**Investigation of Pre-Exposure Protective Role of *Psidium guajava* (Guava) Leaf Extract in Lead Acetate-Induced Reproductive Dysfunction in Male Albino Rats**

 **ABSTRACT**

Guava (*Psidium guajava* L.) is a widely consumed fruit in the tropical region and other parts of the world. The leaves, especially, have been reported to have both nutritional and medicinal benefits due to their rich phytochemical content. Studies have reported ameliorative potentials of guava leaf extract against heavy metal-induced reproductive derangements, particularly when administered after exposure. These reports are mainly post-exposure intervention studies. Therefore, the focus of this study was to investigate the potential of guava leaf extracts in rats before exposure to lead toxicity (pre-exposure). A total of 35 male albino rats weighing 200 grams were randomly divided into 5 groups of 7 rats per group. **Group A** is the negative control, given food and water only *ad libitum*. **Group B is the p**ositive control, treated with 30mg/kg of lead acetate daily for 14 days while **Groups F, G, and H were t**reated with 250, 500, and 750mg/kg treatment of guava leaf extracts respectively daily for 14 days followed by treatment with 30mg/kg of lead acetate daily for another 14 days. After the treatment period, rats were anesthetized, sacrificed, and blood samples were collected into plain bottles through cardiac puncture for assay of luteinizing hormone (rLH), follicle stimulating hormone (rFSH), testosterone, malondialdehyde (MDA) and superoxide dismutase (SOD) using ELISA. Epididymis samples were collected for semen analysis, and testes were processed for histological examination. Statistical analysis was performed using GraphPad Prism, with significance set at p<0.05. The result indicated that flavonoids are the most abundant phytochemicals present in guava leaves, with a concentration of 8.01mg/ml, relatively to saponins, tannins, anthraquinones, alkaloids, and phenols with concentrations of 6.07, 5.03, 0.01, 0.50, and 2.87mg/ml, respectively. The results of measurable sperm parameters indicated significantly higher active motility in Groups A, F, G, and H compared to Group B. However, no significant differences were observed between Groups A, F, G, and H. Sperm count indicated that Group H had significantly lower values than Groups A, F, and G. Also, Group F had significantly higher values than G. LH indicated significantly lower values in Groups A, G, F, and H compared to Group B. In addition, Group H indicated a significantly higher value in FSH than Groups A, F, and G. Regarding testosterone, Groups F and G had significantly higher values than Groups H and B. In the LH-FSH ratio, Group H had significantly lower values than other treated groups except Group B and vice versa in the LH-FSH ratio where Group H had significantly higher values than the other treated groups except Group B. Finally, the T-LH ratio indicated that Group H had significantly lower values compared to Groups F, G, and A. However, Group A had significantly higher values of T-LH ratio compared with Groups B, F, G, and H. Malondialdehyde (MDA) and superoxide dismutase (SOD) indicated significantly higher value in Group H compared to Groups A, G and F, but significantly lower compared to Group B. Conclusively, the study revealed that consumption of guava leaves extract before exposure to lead acetate does not confer protection in a dose-dependent manner against the disruption of male endocrine system. Rather, an unusual lower dose of guava extracts of 250mg/kg was observed to be more protective than 500mg/kg and 750mg/kg.

**Keywords:** *Psidium guajava*, Pre-Exposure, Protective, Guava Leaf Extract, Testosterone, Luteinizing Hormone, Follicle Stimulating Hormone, FSH-LH ratios, Testosterone-LH ratio, Lead, Endocrine disruption, Male

1. **INTRODUCTION**

Guava (*Psidium guajava* L.) is a widely consumed fruit in the tropical region and other parts of the world (Huynh et al., 2025). The leaves, especially, have been reported to have nutritional and medicinal benefits due to their rich composition of phytochemicals such as flavonoids, phenolics, meroterpenoids, and triterpenes as the main bioactive constituents (Bazioli et al. 2020**;** Braga et al. 2022). Bazioli et al., (2020) and Kumar et al., (2021) reported that guava leaves are applicable in the traditional management of several ailments following its anti-cancer, antioxidant, anti-inflammatory, anti-microbial, and anti-diabetic properties. Kumar et al. (2021) and Braga et al. (2022), further reported that guava leaves are used in the treatment of various ailments such as gastrointestinal disorders, wounds, and skin infections. More so, consumption of guava leaf extracts in the form of “drink” has been reported to mitigate or alleviate the severity of heavy metal toxicity after exposure (El-Sesy & Mahran, 2020).

