Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 12 (1)

Histological, Hormonal and Semen Quality Assessment Following Ingestion of Clove Buds (*Syzgium aromaticum*) on the Testes of Adult Male Sprague Dawley Rats

[1]Memudu AE, [1*]Odetola AA, [2]Adeleye OO and [1]Ehebha SE
[1]Department of Anatomy, College of Medicine, Edo University Iyamho, Edo State, Nigeria.
[2]Department of Anatomy, Lead City University, Ibadan, Oyo State.

Corresponding Author: Odetola AA
Email: amosodetola@gmail.com; +2348032072958

ABSTRACT
Environmental pollution, stress, lifestyle, infections and several other factors are major aetiological factors of male infertility. To evaluate the effects of oral ingestion of aqueous extract of *Syzygium aromaticum* (clove buds) on the seminiferous tubules, testicular histology, testosterone level and its effects on semen profile. Twenty adult male Sprague-Dawley rats used for this study were divided into three groups; Group A- control (n=10), Groups B (n=5) and C (n=5) experimental groups treated with 200mg/kg b.w per day of crude extract of *Syzygium aromaticum* for 4 weeks and 8 weeks respectively via oral intubation. Testes stained for histological analysis, serum testosterone level and caudal epididymis excised for semen analysis using Neubauer Chamber. There was a statistical significant ($P<0.05$) increase in body, testicular weights and serum testosterone in 200mg/kg *Syzygium aromaticum* bud treated group as compared to the control. Seminiferous tubules shows spermatogenic cells series, proliferative Leydig as well as normal sperm morphology with no teratozoospermic, oligospermic or azoospermic condition detected. Semen analysis showed a statistical significant ($P<0.05$) increase in sperm count and motility in 200mg/kg *Syzygium aromaticum* extract treated animals. Aqueous extract of *Syzygium aromaticum* has antioxidant and androgenic potential that mediates proliferation of Leydig cell and spermatogenic cells series. Hence, this forms the therapeutic basis of its use in treating male fertility disorders.

Key words: Seminiferous tubules, Spermatogenic cells, Leydig cells *Syzygium aromaticum*

INTRODUCTION
The incidence of male infertility has increased overtime of several factors such as environmental pollution, stress and lifestyle.[1,2] Oxidative stress induced by reactive oxygen species [ROS],[3-5] generated by abnormal sperm cells is an aetiology of male factor infertility.[5-7] Oxidative stress is the causative agent of 25% of male infertility, [8] and it has deleterious effects on spermatozoa.[9] Antioxidant is used in averting this because mammalian sperm membrane is sensitive to radical damage.[10] Treatment of this male reproductive disorder in developing countries is quite expense, enhance people tend to fall back on the use of natural products with therapeutic properties traced by herbal medicine practitioner for their primary healthcare needs.[11-13] *Syzygium aromaticum* (*Myrtaceae*) or Clove flower buds cultivated primarily in Zanzibar, Indonesia, West Africa and Madagascar; it is also grown in India and called Lavang, Pakistan, and Sri Lanka; it belongs to the family *Myrtaceace*. It is generally described as rich brown, dried unopened flower buds of *Syzygium aromaticum.* Its local name in Yoruba is *Kanafuru* in Hausa is *Kaninpar*i.[14,15] It is commonly known as clove, a well-known traditional medicine in Australia, Southeast Asia and in Indian subcontinent, and it is widely used in various disorders including dental, respiratory, headache and sore throat.[16,17] Clove essential oil are used as painkiller for treatment of toothache; as an aphrodisiac, carminative, anti-bacterial, anti-fungal,[18] anti-helmintic agent, prevention of premature ejaculation, bronchial congestion, diabetes, halitosis, fever, oral candidiasis, cough, arthritis, hypertension and stomach upset [prepared as infusion among the Yoruba called "Ogun Jedi-Jedi" [clove infusion.[15, 19] In Ayurvedic and Unani medicines, the *Syzygium aromaticum* is well-known for its aphrodisiac property and is used to treat male sexual disorders.[20-22] Treatment with *Syzygium aromaticum* has been reported to produce a sustained increase in the mounting frequency of normal male rats and mice.[23] Its main chemical contents are *eugenol*, β-caryophylline, vanillin, methylsalicylate (a pain –killer), gallaotannic acid, flavonoid such as eugenin, kaempferol, rhamnetin and eugenitin and triterpenoids like oleanolic acid stigmasterol, and campesterol, including several sesquiterpenes. [24-26] *Eugenol* is responsible for its pungency and aroma [27, 28] has antioxidant activity.[29, 30] Different studies reveal that *Syzygium aromaticum* acts as anti-fungal, [31] anti-inflammatory and anti-microbial,[32] anti-carcinogenic and anti-mutagenic.[19] Excess oral ingestion of clove in its diluted oil form or as clove cigarettes may cause vomiting, sore throat, seizure, sedation, fluid in the lung, kidney failure and liver damage.[33] Dragland *et al* [28] reported that daily

