Drug Discovery

To Cite:
Bassey EI, Davies OB, Malachy AA, Ekong MB, Essien IG. Effects of *Tetrapleura tetraptera* fruit ethyl acetate fraction, apigenin and fluoxetine on the testes of Swiss mice exposed to stress. *Drug Discovery* 2026; 20: e9dd3059
doi: https://doi.org/10.54905/disssi.v20i45.e9dd3059

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Peer-Review History
Received: 21 July 2025
Reviewed & Revised: 16/August/2025 to 27/February/2026
Accepted: 07 March 2026
Published: 21 March 2026

Peer-Review Model
External peer-review was done through double-blind method.

Drug Discovery
pISSN 2278–540X; eISSN 2278–5396

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Effects of *Tetrapleura tetraptera* fruit ethyl acetate fraction, apigenin and fluoxetine on the testes of Swiss mice exposed to stress

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ABSTRACT

Stress significantly influences changes in numerous physiological processes and may contribute to various disorders, including male sub-fertility or infertility. This study aimed at assessing the effects of *Tetrapleura tetraptera* (TT) fruit ethyl acetate fraction, apigenin and fluoxetine on the inflammatory changes in the testes using caspase-3 stains, testicular hormones (testosterone, follicle stimulating hormone, luteinizing hormone) and testes homogenate of malondialdehyde, catalase, and superoxide dismutase following chronic unpredictable mild stress. Twenty five male Swiss mice (20-30g) were divided into five groups (1-5, n=5) for this study: Group 1 served as the control and received 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 had exposure to different specific stressors, administrations given orally, and the experiment lasted for 28 days. The testes were processed and stained with Hematoxylin and Eosin. The results showed that stress led to significant ($p<0.05$) weight loss, while the TT extract resulted in severe 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 testes

1. INTRODUCTION

Exposure of the body to sustained internal or external stressors induce a physiological or psychological response seen as chronic stress. Many chronic stressors exist, but most of them are encountered on a daily basis, affecting multiple systems in the body. These stressors are associated with death or long-lasting effects on an individual's life (Epel *et al*., 2018).

Oxidative stress occurs when free radicals and reactive oxygen species (ROS) damage vital macromolecules in the body. Many tissues in the body suffer from the harm of oxidative stress. The central nervous system is very vulnerable to oxidative stress damage because of it requires high energy, and is rich in lipid and protein

(Sharifi-Rad *et al*., 2021, Malachy *et al*., 2025). Oxidative stress damage is greatly involved in the development of several diseases like diabetes, cancer, cardiovascular problems, rheumatoid arthritis, and neurological disorders.

ROS-induced oxidative stress also contributes to tissue inflammation. For instance, in the Leydig cells, as in many other cell types, ROS are continuously produced during normal aerobic metabolism through the mitochondrial electron transport chain. Also, during steroid hormone synthesis, particularly during steroid hydroxylation reactions mediated by cytochrome P450 enzymes, more ROS are produced (Wang *et al*., 2012).

Some medicinal plants have been used in managing stress-related disorders. *Tetrapleura tetraptera* (Aidan) in West Africa has been known for its traditional medicinal use. The fruits of *Tetrapleura tetraptera* have many bioactive compounds, like tannins, flavonoids, phenolic compounds, and saponins, which are known for their antioxidant, anti-inflammatory, and neuroprotective properties (Adusei *et al*., 2019). It has been reported by many researchers that *Tetrapleura tetraptera* extracts have high activities against oxidative stress. Agbai *et al*. (2019) and Johnlouis (2022) noted that extracts from the plant can reduce the harmful effect of reactive oxygen species (ROS) by enhancing the activities of endogenous antioxidant enzymes. It has been earlier documented that *Tetrapleura tetraptera* is potent in the treatment of various health conditions, such as inflammation, infections, and diabetes. The antioxidant properties of *Tetrapleura tetraptera* can reduce oxidative stress, a significant factor in stress-induced damage to cells and tissues (Aderibigbe *et al*., 2011; Agbai *et al*., 2019).

