

Amaranthus viridis Ameliorates Oxidative Stress and Modulates TNF- β in Tramadol-Induced Testicular Toxicity

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History

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ABSTRACT

Objective: Tramadol, a synthetic opioid analgesic, is associated with reproductive toxicity and testicular oxidative damage following prolonged exposure. Amaranthus viridis (green amaranth), a medicinally valued tropical vegetable, possesses antioxidant and anti-inflammatory properties that may offer protective effects. This study investigated the protective potential of Amaranthus viridis leaf extract against tramadol-induced testicular toxicity in adult male Wistar rats, focusing on oxidative stress biomarkers, reproductive hormones, and inflammatory cytokines.

Methods: Twenty-five adult male Wistar rats were randomly assigned into five groups (n=5). Group 1 received distilled water (control); Group 2 received 200 mg/kg tramadol (toxic control); Groups 3, 4, and 5 were administered combined doses of tramadol (150, 100, and 50 mg/kg, respectively) and Amaranthus viridis extract (100, 200, and 300 mg/kg, respectively) for 21 days via oral gavage. Testicular tissues and serum samples were evaluated for histology, oxidative stress markers (SOD, GPx, MDA), reproductive hormones (LH, FSH, testosterone), and inflammatory cytokines (IL-1, IL-6, TNF- α , TNF- β).

Results: Tramadol administration significantly disrupted testicular histoarchitecture and caused a marked reduction in LH, FSH, testosterone, SOD, and GPx levels, along with elevated MDA and TNF- β levels (p<0.05). Co-treatment with A. viridis extract, especially at 300 mg/kg, significantly restored LH, FSH, testosterone, SOD, and GPx levels and suppressed TNF- β expression, although MDA levels remained elevated. Histologically, A. viridis treatment attenuated germ cell necrosis and improved seminiferous tubule integrity.

Conclusion: A. viridis extract demonstrated a dose-dependent protective effect against tramadol-induced testicular toxicity by enhancing antioxidant defenses, restoring hormonal balance, and suppressing proinflammatory cytokines—highlighting its therapeutic potential in male reproductive health.

Keywords: Tramadol, Amaranthus viridis, Testicular toxicity, Oxidative stress, Reproductive hormones, TNF-B

Amaranthus viridis Tramadol Kaynaklı Testis Toksisitesinde Oksidatif Stresi Azaltır ve TNF- β 'yi Düzenler

Araştırma Makalesi

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Öz

Amaç: Sentetik bir opioid analjezik olan tramadol, uzun süreli maruziyet sonrasında üreme toksisitesi ve testiküler oksidatif hasarla ilişkilidir. Tıbbi değeri yüksek tropikal bir sebze olan Amaranthus viridis (yeşil amarant), antioksidan ve antiinflamatuar özelliklere sahip olup koruyucu etkiler sunabilir. Bu çalışma, yetişkin erkek Wistar sıçanlarında tramadolün neden olduğu testiküler toksisiteye karşı Amaranthus viridis yaprak özütünün koruyucu potansiyelini; oksidatif stres biyobelirteçleri, üreme hormonları ve inflamatuar sitokinler üzerine odaklanarak araştırmıştır.

Yöntem: Yirmi beş yetişkin erkek Wistar sıçanı rastgele beş gruba (n=5) ayrılmıştır. Grup 1 distile su (kontrol), Grup 2 200 mg/kg tramadol (toksik kontrol), Gruplar 3, 4 ve 5 sırasıyla tramadol (150, 100 ve 50 mg/kg) ile Amaranthus viridis özütü (100, 200 ve 300 mg/kg) kombinasyonlarını 21 gün boyunca oral gavaj yoluyla almıştır. Testis dokuları ve serum örnekleri histoloji, oksidatif stres belirteçleri (SOD, GPx, MDA), üreme hormonları (LH, FSH, testosteron) ve inflamatuar sitokinler (IL-1, IL-6, TNF- α , TNF- β) açısından değerlendirilmiştir.

Bulgular: Tramadol uygulaması testis histo-mimarisini önemli ölçüde bozmuş; LH, FSH, testosteron, SOD ve GPx düzeylerinde belirgin azalmaya, MDA ve TNF- β düzeylerinde ise artışa neden olmuştur (p<0.05). Özellikle 300 mg/kg dozda A. viridis özütü ile birlikte tedavi, LH, FSH, testosteron, SOD ve GPx düzeylerini anlamlı biçimde geri kazandırmış ve TNF- β ekspresyonunu baskılamıştır; ancak MDA düzeyleri yüksek kalmaya devam etmiştir. Histolojik olarak A. viridis tedavisi, germ hücreleri nekrozunu azaltmış ve seminifer tübül bütünlüğünü iyileştirmiştir.

Sonuç: A. viridis özütü, tramadol kaynaklı testiküler toksisiteye karşı antioksidan savunmayı güçlendirerek, hormonal dengeyi yeniden sağlayarak ve proinflamatuar sitokinleri baskılayarak doz-bağımlı bir koruyucu etki göstermiştir. Bu bulgular, A. viridis'in erkek üreme sağlığında terapötik potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: Tramadol, Amaranthus viridis, Testiküler toksisite, Oksidatif stres, Üreme hormonları, TNF- β .