dose beyond 2.5mg/kg daily in adult human is lethal. *Syzygium aromaticum* has been shown to increase sex hormone levels and active spermatogenesis in low doses, however, loosening of germinal epithelium or intraepithelial vacuolation were also revealed in some seminiferous tubules while high dose of the extract was shown to be toxic to spermatogenesis.[32, 34] Previous studies had reported the effects of different form of extraction of clove or its active component on testicular histology or its aphrodisiac effects. Tajuddin *et al* [19] reported that oral ingestion of 100 mg/kg, 250 mg/kg and 500 mg/kg ethanolic extracts of clove with 5 mg/kg of Sildenatil citrate (Viagra) improves sexual activity of rats as compared to 5 mg/kg of Sildenatil citrate (Viagra). Chukwuma and Nnaemeka,[35] explained that "cloves are able to prolong ejaculation time and boost sperm count due to their high content of Zinc which control production of testosterone and progesterone which have positive effects on libido". Therefore only food rich in zinc is considered as an aphrodisiac.[35] The different reports on *Syzygium aromaticum* (clove fruit buds) therapeutic mechanism of action on proliferation of spermatogenic cell lines, interstitial cells, semen quality and testosterone activities create a need for further study.[19,29,32] Hence this study was design to study effect of 200mg/kg of *Syzygium aromaticum* (clove fruit buds) on body weight, testicular weight, testicular histology and semen analysis.

MATERIALS AND METHODS

Experimental animals: Twenty healthy adult male Sprague-Dawley rats, of about two month old having an average weight of 120g were used for this study. They were cared for according to ethics in "*Guide for the Care and use of Laboratory Animals"*.[36] The study was approved by Anatomy Department Research ethics committee according to standard procedure layout by National Research Council(NRC), before commencement of the study. The animals were procured at Covenant Farm (Nig.) Enterprises, Ibadan, Oyo state, Nigeria. They were authenticated in the Department of Zoology, University of Lagos, Nigeria. They were bred at Rats Control room of the Department of Anatomy, University of Lagos, Nigeria where they were kept in well ventilated rat metallic cages, in a standard laboratory condition (12hrs light: 12hrs dark cycle; room temperature) and given Pelleted rat feed (UAC, Vital Feeds Lagos, Nigeria) and water *ad libitum*. They were allowed to acclimatize for two weeks before experimentation.

Plant materials: Dried fruits of *Syzygium aromaticum* [Figure1] (clove fruit buds) were procured from Lawanson market; Surulere, Lagos. Spieces were taken to The Botany Department in University of Lagos, Nigeria; for identification and authentication, where voucher specimens were deposited.

Figure1: Shows image of dried clove bud (A) and (B) image of Syzygium aromaticum (dried clove fruits bud) and its ground form used for this study.

Preparation of the aqueous extracts of each spice: *Syzygium aromaticum* dried fruits (Clove buds) were sun-dried and crushed into powdered form using a blender. A 10g (10,000mg) portion of the ground *Syzygium aromaticum* was weighed and macerated in 50ml of distilled water, to obtain final dose concentration of 200mg/ml per animal body weight. Final extract concentration obtained was 200mg/ml per kg of animal body weight. Prepared extract was refrigerated during the period of experiment. Each animal treated with the aqueous extraction of *Syzygium aromaticum* were given one ml via gastric intubation. Clove essential oil is safe at doses lower than 1500mg/kg. WHO established that daily quantity of clove consumption was 2.5mg/kg body weights in human.[37]

Experiment Duration: This is a short-term (28 days) and long – term experiment [56 days]

Experimental Design: Administration was done once daily in the morning (between 7.30a.m – 9.00 a.m)

Euthanasia of experimental animals: The final weights of rats were obtained 24 hours after the last administration. The experimental animals were anaesthesized and euthanized by intraperitoneal injection of 50mg/ml of ketamine (Claris life sciences Ltd, India). The abdominal skin sterilized and incision made to expose the heart for blood collection using cardiac puncture.

Blood collection for serum biochemical analysis: Blood was collected via cardiac puncture using a 5ml syringe inserted into the left ventricle and transferred

into a plain labelled specimen. They were arranged in a centrifuge machine and spun at 2500 r.p.m for 10 minutes. Decanted serum into plain specimen bottles were assayed for serum testosterone level using Enzyme linked immunoassay (ELISA) technique via commercial kit procured from BIOTEC Laboratories Ltd, United Kingdom

Semen analysis: Sperm motility and Count was access using the WHO classification system. Each sample was assessed twice. For consistency all readings were carried out at 37˚C.[38] Left epididymis was placed in pre-warmed Petri-dish containing 5ml of sodium citrate solution (2.9%) at 37 ˚C. The suspension was stirred and one drop placed on a warmed slide with a cover slip placed. At least five microscopic fields were observed at 400X magnification, using a standard light microscope. Motility was reported as an average percent motile. For sperm counting, right epididymis and specimens of right testis were homogenized manually in 0.5 ml of 0.9% NaCl solution. The homogenates were diluted with 1.5 ml of saline, spermatozoa were counted using Neubauer Counting Chamber (haemocytometer) for semen analysis the recommended protocol of the WHO manual at 400X magnification in five squares.[38]

Testicular tissue collection for histopathological analysis and tissue micrography: Incision was made in the lower abdominal wall and the testes push out from the scrotal sac. The testes were excised and wet weight taken using analytical weighing scale. Animals were dissected and testes collected, weighed and fixed in Bouin's fluid. Longitudinal sectioned testicular tissues were processed for histological studies, embedded in paraffin wax and serial section using a Leica Rotary microtome set at 5µm. Testicular tissue sections were stained using Haematoxylin and Eosin stain for general cytoarchitecural appearance of the testicular tissue. Stained sections were viewed using light microscope (Olympus, Germany) at magnification of 400X.

