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EFFECTS OF *Tetrapleura tetraptera* FRUIT ETHYL ACETATE FRACTION, APIGENIN AND FLUOXETINE ON THE TESTES OF SWISS MICE EXPOSED STRESS

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ABSTRACT

Introduction: Stress significantly influences changes in numerous physiological processes and may contribute to various disorders, including male sub-fertility or infertility.
Aim: This study aimed at assessing the effects of *Tetrapleura tetraptera* (TT) fruit ethyl acetate fraction, apigenin and fluoxetine on the body weight, testes histology and semen parameters of the testes following chronic unpredictable mild stress.
Methodology: Twenty five male Swiss mice (20-30g) were randomly assigned into five groups (1-5, n=5) for this study: Group 1 served as the control and was administered 20 mL/kg body weight of distilled water; groups 2-5 were; stress + water, stress + 50 mg/kg body weight ethyl acetate fraction of TT fruit, stress + 50 mg/kg body weight of apigenin, and stress + 50 mg/kg body weight of fluoxetine. The mice were exposed to different specific stressors, administrations given orally and the experiment lasted for 28 days. The animals were sacrificed. The testes were processed and stained with Hematoxylin and Eosin.
Result: The results showed that stress significantly ($p<0.05$) reduced weight, while the TT extract treatment group led to more weight loss. Also, TT, apigenin, and fluoxetine all reduced normal sperm morphology and sperm concentration. There was severe distortion of tissue structures in testes by stress. In conclusion, stress had negative effects on reproductive health.

Keywords: Tetrapleura tetraptera, apigenin, fluoxetine and testis

INTRODUCTION

Chronic stress is the physiological or psychological response induced by a long-term internal or external stressor. The stressor, either physically present or recollected, will produce the same effect and trigger a chronic stress response. There is a wide range of chronic stressors, but most entail relatively prolonged problems, conflicts and threats that people encounter on a daily basis. Several chronic stressors have been identified as associated with disease and mortality, including environment, financial strain, interpersonal stress, work stress and caregiving (Epel *et al*., 2018). Stress responses, such as the fight or flight response, are fundamental. The complexity of the environment means that it is constantly changing. To navigate the surroundings, a system that is capable of responding to perceived threatening and harmful situations is necessary during stress, physiological adaptations counteract with the negative effects of the stressor and maintain homoeostasis

Many studies have shown that long-term excessive stress can impair male reproductive function, leading to failure of sexual function and decreased fertility. The main function of Leydig cells in the testes is to synthesize and secrete androgen.

Oxidative stress, free radicals and reactive oxygen species (ROS) damage the body macromolecules, with the CNS being more prone to oxidative damage due to its increased energy demands, high cellular content of lipids, proteins and decreased levels of anti-oxidants (Sharifi-Rad *et al*., 2020). Oxidative stress is a dangerous pathophysiological mechanism reported in various pathologies like cancer, diabetes, cardiovascular diseases, neurological disorders and rheumatoid arthritis, Oxidative stress induced by reactive oxygen species (ROS) is a crucial trigger of tissue inflammation, ROS are continuously produced in Leydig cells as in other cells during normal cellular aerobic metabolism via the mitochondrial electron transport chain. Additionally, ROS are synthesized as a by-product of steroidogenesis, especially during steroid hydroxylation by cytochrome P450 enzymes (Wang *et al*., 2012).

Medicinal plants have been explored for their potential therapeutic effects in managing stress-related disorders. *Tetrapleura tetraptera*, known as "Aidan" in West Africa, is one such plant with a history of traditional medicinal use. The fruits of *Tetrapleura tetraptera* are rich in bioactive compounds, including flavonoids, tannins, saponins, and phenolic compounds, which are known for their antioxidant, anti-inflammatory, and neuroprotective properties (Adusei *et al*., 2019). Research indicates that extracts from *Tetrapleura tetraptera* exhibit significant antioxidant activity, which can mitigate oxidative stress induced by disorders or disease conditions. For instance, studies have shown that the plant's extracts can enhance the activity of endogenous antioxidant enzymes, thereby reducing the detrimental effects of reactive oxygen species (ROS) (Agbai *et al*., 2019; Johnlouis, 2022). Previous studies have highlighted the therapeutic potential of *Tetrapleura tetraptera* in various health conditions, such as inflammation, infections, and diabetes. Its antioxidant properties may help reduce oxidative stress, a significant factor in stress-induced damage to cells and tissues (Aderibigbe *et al*., 2011; Agbai *et al*., 2019).