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Introduction

The growing reliance on opioid analgesics such as tramadol poses a significant public health concern worldwide, including in Nigeria. Tramadol is a centrally acting synthetic opioid used to manage moderate to severe pain through dual mechanisms: μ -opioid receptor agonism and inhibition of norepinephrine and serotonin reuptake, making it effective in treating postoperative, musculoskeletal, and neuropathic pain.¹ However, its misuse, particularly among young populations in sub-Saharan Africa, has been linked to various adverse health outcomes.²

Chronic tramadol administration has been associated with oxidative stress, marked by elevated reactive oxygen species (ROS) production, leading to tissue damage, especially in the testes.³ It disrupts antioxidant enzyme activity (SOD, CAT, GPx) while increasing malondialdehyde (MDA) levels, a marker of lipid peroxidation.^{4,5} This oxidative damage compromises cell membrane integrity, promotes apoptosis, and impairs spermatogenesis and testosterone synthesis.⁶ Furthermore, tramadol suppresses the hypothalamic-pituitary-gonadal axis, resulting in reduced gonadotropins and testosterone levels⁷, and induces testicular structural changes such as thinning of the germinal epithelium, vacuolization, and sloughing of spermatogenic cells.⁸ Functional impairments in Sertoli and Leydig cells, as well as testicular inflammation and fibrosis, have also been documented.^{3,4}

Amaranthus viridis (green amaranth) is a widely consumed leafy vegetable with notable medicinal properties due to its rich content of flavonoids, phenolic acids, saponins, and antioxidants.⁹ These phytochemicals provide antioxidative and anti-inflammatory effects, which are essential for mitigating tramadol-induced testicular toxicity. Al-Mamun *et al.*¹⁰ and Akowuah *et al.*¹¹ reported that *A. viridis* extracts reduce oxidative biomarkers like MDA while enhancing antioxidant enzymes such as SOD and CAT. Experimental studies have also demonstrated its protective effects on testicular tissue: Adedara *et al.*¹² found that *A. viridis* preserved testicular architecture and reduced apoptosis in cadmium-exposed rats, while Oyewole and Oyeleke¹³ observed improved sperm count and motility in rats under oxidative stress. These findings support the therapeutic potential of *A. viridis* in addressing male reproductive dysfunction.

Methods

Ethical approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, Rev. Fr. Moses Orshio Adasu University, Makurdi, Benue State-Nigeria. The study was conducted in accordance with internationally recognized guidelines for the ethical use of animals in scientific research.

A total of twenty-five (25) healthy adult male Wistar rats, weighing between 160–220 g, were procured and housed in the Animal Facility of the College of Health Sciences, Benue State University, Makurdi. The animals were randomly allocated into five groups (n = 5 per group) and provided with

standard rat pellets (Vital Feed) and distilled water ad libitum throughout the experimental period. The rats were maintained under standard laboratory conditions: a controlled room temperature of $25 \pm 1^\circ\text{C}$, a 12-hour light/dark cycle, and adequate ventilation. The animals were allowed to acclimatize to the laboratory environment for two weeks prior to the commencement of experimental treatments. Hygiene was ensured by daily removal of waste and replacement of food and water.

Drug and Plant Preparation

Pharmaceutical-grade tramadol tablets were purchased from Rovi Pharmaceuticals, Makurdi. A stock solution was prepared by dissolving 1000 mg of tramadol in 250 ml of distilled water, and the solution was administered orally to the animals via gavage.

Fresh *Amaranthus viridis* leaves were harvested from a private garden located in the Genabe district of Makurdi, Benue State, and authenticated by the Department of Biological Sciences, Benue State University, Makurdi. The harvested leaves were cleaned by rinsing thoroughly with tap water, followed by deionized distilled water. Roots and non-leafy parts were discarded. The cleaned leaves were chopped into smaller pieces and air-dried at ambient temperature for two weeks. The dried leaves were subsequently ground into a fine powder, yielding 200 g of powdered material.

The powdered *A. viridis* was macerated in 2 liters of distilled water in a glass container and stirred at two-hour intervals for 24 hours. The resulting mixture was first filtered using muslin cloth and then passed through Whatman No. 1 filter paper to obtain a clear aqueous extract. The filtrate was concentrated using a rotary evaporator at 40°C . Further concentration was achieved using a vacuum oven set at 45°C and a pressure of 700 mmHg. The resulting concentrated extract was weighed, and the percentage yield was calculated. The extract was stored in a sterile, airtight container at 4°C until required for administration.

Experimental Design and Tissue Processing

The animals were divided into five groups and treated as follows for a duration of 21 days: Group 1 (Control): Received distilled water and standard rat feed (Vital Feed) only. Group 2 (Negative Control): Received 200 mg/kg body weight of tramadol administered orally once daily. Group 3: Received 150 mg/kg body weight of tramadol combined with 100 mg/kg body weight of *Amaranthus viridis* extract, administered orally once daily. Group 4: Received 100 mg/kg body weight of tramadol and 200 mg/kg body weight of *A. viridis* extract, administered orally once daily. Group 5: Received 50 mg/kg body weight of tramadol and 300 mg/kg body weight of *A. viridis* extract, administered orally once daily. All administrations were conducted via the oral route using a calibrated gavage.

At the end of the 21-day treatment period, the animals were euthanized by intramuscular injection of ketamine hydrochloride at a dose of 20 mg/kg body weight. The testes were carefully excised, cleared of adherent fat, and weighed using a precision analytical balance. The tissues were then fixed in Bouin's solution and processed for histological

analysis. Routine hematoxylin and eosin (H&E) staining was performed for light microscopic evaluation, and Alcian Blue staining was employed for immunohistochemical assessment of acidic mucopolysaccharides and glycoproteins.