Heavy metals are mainly transitional elements that confer the ability to induce oxidative damage in tissues and cells through their bio-accumulative tendencies (Sobral–Souza et al. 2019; Ben-Chioma et al. 2023). Therefore, their toxicity is usually chronic rather than acute except in accidental or intentional exposure leading to acute attack (Elekima et al. 2024). Heavy metals such as lead, chromium, arsenic, mercury, and so on have been implicated in the contamination of food, water, and the environment and eventually inducing health risks or derangements (Ben-Chioma et al. 2023; Elekima et al. 2024).. In the Niger Delta of Nigeria, particularly in Rivers State environmental pollution in the form of oil spillage and gas flaring have been become a significant global concern, particularly regarding the accumulation of heavy metals and other toxicants in various ecosystems (Tchounwou et al., 2012; Elekima et al., 2020). These continuous oil spills and gas flares over the years have led to contaminated underground waters, drinking water, rivers, seafoods, farmlands, and edible crops, therefore unavoidably exposing residents and communities to heavy metal toxicities (Elekima et al., 2020).

Exposures to these heavy metals have been documented to be associated with some derangements, such as male and female reproductive disorders, particularly endocrine disruption. However, Boskabady et al. (2018) have documented the mitigating effect of guava leaves extracts on oxidative stress, inflammation, and cell damage linked to heavy metals toxicity such as lead and mercury. Ruksiriwanich et al. (2022) further reported ameliorative potentials of guava leaf extract against heavy metal-induced reproductive derangements, particularly when administered after exposure. Ferdinand et al. (2014) also reported improvement in sperm quality, motility, and viability in rats treated with 80, 100, and 120 μl of oil per kg bodyweight. These reports are mainly post-exposure intervention studies. Therefore, the focus of this study was to investigate the potential of guava leaf extracts in rats before exposure to lead toxicity (pre-exposure).

1. **MATERIALS AND METHODS**

**2.1 Materials**

Materials used include guava leaves, lead acetate, pH meter, Olympus microscope, centrifuge, electronic weighing balance, 10% formal saline, Microplate Reader, Shandon AS 325 Rotary Microtome, Leica tissue processor, Haematoxylin and Eosin stain, rLH, rFSH, and testosterone ELISA Kits purchased from Calbiotech, U.S.A. MDA and SOD ELISA kits purchased from Elabscience, India.

* 1. **Experimental Animal**

Male rats were procured from the Department of Anatomy, Rivers State University, Port Harcourt, and were transported in well-ventilated, wired cages to the animal house located in the Department of Animal and Environmental Sciences of the same University. The animals were fed with poultry chow and water *ad libitum*. Rats were acclimatization for 14 days before the start of the experiment.

**2.3 Collection and Preparation of Guava Leaf**

The guava leaves were identified by Dr. M. G. Ajuru at the Department of Plant Science and Biotechnology, Rivers State University, with an accession/collection Number of RSUPbH019. The guava leaves were carefully sorted to remove any dead matter or unwanted particles. The leaves were left to air-dry over a period of 14 days at room temperature, after which they were finely ground using a blender, and 600g were measured into 1000 mL of 80% v/v ethanol for 72 hours. The extraction was done using a Soxhlet machine at a temperature of 60 0C. The yielded crude weighed 15.62g and was transferred into a sample bottle and refrigerated at 4 0C before being used for the study.

**2.4 Experimental Design**

A total of 35 male albino rats weighing 200 grams were randomly divided into 5 groups, 7 rats per group.

**Group A** = Negative control, given food and water only

**Group B** = Positive Control, treated with 30mg/kg of lead acetate daily for 14 days

**Group G** = Treated with 250mg/kg treatment of guava leaf extracts daily for 14 days followed by treatment with 30mg/kg of lead acetate daily for another 14 days

**Group G** = Treated with 500mg/kg treatment of guava leaf extracts daily for 14 days, followed by treatment with 30mg/kg of lead acetate daily for another 14 days.