Statistical Analysis: Data were analyzed using one-way analysis of variance (ANOVA) by the Statistical Processor System Support [SPSS] for Windows software, version 16.0 (SPSS, Chicago, IL), to compare all the treated groups and presented as Mean ± SEM or Mean ± SD (for Weight). This was followed by Tukey post hoc test to ascertain the differences between groups.

RESULTS

Body weights changes: There was a significant increase in final body weights in both control and experimental animal ($P<0.05$) in both 28 days and 56 days treatment as compared to their initial weights. It was observed that there was no statistically significance difference ($P<0.05$) in final weight of Groups B when compared with A1 same as in Group C compared with A2. As seen in Figure 2.

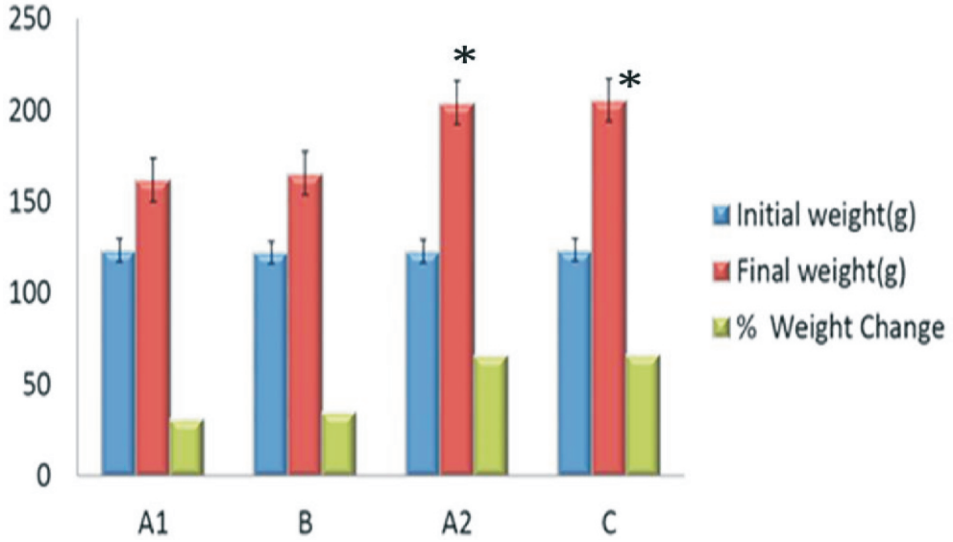

Figure 2: Graphical representation of Mean Initial and Final Body Weights of Experimental animal treated with 200mg/kg of Aqueous Clove bud. Legend: A1: Control 28days, B: 200mg/kg Clove buds for 28days A2: Control for 56 days and C: 200mg/kg of Clove buds for 56 days. Data expressed as Mean±SD.*$P<0.05$ represents statistically significant increase in final body in C as compared with B treated.

Testicular Weight: Results obtained showed a significant increase in testicular weight in B and C as shown in figure 3. Comparing B against C there was a statistical significant increase (P<0.05) in both 28days and 56 days treatment, as seen in Figure 3.

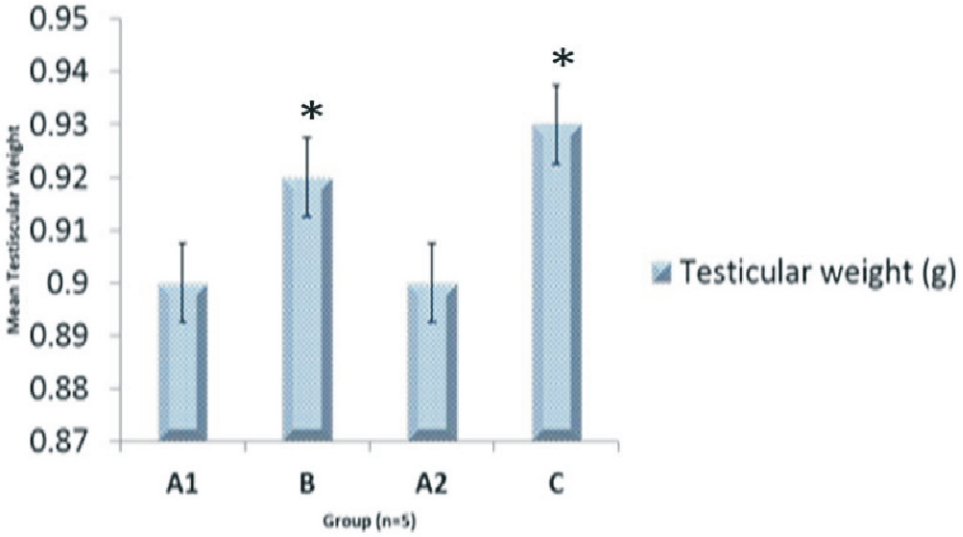

Figure 3: Graphical representation of Mean Testicular Weights of Experimental animal treated with 200mg/kg of Clove bud. Legend: A1: Control 28days, B: 200mg/kg Clove buds for 28days A2: Control for 56 days and C: 200mg/kg of Clove buds for 56 days. Data expressed as Mean±SD *P<0.05 represents statistically significant increase in testicular weights in B* vs A1 and *C vs A2. respectively.