Biochemical Evaluation

Quantification of reproductive hormones, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) was achieved via competitive enzyme immunoassay technique utilizing a polyclonal anti-LH, anti-FSH, and anti-T. These were done with ELISA at 450nm using the microplate readers, according to manufacturer's specifications. Briefly, the blood that was collected into plain containers was allowed to clot. Each sample was centrifuged at 1000 rpm for 10 min to achieve separation. The serum obtained was put into aliquots in each case, labeled, and stored at $-200C$). One aliquot of each specimen was taken at a time, to avoid repeated freezing and thawing, and the samples were analyzed for hormone estimation using enzyme immunoassay (EIA), according to the World Health Organization (WHO) matched reagent program protocol (manual) for EIA kits (protocol/ version of December 1998 for LH, FSH). The kits were supplied by NIADDK – NIH (USA).

Testosterone concentrations in plasma were determined by the enzyme immunoassay technique based on the principle of competitive binding between TT and TT-horseradish peroxidase conjugate for a constant amount of rabbit anti-TT, as previously described.¹⁴

Cytokines and hormones were measured using commercial ELISA kits (Manufacturer, Country; Catalog No.). Detection ranges were approximately IL 1 β : 31.25–2000 pg/mL, IL 6: 12.50–800 pg/mL, TNF α : 15.63–1000 pg/mL, TNF β : 15.6–1000 pg/mL; FSH: 0.625–40 mIU/mL, LH: 1–50 mIU/mL, Testosterone: 0.313–20 ng/mL, with detection limits as specified by the manufacturers. Cytokines were measured in serum, and hormones in serum. Sperm motility was assessed using a manual microscopic method.

Result

Body Weight

The result showed that Group 4 (100mg/kg Tramadol + 200mg/kg Amaranth) had the greatest body weight change (123.50 \pm 8.76 g) while Group 1 (normal saline) had the least body weight change (81.12 \pm 1.21 g). When compared, it was shown that Group 2 (200mg/kg Tramadol), Group 4 (100mg/kg Tramadol + 200mg/kg Amaranth) and Group 5 (50mg/kg Tramadol + 300mg/kg Amaranth) all showed a significant increase ($p < 0.05$) in body weight change when compared to Group 1 whereas, Group 3 (150mg/kg Tramadol + 100mg/kg Amaranth) showed no significant difference at $p < 0.05$. When compared to Group 2, Group 1 (normal saline), Group 3 (150mg/kg Tramadol + 100mg/kg Amaranth) and Group 5 (50mg/kg Tramadol + 300mg/kg Amaranth) all showed a significant decrease ($p < 0.05$) in mean body weight change whereas, Group 4 showed a significant increase in mean body weight change when compared to Group 2.

Table 1. Showing the Mean Body Weight Changes and Standard Deviation (SD) across Groups on the effect of *A. viridis* on tramadol-induced testicular toxicity in adult male Wistar rats

GROUPS (N)	INITIAL BODY WEIGHT (g)	FINAL BODY WEIGHT (g)	BODY WEIGHT DIFFERENCE (g)
GROUP 1	87.40 \pm 10.30	168.52 \pm 29.60	81.12 \pm 1.21 +
GROUP 2	94.92 \pm 7.44	190.68 \pm 14.07	95.76 \pm 6.81*
GROUP 3	90.18 \pm 16.16	169.82 \pm 46.63	79.64 \pm 34.29+
GROUP 4	93.55 \pm 7.70	217.05 \pm 1.06	123.50 \pm 8.76*+
GROUP 5	81.22 \pm 12.73	170.32 \pm 38.88	89.103 \pm 2.17*+

Values are expressed as MEAN \pm SD; N = 5; NS = Normal Saline; NC = Negative Control; TDL = Tramadol; AEA = Aqueous Extract of Amaranth; LD = Low Dose; MD = Medium Dose; HD = High Dose; * = statistically significant difference at $p < 0.05$ compared to the control group; + = statistically significant difference at $p < 0.05$ compared to the negative control group.

Reproductive Hormones

Luteinizing hormone (LH)

The result indicated that Group 5 had the highest mean value of LH (2.1 \pm 0.24 mIU/ml) while Group 2 had the lowest mean LH value (1.42 \pm 0.45mIU/ml). When compared to Group 1, only Group 2 showed a significant decrease ($p < 0.05$) whereas Groups 3, 4 and 5 showed no significant change. When compared to Group 2, both Group 1 and Group 5 showed a significant increase ($p < 0.05$) in mean LH level.

Follicle Stimulating Hormone (FSH)

Group 1 had the highest mean value of FSH (3.6 \pm 0.52 ng/ml) while Group 2 had the lowest mean value of FSH (2.06 \pm 0.16 ng/ml). Compared to Group 1, Groups 2–5 all showed a significant decrease ($p < 0.05$) in mean FSH. When compared to Group 2, Groups 1 and 5 showed a significant increase ($p < 0.05$) whereas Groups 3 and 4 showed no significant change.

Testosterone

The highest mean testosterone level (6.58 \pm 0.48 ng/ml) was observed in Group 1 whereas the lowest value (4.31 \pm 0.04 ng/ml) was observed in Group 2. Groups 2 and 3 showed a significant decrease ($p < 0.05$) when compared to Group 1 whereas Groups 4 and 5 showed no statistically significant difference. When compared to Group 2, Groups 1, 4 and 5 showed a significant increase ($p < 0.05$) while Group 3 showed no significant difference.