Several effects of apigenin in many body systems of mammals in vitro as well as in vivo are traceable to its antioxidant and antigenotoxic effects and its role in scavenging free radicals. Apigenin helps in cancer prevention through induction of apoptosis in various cell lines and animal models (Kaur *et al*., 2008). Also, apigenin is known to reverse the adverse effects of cyclosporine. Research has been conducted to study the effects of apigenin on the reversal of cyclosporine-induced damage and was assessed by immuno-histochemical estimation of Bcl-2, and estimation of apoptosis in histological sections (Chakravarthi *et al*., 2009).

Fluoxetine, which belongs to the family of selective serotonin reuptake inhibitor (SSRI), is widely used in the treatment of various psychiatric disorders, like major depressive disorder (MDD) and anxiety disorders. Over the years, fluoxetine has gained significant attention due to its efficacy, safety profile, and unique pharmacological properties (Patel *et al*., 2018). It reduces corticosterone level, and modulates neurotransmitter concentrations in limbic areas (Estévez-Cabrera *et al*., 2023).

2. MATERIALS AND METHODS

Materials

The fruits of *Tetrapleura tetraptera* were obtained and authenticated at the herbarium of the Department of Botany and ecological study, University of Uyo. Apigenin and Fluoxetine were purchased from a pharmacy store in Uyo, Akwa Ibom state, Nigeria.

Extract Preparation and Partitioning

The pods of *Tetrapleura tetraptera* were dried and ground into fine powder with a grinder. Methanol extraction was then carried out on the *Tetrapleura tetraptera* powder. Crude extract of *Tetrapleura tetraptera* was then dissolved in distilled water, poured into a separation funnel and passed through N-Ethyl acetate solvent to yield N-Ethyl acetic fraction. Air drying of the N-Ethyl acetic fraction was done under room temperature for 72 hours to yield a dried compound of the fraction.

Animals Care and Handling

Twenty-five male Swiss mice weighing between 20g and 30g were used for this experiment. The mice were divided into five groups of five animals each and housed in well-ventilated standard animal cages. The animals had unrestricted access to standard chow pellets and water *ad libitum* at room temperature of 25-28 ℃ and 12:12 light/dark cycle with. The control group was Group 1, while Groups 2-5 served as the test groups.

Chronic mild stress Protocol

Chronic mild stress was induced according to the method described by Szala-Rycaj *et al*. (2023) with modifications (Table 1).

Experimental Design

This study lasted for 28 days. Stress alone was induced in the first 14 days and stress induction with treatment was done for the next 14 days. The mice were shared into five (5) groups of five (5) animals each (n = 5). Group 1 served as the control group, which received

distilled water; Group 2 had exposure to stress without treatment; Group 3 was exposed to stress and received 50 mg/kg body weight of ethyl acetate fraction of *T. tetraptera*; Group 4 was exposed to stress and received 50 mg/kg body weight of Apigenin and; Group 5 was exposed to stress and received 50 mg/kg body weight of Fluoxetine (Table 2).

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)

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

Groups (n = 5)	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

The experiment lasted for 28 days. On the 29th day, the animals were anaesthetized through injection of 50 mg/kg of ketamine intraperitoneally. Blood sample was collected by cardiac puncture into a plain bottle for biochemical analyses. The serum was separated from the blood cells by leaving the blood sample undisturbed for 15 minutes and centrifuged for 20 minutes at 3500 rpm, then kept in plastic vials and immediately stored for biochemical studies. The testes were harvested and stored in an ice pack. Homogenates were obtained from the testes and used for oxidative stress markers analysis.