**Group H** = Treated with 750mg/kg treatment of guava leaf extracts daily for 14 days, followed by treatment with 30mg/kg of lead acetate daily for another 14 days.

The LD50 of lead acetate was achieved using the Kerber-Breham method as described by Isrea et al. (2021), while the dose selection of guava leaf extract was established following Lork’s method as described by Enegide et al. (2013)

**2.5 Specimen Collection, Preparation, and Analysis**

At the end of the experiment, rats were anaesthetized using chloroform and sacrificed. Approximately 5 mL of whole blood specimen was collected through cardiac puncture. Samples were allowed to clot and spun at 4500 rpm for 5 minutes to derive serum. The serum samples were used for the laboratory analysis of LH, FSH, Testosterone, MDA, and SOD using ELISA technique as described by Engvall & Perlmann, (1971). Semen samples were also collected by the excision of the epididymis for semen analysis as described by WHO (2021). Finally, testicular tissues were harvested for histological examination.

**2.6 Statistical Analysis**

Raw data were analysed using GraphPad Prism version 8.0.2**.** Statistical tool such as the O**ne-way ANOVA** was used to compare the groups. Statistical significance was pegged at p<0.05.

**3 RESULTS**

**3.1 Results of the Quantitative Analysis of the Phytochemicals of Guava Leaves**

This result indicates that the most abundant phytochemicals present in guava leaves are flavonoids with a concentration of 8.01mg/ml, relative to saponins, tannins, anthraquinones, alkaloids, and phenols with concentrations of 6.07, 5.03, 0.01, 0.50, and 2.87mg/ml, respectively (Table 1).

**Table 1: Results of the Quantitative Analysis of Phytochemical Components of Guava**

 **Leaves**

|  |  |
| --- | --- |
| **Phytochemical** | **Concentration (mg/ml)** |
| Flavonoid | 8.01 |
| Saponins | 6.07 |
| Tannins | 5.03 |
| Anthraquinones | 0.01 |
| Alkaloids | 0.50 |
| Phenol | 2.87 |

**3.2 Results of Measurable Sperm Parameters**

The results of measurable sperm parameters indicated significantly higher active motility in Groups A, F, G, and H compared to Group B. However, no significant differences were observed between Groups A, F, G, and H. In addition, when the total sperm count was considered, Group H had significantly lower values than Groups A, F, and G but significantly higher values than Group B. Also, Group F had significantly higher values compared to G (Table 2). Finally, the pH of the seminal fluid had significantly higher values in Group B compared to the treated groups and the negative control. However, there were no significant differences in the pH between the treated groups and the negative control at p<0.05 (Table 2).

**3.3 Results of Testosterone, Luteinizing Hormone, Follicle Stimulating Hormone, and**

 **their Ratios**

The result obtained indicated significantly lower values of LH in Groups A, G, F, and H compared to Group B. However, no significant differences were observed between Groups A, F, G, and H. In addition, Group H indicated a significantly higher value in FSH than A, F, and G. No significant difference between H and B was seen. Regarding testosterone, Groups F and G had significantly higher values than Groups H and B. Meanwhile, Group H had significantly higher values of testosterone than Group B. However, in Groups A and F, no significant differences in testosterone were seen. In the LH-FSH ratio, Group H had significantly lower values than other treated grruops except Group B and vice versa in the LH-FSH ratio where Group H had significantly higher values than the other treated groups except Group B. finally, the T-LH ratio indicated that Group H had significantly lower values compared to Groups F, G and A, but significantly higher values compared to Group B. However, Group A had significantly higher values of T-LH ratio compared with Groups B, F, G, and H (Table 3).

**3.3 Results of the Oxidative Stress Markers**

The oxidative stress markers considered were Malondialdehyde (MDA) and superoxide dismutase (SOD) indicated significantly higher value in Group H compared to Groups A, G and F, but significantly lower compared to Group B. However, no significant differences were seen between Groups A, G, and F at p<0.05 (Table 4).