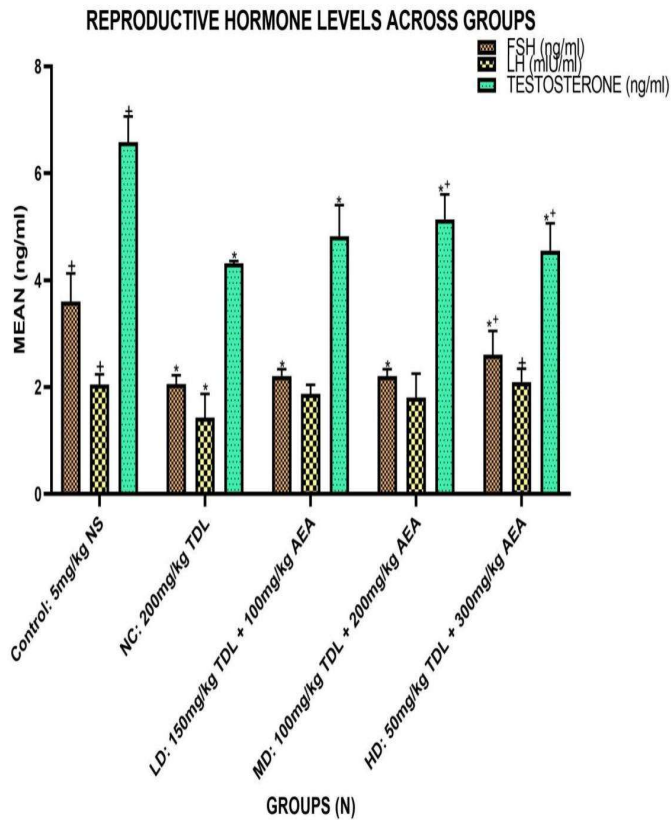


Figure-1. Bar Chart showing the mean levels of reproductive hormones (FSH, LH, and Testosterone). All values were represented as the mean and standard deviation (SD), followed by One Way ANOVA –Tukey’s multiple Comparison test; TDL = Tramadol; AEA = Aqueous Extract of Amaranth.

Oxidative Stress Markers

Super Oxide Dismutase SOD

The highest SOD value (22.53±2.99 U/mgpro) was observed in Group 1 while the lowest (14.93±1.62 U/mgpro) was observed in Group 2. Groups 2–4 showed significant decreases compared to Group 1, whereas Group 5 showed no significant change.

Compared to Group 2, Groups 1 and 5 showed significant increases while Groups 3 and 4 showed no significant change.

Malondialdehyde (MDA)

Group 4 had the highest MDA value (2.15±0.13 nmol/mgpro) while Group 1 had the lowest value (1.70±0.24 nmol/mgpro). All treatment groups showed a significant increase compared to Group 1. Compared to Group 2, only Group 1 showed a significant decrease.

Glutathione Peroxidase (GPx)

The highest GPx value (0.79±0.15 U/mgpro) was observed in Group 5 while the lowest was observed in Group 3. No group showed a significant change compared to Group 1. Compared to Group 2, only Group 5 showed a significant increase.

Table-2. Showing the Mean Oxidative Stress and Antioxidant Markers with Standard Deviation (SD) across all Groups on the effect of *A. viridis* on tramadol-induced testicular toxicity in adult male Wistar rats

GROUPS (N)	SOD (U/mg pro)	GPx (U/mg pro)	MDA (nmol/mg pro)
GROUP 1 (Control: NS)	22.53±2.99 ⁺	0.67±0.06	1.70±0.24 ⁺
GROUP 2 (NC)	14.93±1.62 [*]	0.57±0.03	2.13±0.25 [*]
GROUP 3 (LD)	17.95±0.58 [*]	0.51±0.14	2.02±0.14 [*]
GROUP 4 (MD)	15.62±2.84 [*]	0.58±0.05	2.15±0.13 [*]
GROUP 5 (HD)	19.28±2.73 ⁺	0.79±0.15 ⁺	2.13±0.15 [*]

Values are expressed as MEAN±SD; N = 5; NS = Normal Saline; NC = Negative Control; TDL = Tramadol; AEA = Aqueous Extract of Amaranth; LD = Low Dose; MD = Medium Dose; HD = High Dose; * = statistically significant difference at p<0.05 compared to the control group; + = statistically significant difference at p<0.05 compared to the negative control group.

Inflammatory Markers

Interleukin-1 (IL-1)

Group 1 had the highest IL-1 level while Group 5 had the lowest. Compared to Group 1, only Group 5 showed a significant decrease. No group showed significant difference compared to Group 2.

Interleukin-6 (IL-6)

Group 4 recorded the highest IL-6 level while Group 3 recorded the lowest. No statistically significant difference was observed across groups.

Tumor Necrosis Factor Alpha (TNF-α)

Group 2 recorded the highest TNF-α level while Group 5 recorded the lowest. No statistically significant differences were observed across groups.

Tumor Necrosis Factor Beta (TNF-β)

Group 2 recorded the highest TNF-β level while Group 1 recorded the lowest. Groups 2–5 showed significant increases compared to Group 1. Compared to Group 2, all co-treated groups showed significant decreases.

Table-3. Showing the Mean inflammatory Markers with Standard Deviation (SD) across all Groups on the effect of *A. viridis* on tramadol-induced testicular toxicity in adult male Wistar rats

Groups (N)	Interleukin-6 (pg/ml)	Interleukin-1 (pg/ml)	TNF- α (pg/ml)	TNF- β (ng/g)
GROUP 1	24.50 ± 2.74	29.05 ± 3.88	28.45 ± 1.21	247.60 $\pm 17.28^+$
GROUP 2	24.22 ± 2.25	26.63 ± 1.36	32.44 ± 8.12	902.85 $\pm 83.52^*$
GROUP 3	22.87 ± 2.40	25.55 ± 1.59	28.54 ± 8.13	461.47 $\pm 90.05^{**}$
GROUP 4	25.75 ± 0.63	25.75 ± 0.91	25.60 ± 3.53	552.85 $\pm 13.36^{**}$
GROUP 5	25.02 ± 2.09	24.60 $\pm 2.88^*$	24.92 ± 1.64	530.37 $\pm 170.36^{**}$

Values are expressed as MEAN \pm SD; N = 5; NS = Normal Saline; NC = Negative Control; TDL = Tramadol; AEA = Aqueous Extract of Amaranth; LD = Low Dose; MD = Medium Dose; HD = High Dose;

* = statistically significant difference at $p < 0.05$ compared to the control group
+ = statistically significant difference at $p < 0.05$ compared to the negative control group.