3. RESULTS

Effects of Stress, Ethyl-acetate Fraction of *T. Tetraptera* Fruit, Apigenin, and Fluoxetine on Reproductive Hormones

Control group showed a testosterone level that is significantly higher when compared to other treatment groups (group exposed to 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 apigenin, and group exposed to stress and 50 mg/kg body weight of fluoxetine) respectively at $p<0.05$. Group 2,

exposed to stress and water showed a decrease in testosterone level that is lower when compared to group exposed to stress and treated with 50 mg/kg body weight of ethyl acetate fraction of *T. tetraptera* fruit extract, exposed to stress and 50 mg/kg body weight of apigenin, and group exposed to stress and 50 mg/kg body weight of fluoxetine.

For luteinizing hormone, the group exposed to stress and treated with 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract showed a significant increase ($p<0.05$) in LH level when compared to all other groups. Meanwhile, the group exposed to stress and treated with 50 mg/kg body weight of apigenin, and the group exposed to stress and treated with 50 mg/kg body weight of fluoxetine showed a significant decrease ($p<0.05$) in LH when compared to the control group and the group exposed to stress and water, respectively.

Also, a significant decrease ($p<0.05$) was noted in FSH levels in groups exposed to stress and water, stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract and group 5 exposed to stress and 50 mg/kg body weight of fluoxetine when compared to control group and exposed to stress and 50 mg/kg body weight of apigenin respectively (Table 3).

Table 3. Effects of stress, ethyl-acetate fraction of *T. tetraptera* fruit, apigenin and, fluoxetine on testicular hormones

Groups	Testosterone (ng/mL)	LH (mIU/mL)	FSH (mIU/mL)
1	1.95±0.494	25.20±0.37	3.40±0.25
2	0.10±0.002*a	25.00±0.42	2.06±0.02*a
3	0.56±0.023*a	27.60±0.66*ab	2.56±0.23*a
4	0.51±0.203*a	16.08±0.24*abc	3.44±0.48*bc
5	0.59±0.173*a	14.80±0.37*abc	1.90±0.13*a
	P<0.05	P<0.05	P<0.05
	F=8.297	F=180.9	F=7.789

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.

Table 4. Effects of stress, ethyl-acetate fraction of *T. tetraptera* fruit, apigenin, and fluoxetine on testicular oxidative stress markers

GROUPS	MDA (μM/g)	SOD (μM/mg)	CAT (u/mg)
Control group	4.79±0.15	1.10±0.05	2.22±0.12
Stress + water	4.93±0.15	1.12±0.06	3.06±0.01*a
Stress + ethyl acetate of *T. tetraptera*	4.53±0.03	1.14±0.09	3.25±0.21*a
Stress + apigenin	3.69±0.11*abc	1.08±0.05	3.07±0.01*a
Stress + fluoxetine	3.08±0.29*abc	1.12±0.07	2.45±0.21*bdc
	P<0.0001	P=0.9740	P=0.0002
	F=22.36	F=0.1193	F=9.688

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 stress, Ethyl-acetate Fraction of *T. Tetraptera* Fruit, Apigenin, and Fluoxetine on Oxidative Stress Markers

There was significant reduction ($p<0.05$) in malondialdehyde level in group exposed to stress and 50 mg/kg body weight of apigenin and group exposed to stress and 50 mg/kg body weight of fluoxetine when compared to groups 1, 2 and 3 (control group, group exposed to stress and water, and group exposed to stress and treated with 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract) respectively.

Results from this study showed that there were no significant differences in superoxide dismutase levels across all groups (group exposed to stress and water, group exposed to stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract, group exposed to stress and 50 mg/kg body weight of apigenin and the group exposed to stress and 50 mg/kg body weight of fluoxetine) compared to the control group.

The results showed that group exposed to stress and water, the group exposed to stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit and exposed to stress and 50 mg/kg body weight of apigenin indicated significant increase ($p<0.05$) in CAT levels compared to control group. In addition, the group exposed to stress and 50 mg/kg body weight of fluoxetine showed a significant decrease ($p<0.05$) in CAT level compared to the group exposed to stress and water, the group exposed to stress and 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract and the group exposed to stress and 50 mg/kg body weight of apigenin (Table 4).

