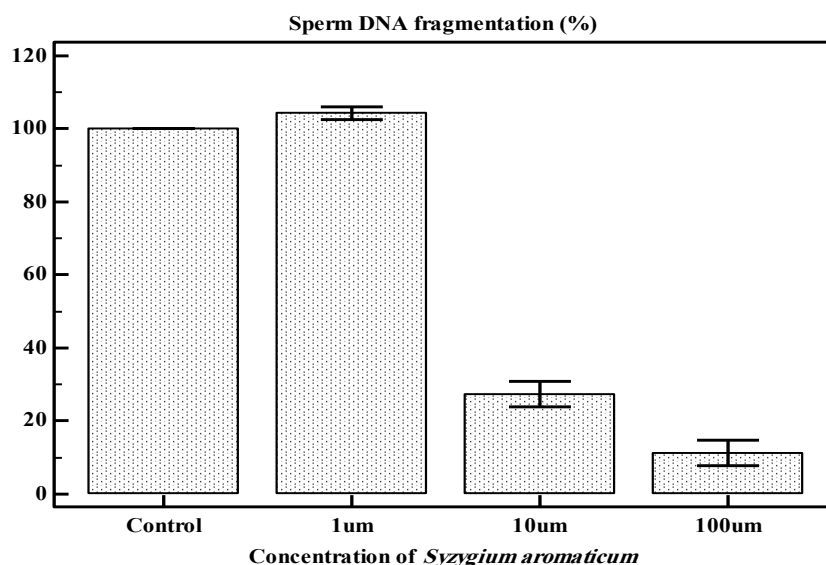


Fig. 4 Effect of treatment with *Syzygium aromaticum* extract on the percentage of TUNEL-positive sperm within the control

Fig. 4 Effect of treatment with *Syzygium aromaticum* extract on the percentage of TUNEL-positive sperm within the control (group of rats not treated with *Syzygium aromaticum* extract), low dose (group of rats force fed with 1µg/ml *Tapisa gercanica* extract) and high dose (group of rats force fed with 100 µg/ml *Syzygium aromaticum* extract). Treatment with the low dose of *Syzygium aromaticum* extract showed a decreasing effect on the percentage of TUNEL-positive sperm whereas the high dose group showed decrease ($P=0.001$) in the percentage of fragmented sperm.

5. DISCUSSION

Clove (flower bud of *Syzygium aromaticum*) oil is widely used for the treatment of various disorders, and the health benefits of clove oil are attributed to its antimicrobial, antifungal, antiseptic, antiviral, aphrodisiac, and stimulating properties.

This study aimed to evaluate the effect of *Syzygium aromaticum* on sperm characteristics of adult rats during 7 weeks supplementation in comparison to as positive control or distilled water as negative control. In order to monitor the health condition of the rats, their body weight was measured every week. After 6 weeks of treatment, the mean of body weight for rats given 240 mg/kg *Syzygium aromaticum* leaf aqueous extract was decline significantly as compared to both control groups. The reduction of body weight of rats supplemented with *Syzygium aromaticum* might be due to its saponin content that has stimulatory effect on testosterone hormone. (Hatami et al., 2019). .reported that long-term treatment with high dose reduce body weight gain. Besides that, a study by showed that *Syzygium aromaticum* significantly reduce body weight gain, total cholesterol, triglyceride, HDL and LDL and up regulated hepatic LDL receptor.

For the purpose of monitoring the fertility of rats, some andrological parameters of the Sprague Dawley rats were evaluated including sperm count, sperm motility, viability and morphology. The mean of sperm counts of rats from *Syzygium aromaticum* treated group showed significant decrease difference when compared with negative control group. The reduction seen in sperm count of rats treated with *Syzygium aromaticum* can be due to the increase in mounting frequency in rats of the same group. This result is consistence with result found by (Mostafa et al., 2023) who observed that the testosterone concentration level and mounting frequency was the highest in rats supplemented with *Phaleria macrocarpa*. Mounting frequency is a strong indicator of the occurrence of sexual performance. In addition, *Syzygium aromaticum* contain Ancistrocladidine, as one of its active compounds. Ancistrocladidine-rich extracts are able to disprove the sexual performance as seen in rat (Pulikottil et al., 2015). In this study, sperm was collected from cauda epididymis, which is the primary sperm storage site prior to ejaculation. During the storage period, the cauda epididymis accumulates sperm to ensure that a sufficient number is available at the time of ejaculation (Pulikottil et al 2019). During the seventh week of the treatment, the female rats were put in the cages of the male rats to enable the assessment of libido behavior. This assessment was carried out for five consecutive days. After 24 hours, the rats were sacrificed to obtain the sperm.