Clove Improves perm count and motility: Average Sperm count and motility increased significantly (P<0.05) in 200mg/kg of aqueous Clove treatment as compared to their controls (A1 and A2) respectively see Figure 4.

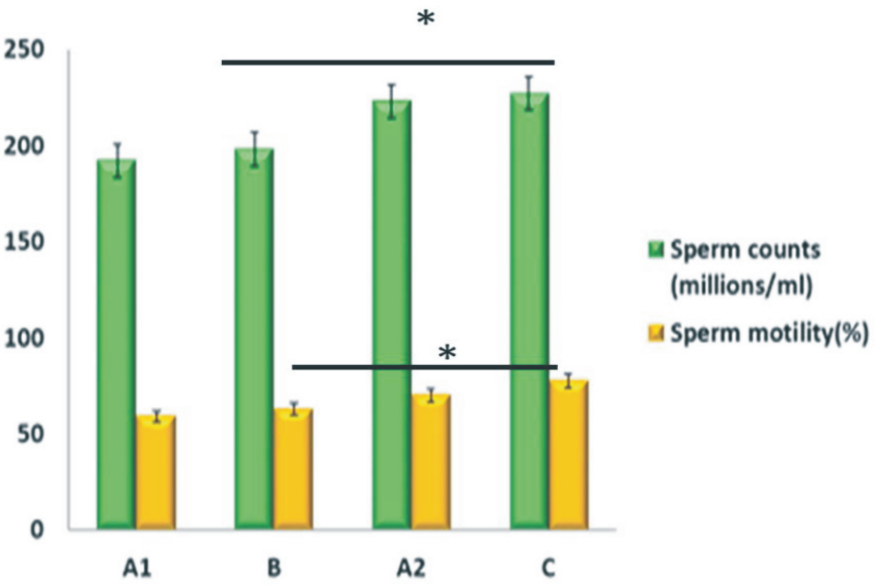

Figure 4: Graphical representation of Mean sperm count and sperm motility of Experimental animal treated with 200mg/kg of Clove bud. Legend: A1: Control 28days, B: 200mg/kg Clove buds for 28days A2: Control for 56 days and C: 200mg/kg of Clove buds for 56 days. Data expressed as Mean±SEM*P<0.05 *P<0.05 represents statistically significant increase in Sperm counts in *C vs B and Sperm motility is *C vs B

Changes in Serum Testosterone secretions: Total Serum testosterone level was increased (P<0.05) in the experimental Groups B and C in comparison with the control group (see, Figure 5). When comparing the increase between the clove treatment, group C had a significant increase (p<0.05) as compared to B.

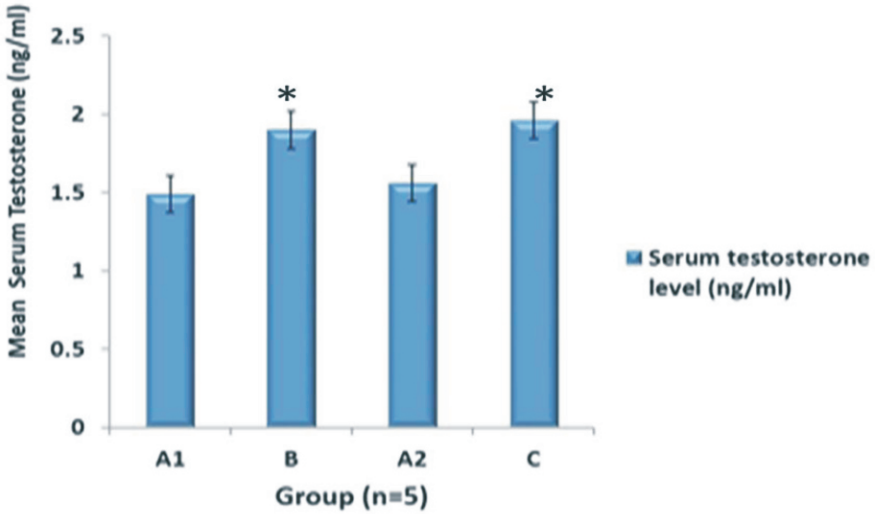

Figure 5: Graphical representation of Mean Serum Testosterone level (ng/ml) of Experimental animals. *Legend: A1- Control 28days, A2: Control 56 days; B= 200mg/kg Clove treated for 28days and C= 200mg/kg Clove treated 56 days.* Data expressed as Mean±SEM *P<0.05 represents statistically significant increase in serum testosterone in *B vs A1 and *C vs A2 respectively

Clove improves testicular cytoarchitecture: There were observable changes in the spermatogenic cell series in the seminiferous tubules of the experimental animals. There was mild distortion of the spermatogenic cell lines in 28 days treatment as seen in Figure 6B compared to the controls but interstitial cells are not totally affected. In 56 days treatment, the seminiferous tubules were well bound together with loose connective tissue, presence of proliferating spermatogenic cell lineage, Leydig cells and Spermatids in adluminal compartment as compared to Controls group A2 and B, see Figure 6). The presence of spermatids indicates the advanced stage of spermatozoa development in spermatogenic cycles.