Sperm Analysis

Sperm Motility

Group 2 recorded the highest sperm motility while Group 1 recorded the lowest. Groups 2, 3 and 5 showed significant increases compared to Group 1 whereas Group 4 showed no significant difference. Compared to Group 2, Groups 1 and 4 showed significant decreases.

Sperm Morphology

Group 4 recorded the highest percentage of normal morphology while Group 1 recorded the lowest. All treatment groups showed significant increases compared to Group 1. Compared to Group 2, only Group 1 showed a significant decrease.

Sperm Count

Group 5 recorded the highest sperm count while Group 1 recorded the lowest. Groups 3–5 showed significant increases compared to Group 1. Compared to Group 2, Groups 3–5 showed significant increases.

Table-4. Showing the mean sperm parameters (count, morphology and motility) across all Groups on the effect of *A. viridis* on tramadol-induced testicular toxicity in adult male Wistar rats.

Groups (N)	Sperm Count ($\times 10^6$ /ml)	Sperm Morphology (%)	Sperm Motility (%)
GROUP 1 (Control: 5mg/kg NS)	12.55 ± 13.42	32.72 $\pm 11.61^+$	30.02 $\pm 29.84^+$
GROUP 2 (NC: 200mg/kg TDL)	14.85 ± 1.61	51.30 $\pm 8.25^*$	49.62 $\pm 15.78^*$
GROUP 3 (LD: 150mg/kg TDL + 100mg/kg AEA)	22.25 $\pm 5.39^{**}$	48.87 $\pm 14.03^*$	45.42 $\pm 12.70^*$
GROUP 4 (MD: 100mg/kg TDL + 200mg/kg AEA)	22.30 $\pm 7.21^{**}$	51.65 $\pm 25.95^*$	31.30 $\pm 6.50^+$
GROUP 5 (HD: 50mg/kg TDL + 300mg/kg AEA)	25.06 $\pm 6.07^{**}$	51.00 $\pm 20.47^*$	44.70 $\pm 16.28^*$

Values are expressed as MEAN \pm SD; N = 5; NS = Normal Saline; NC = Negative Control; TDL = Tramadol; AEA = Aqueous Extract of Amaranth; LD = Low Dose; MD = Medium Dose; HD = High Dose; * = statistically significant difference at $p < 0.05$ compared to the control group; + = statistically significant difference at $p < 0.05$ compared to the negative control group.

Histological Examination

Hematoxylin and Eosin (H&E)

Group 1 revealed normal histomorphology with intact seminiferous tubules, Sertoli cells and Leydig cells. Groups 2 and 3 showed varying degrees of abnormalities including spermatid retention, tubular atrophy, disorganized germ cells, necrosis and karyorrhexis. Groups 4 and 5 showed testicular histomorphology comparable to the control group with abundant spermatocytes and intact seminiferous tubules.

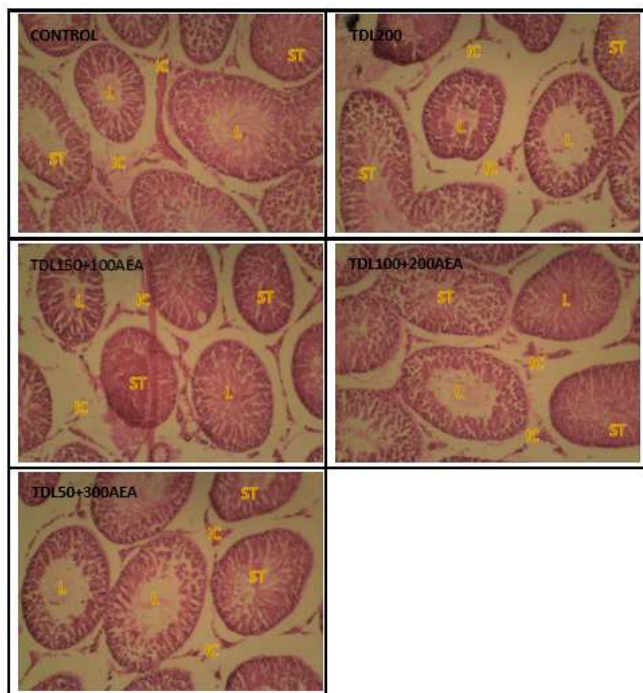


Figure-2. Representative photomicrograph of the testes of Wistar rats revealing a detailed view of seminiferous tubules (ST), Lumen of seminiferous tubule (L), as well as the interstitial compartment (IC). control=normal saline, TDL200=200mg/kg body weight of tramadol, TDL150=150mg/kg body weight of tramadol, TDL100=100mg/kg body weight of tramadol, TDL50=50mg/kg body weight of tramadol, AEA100=100mg/kg body weight of aqueous extract of Amaranth, AEA200=200mg/kg body weight of aqueous extract of Amaranth, AEA300=300mg/kg body weight of aqueous extract of Amaranth. Control group revealed normal testicular morphology with normal spermatic cells lineage in the seminiferous tubules and a well partitioned interstitial tissue. TDL200 presented a testicular architecture with excessive interstitial space and seminiferous tubules with tubular atrophy, and diffuse disorganization of germ cells. TDL150+AEA100, TDL100+AEA200 and TDL50+AEA300 were presented with a testicular architecture with a restoration appearance, specifically, with an increase in the concentration of AEA, the interstitial compartment, the seminiferous tubule and the lumen were progressively becoming similar to what was obtainable in the control group. Heamatoxylin and Eosin stain x100 magnification.