Apigenin, a natural flavonoid found in various plants and fruits, has demonstrated significant therapeutic potential in recent studies. It exhibits anti-inflammatory, antioxidant, and neuroprotective properties (Salehi *et al*., 2019; Azeem *et al*., 2024). Notably, apigenin has shown promise in alleviating stress-related conditions and depressive behaviors. In animal models, chronic oral administration of apigenin reduced immobility time in forced swimming tests and reversed decrease in sucrose intake caused by chronic mild stress. (Yi *et al*., 2008).

Fluoxetine, a widely prescribed selective serotonin reuptake inhibitor (SSRI), has shown efficacy in alleviating stress-induced depressive-like behaviors in animal models. It reduces corticosterone levels, immobility in forced swim tests, and modulates neurotransmitter concentrations in limbic areas (Estévez-Cabrera *et al*., 2023

MATERIALS AND METHODS

Materials

Tetrapleura tetraptera fruits were obtained, identified and authenticated at the department of Botany and ecological study, Faculty of Biological sciences, University of Uyo. Apigenin and Fluoxetine drugs were obtained from a pharmacy store in Uyo metropolis, Nigeria.

Extract Preparation and Partitioning

The pods of *Tetrapleura tetraptera* were dried, carefully cut with a clean knife, seeds removed and dried. Once the pods were sufficiently dried, they were grounded into a fine powder, using a grinder. The Powder was then subjected to standard methanol extraction method.

To arrive at the fraction, crude extract was dissolved in distilled water, poured into a separation funnel then passed through N-Ethyl acetate solvent to yield N-Ethyl acetic fraction. The fraction was then air dried under room temperature for 72 hrs to yield a dried compound of the fraction.

Animals Care and Handling

A total of twenty-five male Swiss mice of body weight 20-30g was used for this study. They were randomly divided into five groups (Groups 1 - 5) each group consists of five animals and housed in well-ventilated standard animal cages. The animals were maintained at room temperature of 25-28 °C and 12:12 light/dark cycle with free access to standard chow pellet and water ad *libitum*. Each group consists of five animals. Group 1 served as the control, while groups 2-5 served as the test groups.

Chronic mild stress Protocol

The CUMS was performed according to the protocols previously described by Szala-Rycaj *et al*. (2023) with modifications (Table1).

Table 1: List of stress factors and the duration of the stimulus

Day	1	2	3	4	5	6	7
Week 1	Wet bedding (24 hours)	Tilted Cage with bedding (24 hours)	Water deprivation (24 hours)	Normal cage with bedding (24 hours)	Introducing predators (rats) in mouse cages (30 minutes)	Introduction of mice in rat bedding (30 minutes)	Food deprivation (24 hours)
Week 2	Wet bedding (24 hours)	Water deprivation (24 hours)	Animal Isolation (2 hours)	Cold restraint test (2 hours)	Heat exposure (30 minutes)	Sucrose preference test (24 hours)	Tail suspension (2 hours)
Week 3 + drugs	Wet bedding (24 hours)	Tilted cage with bedding (24 hours)	Water deprivation (24 hours)	Normal cage with bedding (24 hours)	Introducing predators (rats) in mouse cages (30 minutes)	Introduction of mice in rat bedding (30 minutes)	Food deprivation (24 hours)
Week 4 + drugs	Wet bedding (24 Hours)	Heat exposure (30 minutes)	Cold restraint stress test (2 Hours)	Sucrose preference test (24 Hours)	Physical restraint stress test (2 Hours)	Forced swimming test (30 minutes)	Food deprivation (24 Hours)/Tail suspension test (2 Hours)

Experimental Design

This experiment was conducted for 28 days, stress induction alone for the first 14 days and stress induction and administration for the next 14 days. The animals were divided into five (5) groups. Group 1 served as the control group, which received distilled water; Group 2 was exposed to stress and received distilled water; Group 3 was exposed to stress was administered 50 mg/kg body weight of ethyl acetate fraction of *T. tetraptera*; Group 4 was exposed to stress and administered 50 mg/kg body weight of Apigenin and; Group 5 was exposed to stress and administered 50 mg/kg body weight of Fluoxetine as shown in Table 3.2.