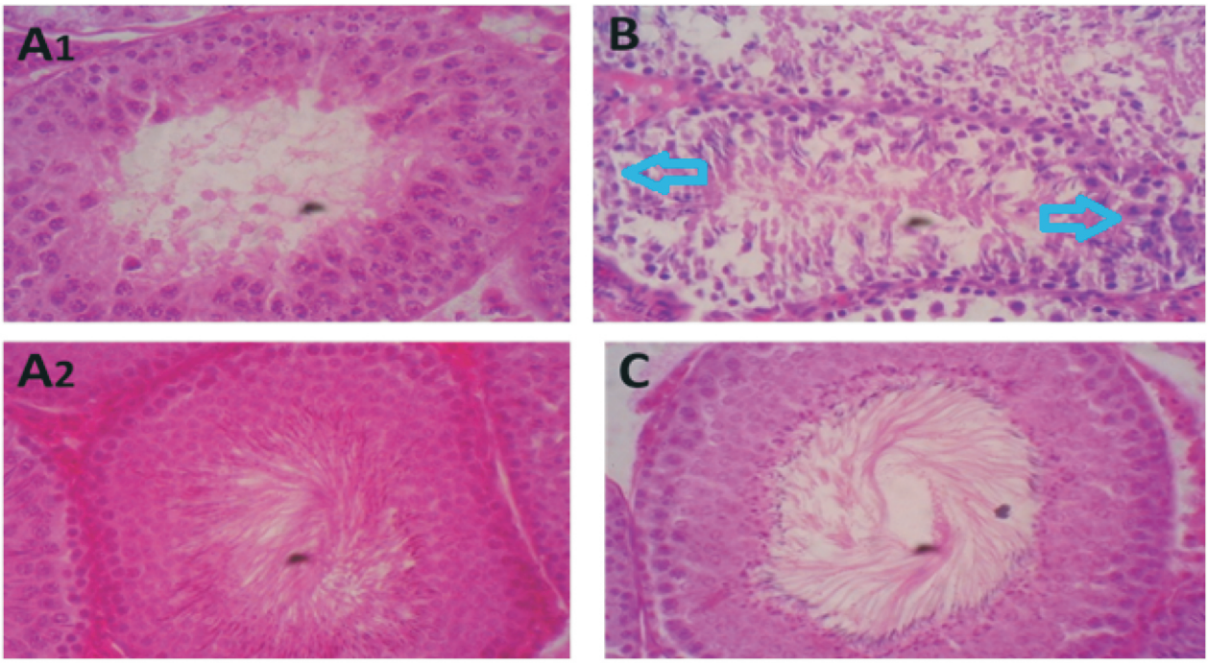

Figure 6: Section of Testicular tissue stained with Haematoxylin and Eosin (H and E) stain. *Mag. 400X. Legend: A1- Control 28days, A2: Control 56 days; B= 200mg/kg Clove treated for 28days and C= 200mg/kg Clove treated 56 days.*

DISCUSSION

This study illustrates the significance of clove [*Syzygium aromaticum*] in body weight gain as well as antioxidant and androgenic role in male rats. Essentials Oil of *Syzygium aromaticum* has been implicated weight gain traced to its terpernoid compound that induces glucose transport into cells given that changes in sugar and hormone insulin correlates to hunger and increase food consumption especially carbohydrate.[39] This supports the role of Clove in increasing weight gain in testes and body as shown in Figure 2 and 3. This finding correlated with study done on clove oil that caused a dose dependent increase in both body and testicular weight.[40] Hence this finding contradicts a study that reported that 200mg/kg/ml of *Syzygium aromaticum* for 2 weeks had no significant increase in body weight as compared to the controls.[41]

Spermatogenesis occurs in the seminiferous tubules in the testes in the presence of androgen [testosterone], its deficiency affects formation of spermatogenic cells leading to male factor infertility. [37] This study showcase that Clove induces proliferation and secretion of testosterone by interstitial cells of Leydigs in both short and long term ingestion of clove. Tajuddin et al [19] reported its aphrodisiac potential linked to ability to increase testosterone secretion. Secretory activities of the testis, epididymis as well as seminal vesicles are androgen dependent. [42] *Syzygium aromaticum* ability to mediate secretory activity of the testis, epididymis and seminal vesicles; because of the androgenic property of the extract components.[43,44]

A good semen quality is attributed to secretion from the epididymis and seminal vesicles that provides quality nourishment for the spermatids during spermiogenesis.[45] The number of sperms and motile sperm increase as compared to the control groups as seen in Figure 4. It has been reported that clove oil improves epididymal sialic acid concentration as reported by Mishra and Singh, [44] that it improves semen quality; this explains its androgenic effect. Eugenol its active compound is found in high percentage in clove flower bud with strong antioxidant property that help mob out cytotoxic compounds such as ROS from epipiymal environment during sperm maturation [30,46] and inhibits lipid peroxidation which prevents damage spermatids via oxidative stress in the testes or epidpiymis.[39] Though eugenol spermicidal activity has been document based on dose given. [47] Baghshahi *et al* [48] linked increased sperm motility and sperm counts to Clove strong antioxidant ability.