Alcian blue

Group 1 showed normal testicular morphology. Group 2 showed severe alterations including diminished interstitial tissue, reduced Leydig cells, vacuolization and enlarged lumen. Groups 3–5 showed progressive restoration of seminiferous tubule structure with Group 5 showing near complete restoration.

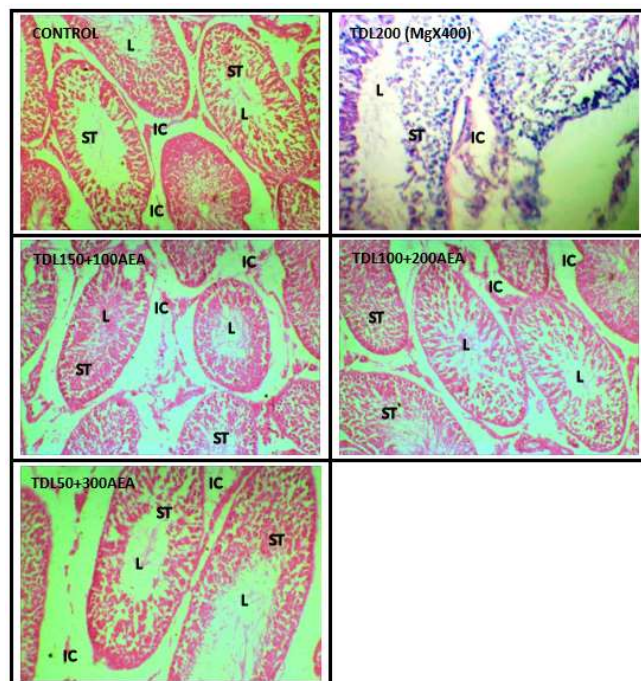


Figure-3. Representative photomicrograph of the testes of Wistar rats revealing a detailed view of seminiferous tubules (ST), Lumen of seminiferous tubule (L), as well as the interstitial compartment (IC). Mg=Magnification, Control=normal saline, TDL200=200mg/kg body weight of tramadol, TDL150=150mg/kg body weight of tramadol, TDL100=100mg/kg body weight of tramadol, TDL50=50mg/kg body weight of tramadol, AEA100=100mg/kg body weight of aqueous extract of Amaranthus viridis, AEA200=200mg/kg body weight of aqueous extract of Amaranthus viridis, AEA300=300mg/kg body weight of aqueous extract of Amaranthus viridis. Alcian blue stain with x100 magnification.

Discussion

Changes in body weight are widely recognized as a non-specific but reliable indicator of systemic toxicity, metabolic dysfunction, and overall physiological well-being following exposure to pharmacological or toxic agents. Alterations in body weight often reflect changes in food utilization efficiency, endocrine regulation, and metabolic homeostasis.^{15,16} In the present study, administration of Amaranthus viridis did not result in a statistically significant adverse effect on body weight in adult male Wistar rats exposed to tramadol-induced toxicity. Notably, Group 4 exhibited the greatest mean body weight change, while Group 1 showed the least increase. The significant increase in body weight observed in Groups 2, 4, and 5 relative to the control group may suggest altered metabolic activity or fluid retention induced by tramadol exposure or phytochemical modulation by A. viridis. Opioids such as tramadol have been reported to influence appetite regulation, insulin sensitivity, and lipid metabolism through central nervous system pathways involving hypothalamic neuropeptides.^{17,18} The lack of weight reduction following A. viridis administration in this study contrasts with findings by Oke et al.¹⁹, who reported body weight reduction associated with the plant's hypolipidemic and appetite-modulating properties. This discrepancy may be attributable to differences in extract preparation, treatment duration, animal model, or dosage regimen, as phytochemical bioactivity is highly dose-dependent.²⁰

Luteinizing hormone (LH) plays a fundamental role in male reproductive endocrinology by stimulating Leydig cells to synthesize and secrete testosterone via activation of steroidogenic enzymes such as cholesterol side-chain cleavage enzyme and 17 β -hydroxysteroid dehydrogenase.²¹ The significant reduction in LH observed in tramadol-treated animals confirms opioid-induced hypothalamic-pituitary-gonadal (HPG) axis suppression. Opioids are known to inhibit gonadotropin-releasing hormone (GnRH) pulsatility through activation of μ -opioid receptors in the hypothalamus, thereby reducing pituitary gonadotropin secretion.^{22,23} The restoration of LH levels observed particularly in Group 5 suggests a dose-dependent endocrine recovery mediated by *A. viridis*. Phytochemicals such as flavonoids, saponins, and phenolic compounds present in *A. viridis* are known to exert steroidogenic and neuroendocrine modulatory effects.^{20,24} These observations agree with Mahmoud af Elghamry²⁵, who reported opioid-induced suppression of gonadotropins and demonstrated partial hormonal recovery following antioxidant therapy.