This study design did not give the rats any sexual rest to allow the cauda epididymis to be sufficiently filled with mature sperm (Sarker et al., 2022) also reported that sperm concentration in the cauda epididymis could depend on factors such as sexual rest and semen collection frequency. Besides that, there is a general agreement that semen volume and sperm concentration increase with prolonged sexual abstinence as reported by some (Somrani et al., 2022). This explained the lower sperm count retained in the cauda epididymis in *Syzygium aromaticum* treated rats. According to (Vijayasteltar et al., et al 2016) sperm motility is one of the most important indices determining the ability of a male to produce viable sperm. It is expressed in a percentage of all moving sperm in a sample. In this study, total mean percentage of motile sperm was changed in all groups of treatments. The mean value of percentage of motile sperm was 50% across all treatment groups. This might be due to the sperm sampling which was collected from cauda epididymis. A study conducted by (Dua Anita et al., 2015). reported that progressive motility for epididymal sperm was lower than ejaculated sperm. A correlation between mitochondrial activities and motile sperm has been shown using cytochemical techniques and flow cytometry (Batiha et al., 2020) also showed rhesus macaques that ATP from mitochondria contributes to sperm motility. Besides that, the ejaculated sperm may have better progressive motility due to the presence of fructose in the fluid secreted by seminal vesicle.

However, the increasing pattern of the percentage of motile sperm from grade 1 to grade 3 motility for rats supplemented with *Syzygium aromaticum* indicated negative influence of *Syzygium aromaticum* in exerting motility of sperm compared to the negative and positive control groups. In order to fertilize an ovum, an ideal sperm should at least have graded as 3, for grade 4 motility, the percentage of sperm for rats treated with *Syzygium aromaticum* was significantly higher than distilled water group. This result indicated that *Syzygium aromaticum* supplementation decreased the sperm motility by decreasing the percentage of the sperm with higher motility grade.

Viable sperm can be defined as sperm that is alive and capable of fertilizing an ovum. In motility analysis, grade 1 sperm are immotile and fail to move. However, in certain cases, immotile sperm are still viable. Therefore, in cases of low motility as well as to further confirm the status of viability of the sperm, viability analysis was carried out. By using the eosin-nigrosin staining method, the viable and dead sperm was able to be differentiated. According to (Batiha et al., 2020 and Alfikri et al., 2020) this classification was based on the degree of membrane permeability of dead spermatozoa which heads showed pink or red coloration due to the breakage of the membrane.

Syzygium aromaticum showed significant decrease in percentage of viable sperm as compared to negative control group. This may be due to *Syzygium aromaticum* potential in decreasing secretion of testosterone hormone through the presence of saponin. This result is in disagreement with who found that saponin has the potency to increase (Mishra et al., 2008). Sperm DNA fragmentation. The percentage of TUNEL-positive sperm representing the percentage of sperm with fragmented DNA was decreased by the low dose (1mg/kg) of *Syzygium aromaticum* extract when compared to the control. Although the decrease in sperm DNA fragmentation was significant ($P=0.001$) Results also show that treatment with the high dose (100mg/kg) of *Syzygium aromaticum* extract caused an increase in DNA fragmented sperm when compared to the control. Again, although this difference was significant ($P=0.012$), an increase in strand breaks could not have been beneficial to the nuclear DNA within the sperm head (Auerbach et al., 2010 and Agbaje et al., 2010). Furthermore, the aqueous extraction of *Syzygium aromaticum* in the high dose (100mg/kg) had a negative effect on the percentage of TUNEL-positive sperm when compared to the effect of the low dose (10mg/kg). In this regard, previous literature supports the concept that ROS production leads to DNA fragmentation, hence a positive relationship between these two parameters was expected (Zari et al., 2021). Morphology can be defined as the structure and form of organisms to include the anatomy, histology and cytology at any stage of its life history Several studies in the literature have reported that percentage of normal sperm morphology is an essential characteristic for in vivo fecundity and in vitro fertilization. (Zari et al., 2021 and Sarker et al., 2022). Most of these studies indicate that morphology is the best predictor among all of the sperm characteristics. Based on the result obtained, the percentage of normal sperm morphology was affected across all treatment groups including the rats given *Syzygium aromaticum*. This indicate that *Syzygium aromaticum* maintain sperm morphology and does caused defect to the morphology of the sperm produced

6. CONCLUSION

Treatment with *Syzygium aromaticum* flower bud causes dose dependent biphasic effect on male reproductive indices in Rats lower dose of *Syzygium* appears stimulatory, while the higher doses have adverse effect on male reproduction. The results suggest that the lower dose of *Syzygium* may have androgenic effect, although high dosage of the *Syzygium aromaticum* extract has a toxic effect on spermatogenesis but further studies needed for a better understanding of the effect of *Syzygium* on the functional physiology of the male reproductive system.

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