Serum testeosterone production have been linked to testicular steroidogenic enzymes such as delta 5,3 beta-hydroxysteroid dehydrogenase (Δ5, 3β-HSD) and 17 beta-hydroxysteroid dehydrogenase (17β-HSD) according to Jane *et al*.[50] Secretory activities of the testis, epididymis as well as seminal vesicles are androgen dependent.[42] In this study, significant increase serum testosterone level ($P<0.05$) correlate with study on *Syzygium aromaticum* ability to mediates secretory activity of the testis, epididymis and seminal vesicles; because of the androgenic property of the extract components.[34,43,44]

Testicular histology shows that 200mg/kg aqueous extract of Syzygium aromaticum has proliferative potentials on spermatogenic cell lineage at 56 days treatment. There was no distortion of the spermatogenetic cell series (seen in plates D), Sertoli and Leydig cells that supports and secretes substances need during spermatogenesis. This histological improvement consequently leads to an increase sperm count. This report opposes a finding that showed *Syzygium aromaticum* administration causing a daily decline in spermatogenesis.[43] Its proliferative potentials seen in 56days treatment [See Figure 6D].

This study contradicts report by Banerjee *et al*[49] that *Syzygium aromaticum* extract administration can lead to significant reduction in the number of proliferating cells and an increase in the number of apoptotic cells though markers for apoptotic cells was not implored in this study. There was mild distortion of spermatogenic cell series [Figure 6B vs 6A1, A2 and C] irrespective of the significant increase sperm count seen in Figure 4.

The extract may influence blood flow to nervous tissue and in this way it may improve reproductive activity thereby increasing spermatogenesis via the activity of gonadotrohic hormones released from the hypohysis.[19] Although, *Syzygium aromaticum* extracts have been reported to affect spermatogenesis, serum testosterone and semen quality in a dose-dependent manner. It was shown that the serum testosterone concentration and spermatogenesis increase in mice treated with low dose of the *S. aromaticum* but they decline in high dose,[34,43,44] use of therapeutic dose in moderation reveals its activity as an aphrodisiac in treating male fertility disorders related to sex hormone secretion impairment.

Phytochemical studies indicate that the clove contains rich phenolic compound such as eugenol, eugenol acetate, caryophyllene, sesqueterpene ester, phenyl propanoid, and β caryophyllene [19,20,51] which are rich in antioxidant activity – basis for their use in producing medicinal drugs.[52] Clove has higher polyphenol compounds as compared to other spices hence higher antioxidant potential.[53]
The earlier study using hydro alcoholic extract (50%) of clove demon-strated the aphrodisiac activity. [54] Recently, the promoting effect of herbal extract on the reproduction was reported.[23]

CONCLUSION

In conclusion, the data of the present study indicates that 200mg/kg of *Syzygium aromaticum* has beneficial effects to increase spermatogenic cell proliferations, sperm density in the seminiferous tubules and semen

quality that is attributed to its ability to mediate gonadal and male accessory gland secretions.

RECOMMENDATIONS

Oxidative stress is the etiology of some kinds of infertility and impairs sperm motility, viability and morphology [55] studies should be carried out on the mechanism of action of clove flower bud on lipid peroxidation.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

We appreciate the Department of Anatomy, University of Lagos for providing equipment for this study. School where the study was carried out has been given full acknowledgement.