Table 2: Schedule of groupings and Administration of Animals in Control and Test Groups

Groups (n = 8)	Treatment/Dosage	Duration (Days)
1	Distilled water (0.2 mL)	28
2	stress + Distilled water (0.2 mL)	28
3	stress + ethyl acetate fraction *T. tetraptera* (50 mg/kg)	28 and 14
4	stress + Apigenin (50 mg/kg)	28 and 14
5	stress + Fluoxetine (50 mg/kg)	28 and 14

Termination of Experiment and Sample Collection

On the 29th day, the animals were anaesthesized through injection of 50 mg/kg of ketamine intraperitoneally, and blood sample was collected by cardiac puncture into a plain bottle for examination of full blood parameters. The serum was separated by allowing blood sample for 15 minutes and centrifuged for 20 minutes, then kept in plastic vials and immediately stored for biochemical studies. After blood collection, the animals were perfused with phosphate buffered saline (for cleansing) and phosphate buffered formalin (for fixing) and the brain tissues and testes were excised.

RESULTS

Effects of Chronic Stress, Ethyl-acetate Fraction of *T. Tetraptera* Fruit Extract, Apigenin and Fluoxetine on Body Weights.

The result showed that control group had significant weight gain (p<0.05). However, groups exposed to chronic stress and water, exposed to stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract, exposed to stress and 50 mg/kg body weight of fluoxetine, and CUMS and 50 mg/kg body weight of apigenin significantly prevented weight gain in the animals and this effect was more pronounced in group exposed to stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract when compared to control group that showed a normal increase in body weight at p<0.05 respectively. Also, group exposed to stress and 50 mg/kg body weight of apigenin showed a moderate weight gain, significantly different from groups 2, 3 and 5 (p<0.05) (Table 4.1).

Table 4.1: Body weights

Groups	Initial Weight (g)	Final Weight (g)	Weight difference (g)
Control group	23.20±0.20	27.00±0.32	3.80±0.12
Stress + water	23.40±0.40	24.20±0.49*a	0.80±0.09
Stress + ethyl acetate of *T. tetraptera* fruit	24.80±0.37	24.00±0.32*a	-0.80±0.05
Stress + apigenin	27.00±0.45	28.80±0.37*abc	1.80±0.08
Stress + fluoxetine	24.40±0.51	24.80±0.37*ad	0.40±0.14
	P<0.05	P<0.05	
	F=14.43	F=29.92	

Values are expressed in Mean±SEM. * indicate significant differences at p<0.05. a = indicating significant difference in relation to Group 1; b = indicating significant difference in relation to Group 2; c = indicating significant difference in relation to Group 3; d = indicating significant difference in relation to Group 4.

Group 1- Control; Group 2- Stress + water; Group 3 = Stress + 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract; Group 4= Stress + 50 mg/kg body weight of apigenin; and Group 5= Stress + 50 mg/kg body weight of fluoxetine.

Effects of CUMS, Ethyl-acetate Fraction of *T. Tetraptera* Fruit Extract, Apigenin and Fluoxetine on Sperm Morphology and Concentration.

Results showed that there was a significant decrease (p<0.05) in % of normal-neck sperm cells in group exposed to Stress and 50 mg/kg body weight of apigenin compared to control group. Group exposed to Stress and 50 mg/kg body weight of fluoxetine showed a significant increase (p<0.05) in % of bent-neck sperm cells when compared to groups exposed to Stress and water and group exposed to Stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract respectively (Table 4.3).

Although, there was no significant differences in % of curved-neck sperm cells across all groups. But the group exposed to stress and 50 mg/kg body weight of apigenin showed a significant decrease ($p<0.05$) in sperm concentration when compared to control group and group exposed to stress and water respectively (Table 4.3).