**Table 2:** **Results (Mean±SD) of Different Groups of Lead-Induced Testicular Toxicity in Rats Pre-Treated with Guava Leaf**

 **Extract**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | GROUPA | GROUPB | GROUPF | GROUPG | GROUPH | F value | P Value | Remark |
| rLH (miu/ml) | 2.40±1.08a | 5.07±0.21b | 2.57±0.41a | 2.17±0.24a | 2.95±1.06a | 14.98 | <0.0001 | S |
| rFSH (miu/ml) | 1.72±0.44a | 4.40±0.38b | 1.77±0.30a | 1.90±0.36a | 4.32±0.37b | 70.80 | <0.0001 | S |
| rTESTO (ng/ml) | 4.57±0.81a | 0.20±0.09b | 4.5±0.76a | 3.88±0.93c | 1.50±0.60d | 43.60 | <0.0001 | S |
| rLH/rFSH | 1.49±0.75a | 1.16±0.15a | 1.48±0.31a | 1.17±0.23a | 0.67±0.20b | 4.059 | 0.0034 | S |
| rFSH/rLH | 0.86±0.45a | 0.87±0.11a | 0.70±0.15a | 0.88±0.17a | 1.58±0.43b | 7.531 | <0.0001 | S |
| T/rLH | 2.14±0.65a | 0.04±0.02b | 1.79±0.44d | 1.808±0.65d | 0.56±0.23c | 32.76 | <0.0001 | S |

**Keys:**rLH= Rat Specific-Luteinizing Hormone, rFSH= Rat Specific-Follicle Stimulating Hormone, rTESTO= Rat Specific-Testosterone, T/LH= Testosterone-Luteinizing Hormone. **Post Hoc (Tukey’s):** Values in the row with different superscripts differ significantly at p<0.05. Group A= negative control group, Group B= positive control group, Group F= Received 250mg/kg of guava leaf extract for the first two weeks and given 30mg/kg of lead acetate for another two weeks, Group G=Received 500mg/kg of guava leaf extract for the first two weeks and given 30mg/kg of lead acetate for another two weeks, Group H= Received 750mg/kg of guava leaf extract for the first two weeks and given 30mg/kg of lead acetate for another two week

**Table 3:** **Results (Mean±SD) of Measurable Sperm Parameters in Different Groups of Lead-Induced Testicular Toxicity in**

**Rats Pre-Treated with Guava Leaf Extract**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | GROUPA | GROUPB | GROUPF | GROUPG | GROUPH | F value | P value | Remark |
| Active Motile (%) | 81.67±12.1a | 3.75±2.7b | 89.0±6.5a | 86.2±9.47a | 71.67±16.1a | 48.69 | <0.0001 | S |
| Sluggish(%) | 11.33±6.53a | 3.75±2.7b | 8.2±4.66a | 10.0±7.07a | 19.3±11.02c | 1.987 | 0.0112 | S |
| Dead(%) | 7.0±6.48a | 92.5±9.6b | 2.8±2.17e | 3.75±2.5e | 9.0±6.56a | 121.5 | <0.0001 | S |
| Count (cells/ml)x107 | 2.28±1.25a | 0.88±0.8b | 3.29±1.7d | 2.34±1.09a | 1.29±0.09b | 3.147 | 0.0221 | S |
| pH | 7.41±0.37a | 8.50±0.6b | 7.40±0.4a | 7.25±0.29a | 7.67±0.29a | 4.875 | 0.0026 | S |

**Post Hoc (Tukey’s):** Values in the row with different superscripts differ significantly at p<0.05

**Table 4:****Results (Mean±SD) of Oxidative Stress Parameter of Different Groups of Lead-Induced Testicular Toxicity in**

 **Rats Pre-Treated with Guava Leaf Extract**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameters | GROUPA | GROUPB | GROUPF | GROUPG | GROUPH | F value | P Value | Remark |
| MDA (ng/ml) | 193.2±26.38a | 397.3±38.21b | 200.7±30.59a | 228.2±21.30a | 258.2±19.82e | 40.98 | <0.0001 | S |
| SOD(ng/ml) | 36.0±6.36a | 12.67±2.42b | 36.0±7.16a | 31.17±2.04a | 27.50±2.07c | 23.84 | <0.0001 | S |

**Keys:** MDA = Malondialdehyde, SOD=Superoxide Dismutase. **Post Hoc (Tukey’s):** Values in the same row with different superscripts differ significantly at p<0.05

 **3.4 Histological Analysis**

Histological investigation of the testis of lead acetate induced testicular toxicity was performed as seen in plate A, B, E, and F.