Follicle stimulating hormone (FSH) is essential for Sertoli cell proliferation, maintenance of the seminiferous epithelium, and regulation of spermatogenesis through stimulation of androgen-binding protein synthesis and germ cell nourishment.²⁶ The marked reduction in FSH levels observed in tramadol-treated animals further confirms HPG axis suppression and impaired Sertoli cell functionality. The finding that only the highest dose of *A. viridis* restored FSH levels suggests a threshold-dependent protective activity likely mediated through enhanced antioxidant defense and endocrine modulation. Oxidative stress has been shown to disrupt Sertoli cell junctional complexes and impair germ cell maturation.²⁷ While these findings align with Mahmoud and Elghamry²⁵, they contrast with Adeyemi et al.²⁸, who reported no significant alteration in reproductive hormone levels following *A. viridis* treatment. Such variations may reflect differences in plant extraction methods, environmental phytochemical variability, or duration of exposure.

Testosterone is indispensable for spermatogenesis, libido, maintenance of secondary sexual characteristics, and structural integrity of the seminiferous tubules.²⁹ The significant reduction in testosterone observed in tramadol-treated rats corroborates previous reports linking opioid administration to Leydig cell dysfunction and steroidogenic enzyme inhibition.^{23,30} Restoration of testosterone levels in Groups 4 and 5 suggests improved Leydig cell function and possible antioxidant-mediated protection against oxidative cellular injury. Antioxidants can preserve mitochondrial integrity within Leydig cells, thereby sustaining steroidogenesis.³¹ However, Ojo et al.³² reported biphasic testosterone responses at higher phytochemical concentrations, indicating that excessive phytochemical exposure may exert inhibitory endocrine effects, a phenomenon commonly observed in phyto-androgenic compounds.

Oxidative stress plays a major role in tramadol-induced reproductive toxicity through excessive generation of reactive oxygen species (ROS) leading to lipid peroxidation, DNA

damage, and protein oxidation.³ The reduction in superoxide dismutase (SOD) activity and elevation in malondialdehyde (MDA) levels observed in tramadol-treated animals in this study confirms oxidative tissue injury. SOD represents the primary enzymatic defense against superoxide radicals, while MDA serves as a reliable biomarker of lipid peroxidation and membrane damage.³³ Restoration of SOD and glutathione peroxidase (GPx) activities by the highest *A. viridis* dose demonstrates potent antioxidant capacity. These findings support reports by Al-Amin et al.³⁴ and Ezekwe et al.³⁵, who demonstrated enhancement of endogenous antioxidant enzymes following *A. viridis* administration. The inability of *A. viridis* to significantly reduce MDA levels in the present study contrasts with findings by Adedapo et al.³⁶, suggesting that while enzymatic antioxidant systems were improved, lipid peroxidation processes may require longer treatment duration or higher phytochemical concentration for complete reversal. The findings however align with Barakat et al.³⁷ and Ezzat et al.³⁸, who reported elevated MDA levels with tramadol exposure. GPx reduces hydrogen peroxide and plays a vital role in cellular defense. The highest GPx activity was found in Group 5, and the lowest in Group 3. No significant changes were observed among groups compared to the control. However, Group 5 showed a significant increase in GPx levels relative to Group 2, suggesting that only the 300 mg/kg dose of *A. viridis* exerted a protective effect. These findings diverge from El-Naggar³⁹, who documented tramadol-induced GPx suppression.

Inflammatory responses are closely associated with oxidative stress and reproductive dysfunction. Cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factors are key mediators of testicular inflammation and germ cell apoptosis.⁴⁰ The reduction of IL-1 observed in the highest dose group suggests anti-inflammatory activity of *A. viridis*, possibly through inhibition of nuclear factor-kappa B (NF- κ B) signaling pathways, which regulate pro-inflammatory cytokine expression.⁴¹ However, the absence of significant changes in IL-6 and TNF- α levels suggests partial immunomodulatory activity rather than complete inflammatory suppression. The significant reduction of TNF- β in co-treated groups supports immune regulatory activity and aligns with Okwuonu et al.⁴², who reported immunomodulatory polysaccharides in *A. viridis*. Variability in cytokine response may reflect differences in local versus systemic inflammatory processes within the testicular microenvironment. The difference between IL-6 and IL-1 levels across the groups may be explained by their distinct inflammatory roles and regulatory mechanisms. IL-1 is primarily an early-response cytokine that reacts rapidly to oxidative stress and tissue injury, making it more sensitive to antioxidant interventions such as *Amaranthus viridis*. In contrast, IL-6 exhibits both pro- and anti-inflammatory properties and is regulated through multiple signaling pathways, including NF- κ B and JAK/STAT pathways, making it less responsive to single-pathway antioxidant modulation.^{43,44} Additionally, IL-6 is produced by a wider variety of immune and non-immune cells and is often associated with sustained inflammatory responses, whereas IL-1 is more closely linked to acute inflammatory reactions,

which may explain the greater variability observed in IL-1 levels compared to IL-6.⁴⁵

Sperm quality parameters serve as direct indicators of male fertility potential. The observed improvements in sperm count and morphology following *A. viridis* treatment suggest enhanced spermatogenic activity and improved germ cell maturation. Antioxidant protection of sperm membrane lipids and DNA integrity likely contributed to these improvements.⁴⁶ Interestingly, sperm motility demonstrated inconsistent improvement across treatment groups, suggesting a biphasic dose-response phenomenon. Similar biphasic responses have been documented in phytotherapeutic agents where moderate doses enhance sperm motility while excessive antioxidant exposure may impair mitochondrial ATP production necessary for sperm motility.^{47,48} The observed increase in sperm count may be attributed to restoration of HPG axis signaling and improved Sertoli-Leydig cell interaction, as previously reported by Ogundare et al.⁴⁹

The unexpectedly higher percentage of sperm motility observed in Group 2 (200 mg/kg tramadol) may be attributed to a compensatory physiological response to moderate toxic stress. Exposure to sub-lethal toxic doses can sometimes stimulate adaptive cellular mechanisms, a phenomenon described as hormesis. At moderate toxicity levels, the testes may temporarily enhance spermatogenic activity or epididymal sperm maturation in an attempt to maintain fertility potential despite endocrine and oxidative challenges.^{50,51} This adaptive response may transiently preserve or even improve sperm motility before severe toxic damage becomes evident.