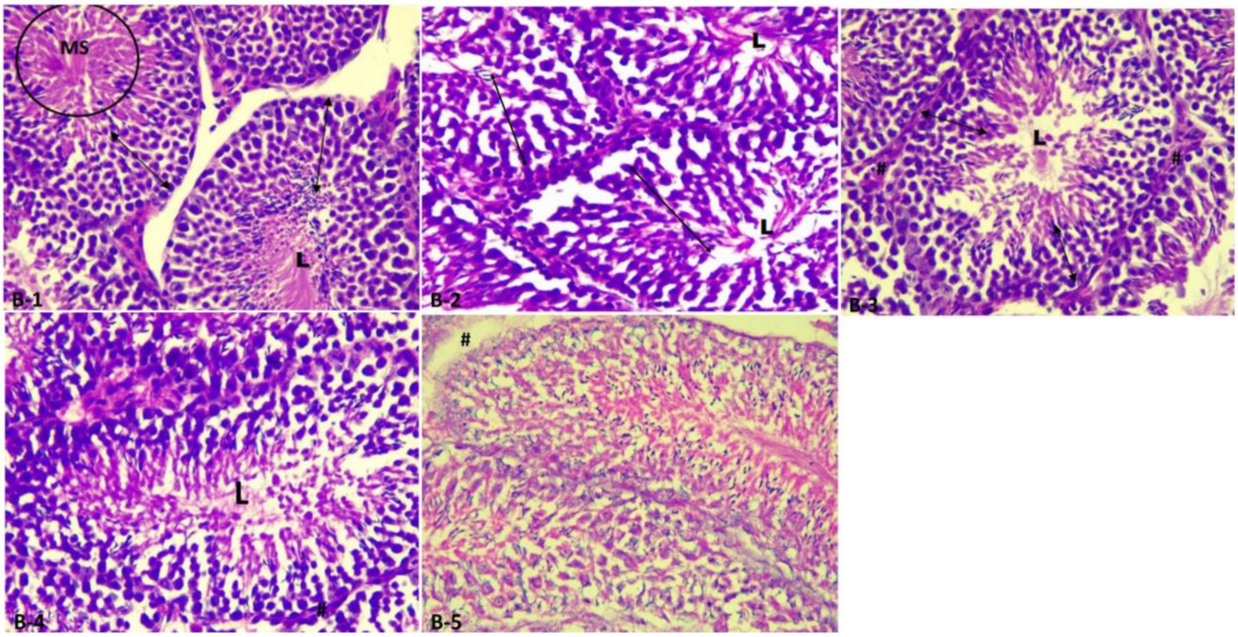
Table 4.3: Effects of Stress, Ethyl-acetate Fraction of *T. Tetraptera* Fruit, Apigenin and Fluoxetine on Sperm Morphology and Concentration.

GROUPS	% Normal Neck	% Bent Neck	% Curved Neck	Concentration (cell x 10^6)
GRP 1	58.00±2.55	25.00±2.24	19.00±1.00	138.30±14.21
GRP 2	54.00±1.87	22.00±2.00	25.00±2.24	150.90±9.32
GRP 3	49.00±3.32	22.00±2.00	23.00±2.00	118.00±5.88
GRP 4	48.00±3.74*a	26.00±1.87	24.00±1.87	106.20±7.09*ab
GRP 5	49.00±2.45	32.00±2.00*bc	20.00±1.58	118.70±10.95
	P=0.102	P=0.014	P=0.120	P=0.033
	F=2.232	F=4.098	F=2.094	F=3.245

Values are expressed in Mean±SEM. * indicate significant differences at $p<0.05$. a = indicating significant difference in relation to Group 1; b = indicating significant difference in relation to Group 2; c = indicating significant difference in relation to Group 3.
Group 1- Control; Group 2- Stress + water; Group 3 = Stress + 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract; Group 4= Stress + 50 mg/kg body weight of apigenin; and Group 5= Stress + 50 mg/kg body weight of fluoxetine.

Histological Findings on Testes Using Haematoxylin and Eosin Staining

Histological findings of testicular tissue of control group using haematoxylin and eosin' photomicrograph, group 3 exposed to stress and administered 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract, and group 4 exposed to stress and administered 50 mg/kg body weight of apigenin showed seminiferous tubule lined by germinal epithelium composed of spermatogenic cells at various stages of development, arranged peripherally along the basement membrane with lumen centrally located, containing mature spermatozoa respectively. On the other hand, group 2 exposed to stress + water and group 5 exposed to stress and administered 50 mg/kg body weight of fluoxetine showed some seminiferous tubule with disorganized epithelium and loss of stratification; cells appear irregular and loosely packed, pale-stained cytoplasm and loss of nuclear components and increased interstitial fibrosis with widened spaces respectively.