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**Plate A:** H&E Stain, Mag., x200, Group A. Treatment: NIL. Well-defined basement membrane (BM) lined with Sertoli cells and spermatogonia. Distinct Leydig cells (LC) areas without clusters. A photomicrograph showed spermatocytes with defined flagellation in the lumen (F) of the seminiferous tubules migrating towards the basement membrane (BM). Also indicated were well-stained Sertoli cells (arrow) lining the basement membrane. Inference: Normal testicular tissue. **Plate B:** H&E Stain, Mag., x200, Group B. Treatment: 30mg/kg of Pb for 2 weeks. Distorted basement membrane (circled area). Distorted and poorly differentiated Leydig cells (LC). Gross loss of Sertoli cells, spermatogonia (sp), and spermatids in their respective layers alongside vacuolations (V). Absence/severe loss of flagellation with aggregated nuclear pycnosis (arrows) in the spermatogonia and spermatids layers of the lumen (F) of the seminiferous tubules. Inference: Histology of a degenerated testicular tissue. **Plate F:**H&E Stain, Mag., x200, Group F. Treatment: 250mg/kg of Guava Leaf Extract for 2 weeks + 30mg/kg of Pb for 2 weeks. Distorted basement membrane (BM) lined with clustered Sertoli cells and spermatogonia. Poorly differentiated Leydig cells (LC). Mildly reduced presence of spermatogonia (sp) and spermatids alongside poor/reduced flagellation (F) as well as nuclear pycnosis (circled area) in the spermatids region of the lumen. There are losses of testicular parenchymal materials with vacuolation (V). The lumen of the seminiferous tubules (F) indicated some form of distortion. Inference: Degenerating testicular tissue with nuclear pycnosis. **Plate H:**H&E Stain, Mag., x200, Group H. Treatment: 750mg/kg of Guava Leaf Extract for 2 weeks + 30mg/kg of Pb for 2 weeks. Intact basement membrane (BM) lined with Sertoli cells and spermatogonia. Hypo-chromatic and poorly differentiated Leydig cells (LC) with clusters of non-Leydig cells. Reduced number of spermatogonia (SP) and spermatids with mild flagellation in the lumen of the seminiferous tubules (F). There are losses of testicular parenchymal materials with vacuolation (V). Inference: Degenerating testicular tissue with associated with loss of spermatogonia and deflagellation of spermatocytes.

**4. DISCUSSION**

The result of flavonoids as the most abundant phytochemical in the extract, as seen in our study, concurs with the reports of Arima & Danno (2002), who also documented that guava leaves are rich in flavonoids. The high amount of flavonoids in the extract indicates that guava leaves may be an effective agent against oxidative stress and inflammation. The presence of saponins and tannins in the extract was also in line with the findings of Akinmoladun et al. (2010), who reported that saponins and tannins in guava extracts contribute to the disruption of bacterial membranes and therefore exert antimicrobial effects. Meanwhile, anthraquinones, alkaloids, and phenols are also phytochemicals with antioxidant properties quantified in our study. Adeyemi et al. (2006) documented that alkaloids, such as guavacine present in guava leaves extract exhibit antibacterial properties against various pathogens.

The non-significant differences observed in the LH values of Groups F, G, and H compared to A (negative control) could be due to the enormous presence of sufficient anti-oxidants supplemented by the guava extract in the rats. Lead induced a significantly higher value of LH in the rats following the disruption of the hypothalamic-pituitary-gonadal (HPG) axis causing impairment of the testicular function as seen in the positive control administered with lead acetate. However, the presence of sufficient anti-oxidant supplementation probably from the guava extract conferred a an ameliorative function in the Groups, F, G, and H treated with 250, 500, and 750mg/kg of guava leaf extract before exposure to 30mg/kg of lead acetate. Luteinizing hormone plays an important role in regulating testosterone synthesis in males (Chen et al., 2019). Regarding FSH, Groups F and G were not significantly different from Group A, which could also account for as the anti-oxidant supplementation from the guava leaves extract. However, Group H values were higher compared to Groups F and G. The higher values of FSH observed in our study as seen in Group H treated with 750mg/kg indicates that probably the guava leaves did not confer any protective role against lead acetate at a higher dose unlike Group s F and G, treated with 250 and 500mg/kg respectively. The results further suggest poor Sertoli cell activities and viz-a-viz reduced spermatogenesis. The increase in FSH is a direct compensatory indication of poor response of the Sertoli cells to the stimulation of FSH and LH. These results are suggestive of a dose-dependent restoration of reproductive function, particularly in the activities of FSH. Our findings with respect to the results of 250 and 500mg/kg are similar to the finding of **Ogli et al., (2022) who reported that t**he administration of 250mg/kg and 500mg/kg of the guava leaf extracts induced an improvement in the FSH and LH level of male reproductive hormones in a dose-dependent manner and potentially boosting male fertility. More so, Ekaluo et al., (2013) also reported dose-depemdent increase in FSH and LH of rats treated with 100, 200 and 300mg/kg of guava leaves extract for 70 days.