Immunoreactivity Findings on Testes Using Caspase-3 Staining

Findings of testicular tissue of control group immunostained for caspase-3 showed no caspase-3 expression of apoptosis, while group 2 exposed to stress and water, group 3 exposed to stress and administered 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract, and group 5 exposed to stress and administered with 50 mg/kg body weight of fluoxetine revealed severe caspase expression of apoptosis respectively, and group 4 exposed to stress and administered with 50 mg/kg body weight of apigenin showed moderate expression of apoptosis (Figure 1).

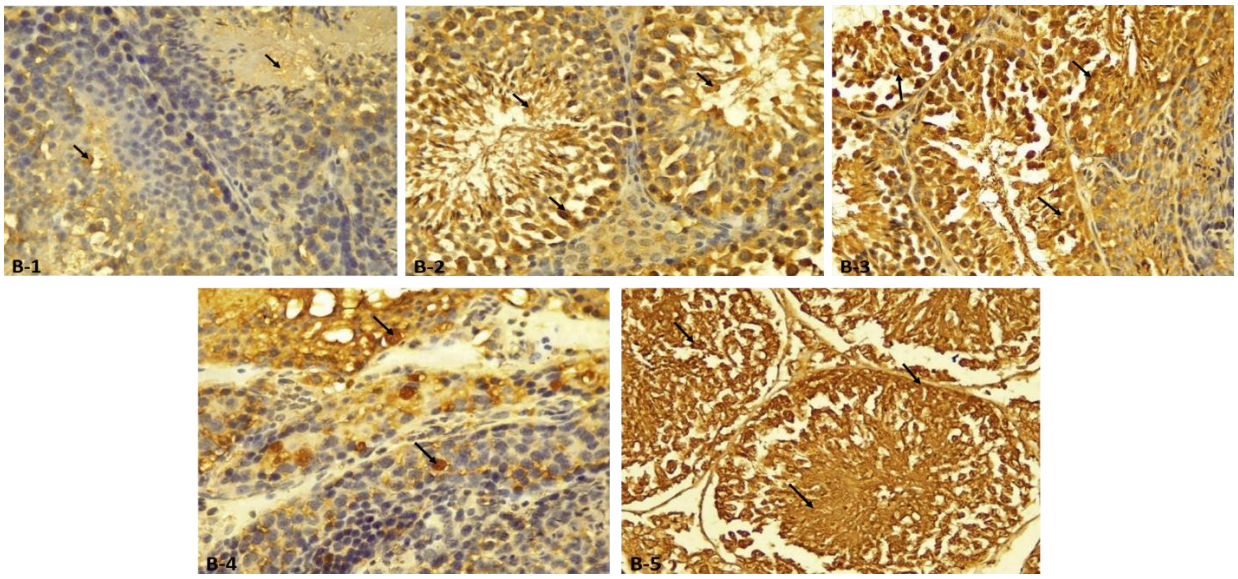


Figure 1. Photomicrographs of the cross-sections of testicular tissue of the control group (B-1) and experimental groups (B-2 to B-5).

B-1: Photomicrograph of the cross-section of testicular tissue of the control group, immuno-stained for caspase-3. It depicts seminiferous tubules with negative caspase-3 immunoreactivity (**black arrow**). The surrounding stromal tissue showed negative caspase-3 expression. Caspase stain, x400 magnification. **Inference: No expression**

B-2: Photomicrograph of the cross-section of testicular tissue exposed to stress and water showing seminiferous tubules with widespread expression of cleaved caspase-3 (**black arrow**). Brown cytoplasmic staining indicating caspase activation, predominantly in germ cells within the seminiferous epithelium. Caspase stain, x400 magnification. **Inference: Severe expression**

B-3: Photomicrograph of the cross-section of testicular tissue exposed to stress and administered with 50 mg/kg body weight ethyl acetate fraction of *T. tetraptera* fruit extract, immune-stained for caspase-3. It demonstrates strong caspase expression in the seminiferous tubules, as indicated by intense brown cytoplasmic and nuclear staining (**black arrow**) of the germinal epithelium. Caspase stain, x400 magnification. **Inference: Severe expression.**