B-1: Photomicrographs of cross section of testicular tissue of control group showing seminiferous tubules (ST). Each seminiferous tubule is lined by a germinal epithelium composed of spermatogenic cells at various stages of development, arranged peripherally along the basement membrane (double-headed arrow). The lumen (L) is centrally located and contains mature spermatozoa (MS). H&E stain. ×400 magnification.

B-2: Photomicrographs of cross section of testicular tissue exposed to Stress and water. Some seminiferous tubules (ST) showed disorganized epithelium with loss of stratification; cells appear irregular and loosely packed (doublehead arrow). H&E stain. x400 magnification.

B-3: Photomicrographs of cross section of testicular tissue exposed to stress and administered 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract showed seminiferous tubules (ST) and normal interstitial connective tissue (#). Each seminiferous tubule consists of normally arranged germinal epithelium (doublehead arrow), the lumens (L) having complete and partly filled mature spermatocytes. pathological changes seen. H&E stain. x400 magnification.

B-4: Photomicrographs of cross section of testicular tissue exposed to stress and administered with 50 mg/kg body weight of Apigenin showing seminiferous tubules (**ST**) and normal interstitial connective tissue (**#**). Each seminiferous tubule consists of normal arranged germinal epithelium (doublehead arrow), the lumens (L) with complete and partly filled mature spermatocytes. H&E stain. x400 magnifications.

B-5: Photomicrograph of cross section of testicular tissue exposed to stress and administered 50 mg/kg body weight of Fluoxetine showing increased eosinophilia of the seminiferous tubules (ST). Pale-stained cytoplasm and loss of nuclear components. Increased interstitial fibrosis with widened spaces (#). H&E stain. x400 magnifications.

DISCUSSION

The present study investigated the effects of *Tetrapleura tetraptera* fruit extract, apigenin and fluoxetine on the histological and semen analysis of the testes of Swiss mice exposed to chronic unpredictable mild stress. It was observed that stress had significant impact on body weight. Consistent with previous findings, stress has been shown to induce an imbalance in energy homeostasis, leading to decreased food intake and changes in metabolic rate (Willner, 1997; Rygula *et al*., 2005). When subjected to chronic stress, animals often exhibit a decrease in appetite and increased physical inactivity, thereby leading to a reduction in weight gain over time. This may be attributed to increased levels of stress hormones such as cortisol and noradrenaline, which promote catabolic activity and suppress appetite (Bjorntorp, 2001; Razzoli *et al.*, 2017). In contrast to the effects observed from stress, the administration of *T. tetraptera* extract appears to provoke a different metabolic response. The present findings confirm that treatment with *T. tetraptera* extract results not in protection against the weight-reducing effects of stress, but was more pronounced in preventing weight gain. *T. tetraptera* used traditionally for its medicinal properties, has been investigated for its pharmacological effects, particularly its potential role as an anti-inflammatory and antioxidative agent (Oduola *et al.*, 2018; Odeku *et al.*, 2022). It is conceivable that the bioactive compounds within the extract might enhance metabolic activity, leading to increased energy expenditure or potentially stimulate thermogenesis, which would contribute to weight loss (Chukwuebuka *et al.*, 2019). Additionally, the possibility of altered appetite regulation due to phytochemicals present in *T. tetraptera* cannot be overlooked, as such compounds might affect the central nervous system or peripherally interfere with hunger signaling pathways.

The findings from this study suggested that treatment with apigenin facilitates weight recovery in animals exposed to stress, although this recovery does not reach baseline or normal weight levels. The therapeutic action of apigenin has been supported by various studies that indicate its potential to ameliorate weight changes induced by high-fat diets through mechanisms such as the inhibition of adipocyte differentiation, modulation of adipogenic pathways, and improvement in metabolic parameters (Gentile *et al.*, 2018; Younatan *et al.*, 2023). Specifically, research indicates that apigenin affects lipid accumulation in 3T3-L1 adipocytes by activating the AMPK pathway and downregulating key genes involved in fat storage and differentiation, thereby reducing body fat content without normalizing weight (Younatan *et al.*, 2023). Additionally, Gentile *et al.* (2018) reported that apigenin helped mitigate increases in body fat and improved glucose homeostasis in obese mice models. These findings strengthen the argument for apigenin's effectiveness in promoting weight recovery under conditions of stress, although aspects of its action may leave weight levels below pre-stress baselines—a reflection of lingering metabolic dysregulation despite pharmacological intervention (Gentile *et al.*, 2018). In contrast, fluoxetine demonstrated a modest impact on weight recovery in the same CUMS model. Although primarily recognized for its antidepressant properties, the efficacy of fluoxetine concerning weight recovery from stress appears limited. As part of its mechanism, fluoxetine impacts serotonin levels, which can influence appetite and energy metabolism. However, in the context of stress-induced weight loss, its action does not completely counteract the effects of stress (Dennis *et al.*, 2019; Dennis *et al.*, 2020). Previous studies have shown that fluoxetine may induce slight weight recovery, yet its effects are less pronounced when compared to apigenin. For instance, while fluoxetine may enhance certain functional outcomes in other contexts, such as neuropsychological recovery post-stroke, the specific benefits related to weight normalization in stressed subjects have not been conclusively established (Dennis *et al.*, 2019; Dennis *et al.*, 2020).