REFERENCES

1. Santi D, De Vincentis S, Magnani E, Spaggiari G. Impairment of sperm DNA methylation in male infertility: a meta-analytic study. *Andrology*. 2017; 5(4):695-703.
2. Awad AB, Fink CS. Phytosterols as anticancer dietary components: evidence and mechanism of action. *J Nutr.* 2000; 130:2127-2130
3. Bui AD, Sharma R, Henkel R, Agarwal A. Reactive oxygen species impact on sperm DNA and its role in male infertility. *Andrologia*. 2018; 50(8):e13012.
4. Silberstein T, Har-Vardi I, Harlev A, Fridger M, Hamou B, Barac T, Levitas E,Saphier O. Antioxidant and polyphenols: concentration and relation to male infertility and treatment success. Oxid.Med.Cell.Longev. 2016.
5. Agarwal A, Makker K, Sharma R. Clove relevance of oxidative stress in male factor infertility: an update. *Am.J. Reprod.Immuno.,* 2008; 59(1): 2 -11.
6. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev.. 2016; 28(1-2):1-10.
7. Chen SJ, Allam JP, Duan YG, Haidl G. Influence of reactive oxygen species on human sperm functions and fertilizing capacity including therapeutical approaches.*Arch Gynecol Obstet.,* 2013; 288(1):191-9.
8. Singh A, Jahan N, Radhakrishnan G, Banerjee BD. To evaluate the efficacy of combination of antioxidants therapy on oxidative stress parameters in seminal plasma in male infertility.J.Clin. Diagn. Res., 2016; 10 (7): QC14-17.
9. Agarwal A, Raychendhny S, Bjugstad, KB, Cho CL. Oxidation- reduction potential odf semen: what is its's role in the treatment of male infertility.ther. Adv.Urol., 2016; 8(5): 302 -318.
10. Maneesh M, Jayalekshmi H. Role of reactive oxygen species and antioxidant an pathophysiology of male reproduction .Indian J.Clin.Biochem., 2006; 21(2): 80 -89.
11. Liu H, Schmitz JC, Wei J, Cao S, Beumen JH, Strchor S, Cheng L, Liu M, Wang C, Wu N, Zhao X, Zhang Y, Lia OJ, Chu E, Lin X. Clove extracts inhibits tumor growth and promotes cell cycle arrest and apoptosis.Oncol. res., 2014; 21 (5): 247 – 259.
12. Choi D, Roh HS, Kang DW, Lee JS. The potential regressive role of Syzygium aromaticum on reproduction of Male Golden Hamster. Dev. Reprod., 2014; 18 (1): 57-64.
13. Choi D. Reproductive physiology of pineal hormone melatonin. Korean J Zool., 1996; 39:337-351.
14. Turner J . Spice: The History of a Temptation. Vintage Books, p. Xv. 2004
15. Ogungbemi T. Outlines and pictures of medicinal plants in Nigeria.1st edition, 2006; 84 – 255.
16. Choi D, Han E-H. The impacts of photoperiods on hypothalamic proteins in the reproductive activities of golden hamsters. *Dev Reprod.,* 2010; 14:185-197.
17. Choi D, Lee S-H. Neuroendocrine system in seasonal breeder: focusing on the reproductive activity of male golden hamster. *Dev Reprod.,* 2012; 16:1-8.
18. Thosar N, Basak S, Bahadure RN, Rajurkar M. Antimicrobial efficacy of five essential oils against oral pathogens: An in vitro study. *European Journal of Dentistry*. 2013; 7(Suppl 1):S71–S77. doi:10.4103/1305-7456.119078
19. Tajuddin A, AhmadS, Latif A. Aphrodisiac activity of 50% ethanolic extracts of Myristica Fragans Houtt (Nutmeg) and Syzygium Aromaticum (clove) in male mice. A comparative study. *BMC. Complement Alternative Medicine.* 2003; 3(1): 6
20. Criddle DN, Madeira SV, Soares de Moura R. Endothelium-dependent and - independent vasodilator effects of eugenol in the rat mesenteric vascular bed. *Journal of Pharmacy and Pharmacology*. 2003; 55:359-365
21. Dwivedi V, Shrivastava R, Hussain S, Ganguly C, Bharadwaj M. Comparative anticancer potential of clove (*Syzygium aromaticum*) - an Indian spice - against cancer cell lines of various anatomical origin. *Asian Pacific J Cancer Prev.,* 2011; 12:1989-1993.
22. Anushri M, Yashoda R, Manjunath PP. Herbs: A Good Alternatives to Current Treatments for Oral Health Problems.*Int. J Advanced Health Sciences.* 2015; 1(12)
23. Lee JS, Oh HA, Kwon JY, Jeong MH, Kang DW, Choi D. The effects of *Cynomorium songaricum* on the reproductive activity in male golden hamsters. Dev Rep., 2013; 17:37
24. Sharma A, Rajendran S, Srivastava A, Sharma S, Kundu B. Antifungal activities of selected essential oils against Fusarium oxysporum f. sp. lycopersici 1322, with emphasis on Syzygium aromaticum essential oil.*Journal of Bioscience Bioengineering.* 2017; 123(3):308-313.