B-4: Photomicrograph of the cross-section of testicular tissue exposed to stress and administered 50 mg/kg body weight of Apigenin, showing prominent nuclear caspase expression with intense dark brown staining (**black arrow**) localized within the nuclei of apoptotic germ cells. Caspase stain, x400 magnification. **Inference: Moderate expression**

B-5: Photomicrographs of the cross-sections of testicular tissue exposed to stress and administered 50mg/kg body weight of Fluoxetine, showing diffuse caspase expression (Immunohistochemistry). It reveals widespread and intense caspase expression within the seminiferous tubules, as evidenced by the strong brown cytoplasmic staining (**black arrow**). Caspase stain, x400 magnification. **Inference: Severe expression**

4. DISCUSSION

The present study investigated the effects of *Tetrapleura tetraptera* fruit extract, apigenin, and fluoxetine on the expression of apoptosis using caspase-3 stain in testicular tissues across various treatment groups. The control group, exhibited minimal caspase-3 expression, which suggested normal regulation of apoptosis, indicative of healthy testicular tissue. This minimal expression of caspase suggests that cellular turnover is appropriately managed, and that spermatogenesis is not adversely affected (Al-Yasi *et al*., 2021). In contrast, the other groups subjected to chronic stress, and those treated with the ethyl acetate fraction of *T. tetraptera* fruit extract, apigenin, and fluoxetine, all demonstrated a significant increase in caspase-3 expression. This suggests pronounced apoptotic activity triggered by stress and drug exposure (Omar *et al*., 2021; Abdelmonem *et al*., 2024). The high staining in caspase-3 immunostaining seen in the groups exposed to stress points to a marked elevation in germ cell apoptosis, which causes serious implications for spermatogenesis and ultimately male fertility. Caspase-3 is a key executioner of apoptosis, involved in the cleavage of various cellular substrates leading to the characteristic morphological and biochemical changes which are associated with programmed cell death (Aydos *et al*., 2014). The observed upregulation in caspase-3 expression due to chronic stress is in line with previous findings showing that stress as a very good inducer of oxidative stress and apoptosis within germ cells. This is seen in studies that have linked stress-induced apoptosis to alterations in cellular integrity and hormone regulation (Elgawish and Abdelrazek, 2014; Elsawy *et al*., 2024). From the findings from this study, while the ethyl acetate fraction of *T. tetraptera* and apigenin, administered to stressed groups reduced the impact of stress on apoptosis, potentially through direct antioxidant effects or by modulating apoptotic pathways, fluoxetine was seen to increase apoptotic signaling. Generally, Fluoxetine increases reactive oxygen species (ROS) levels and supports caspase-dependent pathways that ultimatelyresults in sperm apoptosis (Omar *et al*., 2017). Thus, the contrasting effects of *T. tetraptera* and fluoxetine on caspase-3 expression shows the potential of natural extracts to protect against stress-related reproductive dysfunction, widely because used SSRIs may pose risks to male reproductive health due to their effects on apoptotic mechanisms. The findings agrees with older studies showing that external stressors, such as toxins and psychological stress, can cause apoptosis through pathways involving caspase-3 activation, ultimately leading to detrimental outcomes on spermatogenesis (Madekurozwa, 2013; Elgawish and Abdelrazek, 2014).

The observed reduction in LH levels following Apigenin administration may likely be due to a feedback mechanism linked to the enhanced levels of estrogen and progesterone or a direct effect on the hypothalamus and pituitary gland (Baker *et al*., 2016). Fluoxetine also provides a significant impact on reproductive hormones. Fluoxetine demonstrated a pronounced suppression of FSH even under Chronic stress. The reduced levels of FSH noted in the stress + Fluoxetine administration suggest possible reproductive suppression. FSH is needed for the development of ovarian follicle and the production of estrogen. Alterations in the neurotransmitter is likely the mechanism through which Fluoxetine suppresses FSH levels that affect pituitary function and disrupt the delicate balance of the HPG axis (Harlow and Wysowski, 2002).