Chronic Stress has been observed to have minimal effects on sperm morphology while slightly increasing sperm concentration (Oguejiofor *et al.*, 2022). This finding suggests that, while stressors may impact sperm characteristics, the overall morphology remains relatively stable, and in some instances, parameters like concentration can even improve. This might be attributed to complex physiological adaptations or compensatory mechanisms in response to stress, potentially leading to increased production of sperm in certain conditions (Oguejiofor *et al.*, 2022). Conversely, the effects of *T. tetraptera*, apigenin, and fluoxetine on sperm quality have been far less favorable. Study has indicated that all these treatments significantly reduced normal sperm morphology and sperm concentration. *T. tetraptera*, in particular, has shown a pronounced negative impact on both morphological integrity and the number of viable sperm. Studies have illustrated that *T. tetraptera* can adversely affect cellular functions by inducing oxidative stress and cellular damage, thereby impairing sperm health (Lin *et al.*, 2019; Koukoui *et al.*, 2023). The negative effects observed with *T. tetraptera* treatment may be linked to its intrinsic properties, which include potential cytotoxicity against reproductive cells (Oguntimehin *et al.*, 2021; Koukoui *et al.*, 2023). Fluoxetine and apigenin, both of which are used for their various therapeutic benefits, have similarly demonstrated detrimental effects on sperm parameters. Fluoxetine, despite being an SSRI with potential mood-improving effects, has been associated with reduced sperm quality, characterized by lowered concentrations and increased morphological anomalies (Boitrelle *et al*., 2012; Nakata *et al*., 2015). Apigenin, although often valued for its antioxidant properties, has also been reported to negatively impact sperm characteristics, leading to poor morphology and reduced concentrations (Adeoye-Isijola *et al*., 2022; Koukoui *et al*., 2023).

On the histological examination of testicular tissue, the control group displayed normal seminiferous tubules lined by organized germinal epithelium, indicative of healthy spermatogenesis and sperm morphology. In contrast, groups exposed to chronic stress without treatment or treated with fluoxetine presented significant degeneration in their seminiferous tubule architecture, including disordered epithelium, loss of cell stratification, and increased interstitial fibrosis, signs suggesting impaired testicular function and potential sperm abnormality (Choowong-In *et al*., 2021). The group administered with 50 mg/kg body weight of ethyl acetate fraction from *T. tetraptera* fruit extract exhibited seminiferous tubules resembling those of the control group, suggesting a protective or restorative effect against the detrimental impacts of stress on reproductive tissues. Studies indicated that *T. tetraptera* is rich in flavonoids, which may exert antioxidant properties, thereby mitigating oxidative stress-induced damage to the testicular cells and preserving spermatogenic functionality (Adienbo and Ezeala, 2021; Okechukwu *et al*., 2022; Koukoui *et al*., 2023). Furthermore, the presence of beneficial compounds within the extract may lead to an improvement in reproductive parameters, underscoring the health benefits associated with *T. tetraptera* fruits (Alaribe *et al*., 2020; Adienbo and Ezeala, 2021). Conversely, the adverse effects observed in the groups treated solely with fluoxetine or subjected to stress without any protective adjunct underline the neuroendocrine impact of chronic stress on male reproductive health and the potential side effects of SSRIs. The identified histological changes, such as irregular cell arrangements and pale-staining cytoplasm associated with increased nuclear detail loss, can be attributed to fluoxetine's known effects on reproductive hormones and spermatogenesis (Choowong-In *et al*., 2021; Adienbo and Ezeala, 2021

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