Additionally, tramadol has been reported to alter neurotransmitter activity, particularly serotonin and norepinephrine pathways, which can indirectly influence reproductive hormonal regulation and epididymal sperm maturation. Moderate alterations in these pathways may transiently enhance sperm motility by affecting epididymal secretory function and sperm membrane stability.^{25,30} However, prolonged or higher-dose exposure typically overwhelms antioxidant defenses and disrupts mitochondrial function in sperm cells, ultimately leading to reduced motility.

Another possible explanation is selective vulnerability of sperm parameters. Sperm count, morphology, and motility do not deteriorate uniformly during toxic exposure. Motility is strongly dependent on mitochondrial ATP production and epididymal microenvironment, which may remain functionally preserved during early or moderate toxic insult, whereas spermatogenic cell proliferation and morphology are more sensitive to direct testicular damage.^{46,52}

Tramadol administration (200 mg/kg) resulted in significant alterations in sperm morphology, as evidenced by increased percentages of abnormal sperm in the negative control group. The observed morphological defects included head abnormalities, tail defects, and midpiece malformations, which are indicative of impaired spermatogenesis and disrupted germ cell differentiation. These changes are likely mediated by oxidative stress and hormonal imbalance, particularly reduced testosterone and FSH levels, which are critical for Sertoli cell

support and proper sperm development.^{25,30} The oxidative stress induced by tramadol may lead to lipid peroxidation of sperm membranes and DNA damage, contributing to the morphological anomalies observed.^{32,34}

Co-administration of *Amaranthus viridis* at increasing doses progressively improved sperm morphology. Groups 4 (100 mg/kg Tramadol + 200 mg/kg *A. viridis*) and 5 (50 mg/kg Tramadol + 300 mg/kg *A. viridis*) exhibited near-normal sperm morphology, suggesting that the phytochemical components of *A. viridis*, including flavonoids and polyphenols, exerted protective effects on the spermatogenic cells. The improvement is likely mediated by a combination of antioxidant activity, anti-inflammatory effects, and restoration of the hypothalamic-pituitary-gonadal axis, which collectively supports proper differentiation and maturation of spermatozoa.^{49,53}

Interestingly, lower doses of *A. viridis* (Group 3, 150 mg/kg Tramadol + 100 mg/kg *A. viridis*) produced partial improvement, indicating a dose-dependent protective effect. This suggests that sufficient bioactive concentration is required to counteract tramadol-induced oxidative and hormonal insults to germ cells. These findings are consistent with previous reports that *A. viridis* can enhance sperm quality, including morphology, by mitigating oxidative damage and supporting spermatogenesis.^{54,55}

In summary, tramadol-induced sperm morphological defects were significantly ameliorated by *A. viridis* in a dose-dependent manner, highlighting its therapeutic potential in preserving male reproductive function under drug-induced toxicity.

Histological findings strongly supported the biochemical and hormonal outcomes observed in this study. Tramadol exposure resulted in seminiferous tubular degeneration, germ cell disorganization, Leydig cell depletion, and evidence of necrosis, all of which are classical histopathological features of drug-induced testicular toxicity.⁵⁶ Co-administration of *A. viridis* resulted in progressive restoration of testicular architecture characterized by improved seminiferous tubular organization, restoration of germinal epithelium, and increased spermatogenic cell population. These findings corroborate Gupta and Sharma⁵³, who demonstrated phytochemical-mediated histological restoration in toxin-induced testicular injury models. Furthermore, the restoration of extracellular matrix organization observed in this study supports findings by Choudhary et al.⁵⁷, who highlighted the role of *A. viridis* in promoting tissue repair and spermatogenic recovery through collagen stabilization and cellular regeneration pathways.

Conclusion

The results of this study have demonstrated that *A. viridis* exerts a dose-dependent protective effect against tramadol-induced reproductive toxicity. Restoration of hormonal balance, antioxidant defenses, and sperm quality highlights its potential as a phytotherapeutic agent. *Amaranthus viridis* ameliorates tramadol-induced testicular toxicity via antioxidant, anti-inflammatory, and hormone-regulating mechanisms. Further studies are hence, recommended to

elucidate its molecular pathways and potential clinical applications.

Limitation

A limitation of this study is the variation in tramadol doses across treatment groups, while *A. viridis* doses were incrementally increased. This design, intended to assess both the dose-dependent toxicity of tramadol and the protective effects of the extract, makes it difficult to fully isolate the individual contribution of each treatment. Future studies using a fixed tramadol dose and varying extract concentrations are recommended to determine the extract's protective potential more precisely.

Although co-administration of *A. viridis* improved several oxidative stress and inflammatory markers, not all parameters (e.g., MDA, IL-6, TNF- α) returned to control levels. Therefore, the observed protective effects should be interpreted as partial rather than complete restoration, highlighting the need for further dose-optimization studies.

Declarations

Ethics approval: Ethical approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, Rev. Fr. Moses Orshio Adasu University, Makurdi, Benue State-Nigeria.

Consent to participate: Not applicable

Consent for publication: Not applicable

Conflicts of interest statement: The researchers declare that there was no conflict of interest in this study.

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