Testosterone also indicated a dose-dependent ameliorative effect on the concentration of guava leaf extract administered. Group H had significantly lower values of testosterone compared to Group G and F. Meanwhile, Group G had a significantly lower value compared to Group F whose values are similar with the negative control (Group A). Testosterone values indicated an inverse relationship with the dose of guava extract administered. Though, it is believed the lower values of testosterone could be due to poor responses of Leydig cells may be due to oxidative stress. The mechanism at which higher doses of guava leaf extracts were less effective than lower concentrations of the extract cannot be explained yet. However, these results suggest that excessive intake of guava leaf extract might have induced mild oxidative stress or hormonal dysregulation, highlighting the importance of dose optimization. This observation are in line with he report Manekeng et al., (2019) who observed that intake of a single high dose of the Psidium guajava bark extract may be nontoxic, but repeat administration could exhibit mild organ toxicity. The lower values of testosterone could also have accounted for the lower concentration or count of sperm cells in the Group H. Our findings concerning the results of 250 and 500mg/kg are similar to the finding of Ogli et al., (2022) who reported that **t**he administration of 250mg/kg and 500mg/kg of the guava leaf extracts induced an improvement in the testosterone level of male reproductive hormones and potentially boosting male fertility. Our results are also in line with out histological findings. The histology indicated hypo-chromatic and poorly differentiated Leydig cells with clusters of non-leydig cells. There were also reduced number of spermatogonia and spermatids with mild flagellation in the lumen of the seminiferous tubules which is an indication of reduced spermatogenesis. In addition, there were losses of testicular parenchymal materials with vacuolations.

The lower and higher values of LH/FSH and FSH/LH ratios respectively in the Group H compared to other treated Groups F and G is a further indication of poor Leydig and Sertoli cells response to the presence of FSH and LH. In addition, the lower values of T/LH values in Group H compared to other treated further confirms the poor response of production of testosterone by the Leydig cells in response to the stimulation of LH. These results further indicate dose-dependent ameliorative tendencies.

The MDA and SOD results also indicated significantly higher and lower values in the Group H compared with other treated groups, which further suggest higher intensity of oxidative stress in the 750mg/kg (Group H) treated group. MDA is a marker of lipid peroxidation resulting from oxidative stress. Therefore, the higher the MDA value, the higher the lipid peroxidation which is an indication of enhanced oxidative stress. On the other hands, SOD is an anti-oxidant enzyme that mop ups free radicals. Therefore, there lower values indicate depletion of this enzyme mostly due to overwhelming activities of free radicals. The poor anti-oxidant status as seen in group H could also account for the significantly higher values of the dead sperm cells compared to other treated groups, F and G. Our biochemical findings also concur with the resut of the histology where loss of testicular parenchymal materials, loss of spermatogonia, and deflagellation of spermatocytes were observed.

**5. CONCLUSION**

This study revealed that consumption of guava leaves extract before exposure to lead acetate does not confer protection in a dose-dependent manner against the disruption of male endocrine system. Rather an unusual lower dose of guava extracts of 250mg/kg were observed to be more protective than 500mg/kg and 750mg/kg.

**DISCLAIMER (ARTIFICIAL INTELLIGENT)**

Author(s) hereby declare that no generative AI technology and text-to-image generators have been used during the writing and editing of this manuscript.

**CONSENT**

It is not applicable.

**ETHICAL APPROVAL**

Animal Ethic committee approval has been collected and preserved by the author (s).

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