25. Yang YC, Wei MC, Hong SJ . Ultrasound-assisted extraction and quantitation of oils from Syzygium aromaticum flower bud (clove) with supercritical carbon dioxide. *J Chromatogr A*. 2014; 1323:18-27.
26. Bensky D, Clavey S, Stoger E, Gamble A. *Chinese Herbal Medicine: Materia Medica*, Third Edition. 2004.
27. Krishnaswamy, K. Raghuramalu, N. Bioactive phytochemicals with emphasis on dietary practices. *Indian J. Med. Res.,* 1998; 108:167 – 181
28. Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several culinary and medicinal herbs are important source of dietary antioxidants. *Journal of Nutrition.* 2003; 133 (5):1286 – 1290
29. Abdel-Wahhab MA, Aly SE . Antioxidant property of *Nigella sativa* (black cumin) and *Syzygium aromaticum* (clove) in rat during aflatoxicosis. *Journal of Applied Toxicology*. 2005; 25(3): 218-23.
30. Gulcin I. Antioxidant activity of eugenol: a structure-activity relationship study. *Journal of Medicinal Food*. 2011; 14 (9): 975-85.
31. Pinto E, Vale-Silva L, Cavaleiro C, Salgueiro L. Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and *Dermatophyte* species. *Journal of Medical Microbiology*. 2009; 58 (11):1454-62
32. Chaieb K, Hajlaoui H, Zamantar T, Kahla-Nakbi AB, Rouabhia M, Mahdouani K, Bakhrouf F. The chemical composition and biological activity of clove essential oil, Eugenia caryophyllata (*Syzugium aromaticum* L Myrtaceae): a short review. *Phytotherapy Research*. 2007; 21(6): 501–506
33. Consolini AE, Sarubbio MG. Pharmacological effects of *Eugenia uniflora* (Myrtaceae) aqueous crude extract on rats' rat. *Journal of Ethnoparmacology.* 2002; 81: 57 – 63.
34. Mishra RK, Singh SK. Biphasic effect of Syzygium aromaticum flower bud on reproductive physiology of male mice. *Andrologia*. 2016; 48(9):923-932. doi: 10.1111/and.12533.
35. Chukwuma M, Nnaemaka O. Nutmeg, clove prevents premature ejaculation, boost sperm count. Guardian Newspaper. 2006.
36. Guide for the Care and use of Laboratory Animals (1985)
37. Gulcini I, Elmastasta M, Aboul-Enan HY. Antioxidant activity of Clove oil. A powerful antioxidant saource.Arab. J. Chem., 2012; 5(4): 489 -499.
38. World Health Organization. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction (4th ed.), Cambridge University Press, Cambridge. 1999.
39. Shodehindel, Oboh G. "Antioxidant Properties of Aqueous Extracts of Unripe Musa paradisiaca on Sodium Nitroprusside Induced Lipid Peroxidation in Rat Pancreas in Vitro Sidiqat Adamson," Asian Pacific Journal of Tropical Biomedicine. 2013; 3(6): 449-457
40. Ahmed M . The Effect of Dietary Clove Oil on Broiler Performance. *Journal of Applied Sciences Research*. 2011; 7(7):49
41. Shama IYA, Suha BM, Warda SA. Effects of the Aqueous Extract of Clove (Syzygium aromaticum) on Wistar Rats. British Journal of Pharmacology and Toxicology. 2013; 4(6):262-266, 2013ISSN:2044-2459; e-ISSN: 2044-2467.
42. Lo KC, Lamb DJ. The testis and male accessory sex organs. In: Strauss LF, Barbieri RL, editors. Yen and Jaffe's Reproductive Endocrinology. Vol. 5. Pennsylvania: Elsevier Saunders. 2004; pp. 367–87.
43. Mishra RK, Singh SK. Safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice. *Food and Chemical Toxicology*. 2008; 46 (10): 3333–3338..
44. Mishra RK, Singh SK. Reproductive effects of lipid soluble components of *Syzygium aromaticum* flower bud in male mice J Ayurveda Integr Med., 2013; 4(2): 94–98
45. Rajalakshmi M. Regulation of male fertility: Epididymis as a potential extragonadal site. In: Ghosh D, Sengupta J, editors. Frontiers in reproductive physiology. New Delhi: Wiley Eastern Limited. 1992; 63-66.
46. Ben Abdallah F, Fetoui H, Zribi N, Fakfakh F, Ammar-Keskes L. Antioxidant supplementations in vitro improve rat sperm parameters and enhance antioxidant enzyme activities against dimethoate-induced sperm damages. *Andrologia*. 2012; doi: 10.1111/j.1439- 0272.2011.01177.
47. Carrasco FR, Schmidt G, Romero AL, Sartoretto JL, Bersani-Amado CA, Cuman RK. Immunomodulatory activity of *Zingiber officinale Roscoe, Salvia officinalis L.* and *Syzygium aromaticum L.* essential oils: evidence for humor- and cell-mediated responses. *Journal of pharmacology & pharmacotherapeutics*. 2009; 61(7): 961-7.
48. Baghshahi H, Riasi A, Mahdavi AH, Shirazi A. Antioxidant effects of Clove bud (*Syzygium aromaticum*) extract used with different extenders on ram spermatozao during cryptopreservation. Cryobiology. 2014; 69(3): 482- 487.
49. Baberjee S, Panda CK, Das S. Clove (*Syzygium aromaticum* L.), a potential chemopreventive agent for lung cancer. *Carcinogenesis*. 2006; 27(8): 1645-54.
50. Jane K, Jana S, Samanta PK. "Effects of Chronic Exposure to Sodium Arsenite on Hypothalamic PituitaryTesticular Activities in Adult Rats: Possible Anestrogenic Mode of Action," Reproductive Biology and Endocrinology. 2006; 4: 9.
51. Tanko Y, Mohammed A, Okasha MA, Umar AH, Magaji RA. Anti-nociceptive and anti-inflammatory activities of ethanol extract of *Syzygium aromaticum* flower bud in wistar rats and

mice. *African Journal of Traditional Complementary and Alternative Medicine*. 2008; 5:209-212.

52. Cortes-Rojas DF, do-Souza CR, Oliveira WP. Clove (*Syzugium aromaticum): a precious spice. Asian Pac.J. Trop. Biomed., 2014; 4 (2): 90- 96.*
53. *Shan B, Cai Y, Sun M, Cork H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituent. Journal of Agricultural and Food Chemistry. 2005; 53: (20): 7749 -7759*
54. Tajuddin, Ahmad S, Latif A, Qasmi IA. Effect of 50% ethanolic extract of *Syzygium aromaticum* (L) Merr. and Perry. (clove) on sexual behavior of normal male rats. BMC *Complementary and Alternative Medicine.* 2004; 4:17.
55. Micinski P, Pawlicki K, .Wielgus E, Bochenek M, Gogol P, Ficek B. Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters. *Reproductive Biology*. 2011; 11 (2): 135-44.