The study highlighted the antioxidant effects of Fluoxetine, Apigenin, and *T. tetraptera* extract in mitigating oxidative stress under conditions of chronic stress. Fluoxetine demonstrated significant antioxidant activity, as evidenced by its ability to reduce the level of malondialdehyde (MDA), which is a biomarker associated with oxidative stress and inflammation. Moreover, Fluoxetine was able to bring the catalase (CAT) level closer to normal, indicating that it effectively improves the antioxidant defense system in the body (Kashyap *et al*., 2021; Al-Amarat *et al*., 2022). The role of Fluoxetine as an antioxidant is supported by evidence that it can modulate oxidative stress and enhance endogenous antioxidant mechanisms (He *et al*., 2015). Similarly, apigenin exhibited antioxidant properties by reducing MDA levels, suggesting a potential imbalance in the antioxidant defense mechanism showing the complexity of apigenin's effects on oxidative stress (Saima *et al*., 2023; Sławińska *et al*., 2023). A study indicated that apigenin interacts with antioxidant enzymes, which may enhance its protective capabilities. However, the specific effects of these interactions in vivo require further clarification (Saima *et al*., 2023). This dual nature underscores the necessity for a nuanced understanding of phytochemical interactions with cellular mechanisms. An examination of the efficacy of *T. tetraptera* indicated that the extract does not effectively reduce the level of MDA but it significantly increases CAT level. These findings imply that *T. tetraptera* may have protective properties against oxidative stress induced by chronic stress (Oguntimehin *et al*., 2021). Although earlier studies have suggested that the high phenolic content of *T. tetraptera* contributes to its antioxidant activity (Adusei *et al*., 2019; Kadiri *et al*., 2020), the specific results of the current investigation indicate that its effects may be beneficial in combating oxidative damage and could potentially exacerbate oxidative stress.

5. CONCLUSION

In this study, apigenin appeared to be the most effective treatment for restoring hormonal balance, whereas fluoxetine had mixed effects, and *T. tetraptera* had weaker reproductive benefits. Fluoxetine is the most effective at reducing oxidative stress, normalizing antioxidant enzyme levels and lowering oxidative markers. Apigenin has moderate antioxidant effects. The *T. tetraptera* extract does not appear beneficial for oxidative stress reduction and might even exacerbate certain stress markers. Also, apigenin and *T. tetraptera* extract were able to reduce apoptosis in the testicular tissue while fluoxetine exacerbated the apoptotic process. Thus some SSRIs like fluoxetine may adversely affect male reproductive health due to their effects on the mechanisms of apoptosis.

Acknowledgements

We thank the authors who contributed to the conduct of this study.

Author Contributions:

Moses B. Ekong: Designed the research and supervised the research work.
Eno-Obong I. Bassey: Proposed the idea and supervised the experiment
Osayuwame B. Davies: Carried out the research and analyzed the data obtained
Akpanabasi Alexis Malachy: Reported the research and developed the manuscript.
Iniobong G. Essien: Collected the data and drafted the manuscript.
All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Ethical Approval

In this article, the animal regulations are followed as per the ethical committee guidelines of Faculty of Basic Medical Sciences, University of Uyo, Uyo, Akwa Ibom State, Nigeria; the authors observed the effects of *Tetrapleura tetraptera* fruit ethyl acetate fraction, apigenin and fluoxetine on the testes of Swiss mice exposed to stress. The Animal ethical guidelines are followed in the study for observation, identification & experimentation.

Informed Consent

Not applicable.

Conflicts of interests

The authors declare that they have no conflicts of interest, competing financial interests or personal relationships that could have influenced the work reported in this paper.

Funding

This research did not receive any external funding like specific grant from funding agencies in the public, commercial, or nonprofit sectors.

Data and materials availability

All data associated with this study will be available based on the reasonable request to corresponding author.

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