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# Protective Effects of Aqueous Extract of *Persea Americana* Leaves Against Secondary Sexual Traits and Histopathological Damages Induced by Antouka Super® in Male Japanese Quails (*Coturnix sp)*

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A B S T R A C T

The control of pesticide's toxicological properties in the food chain could be a benefit to farmers by increasing animal productivity. The current study's objective was to evaluate the protective effects of aqueous extract of *Persea americana* leaves (AEPAL) on secondary sexual traits and testis histology damages induced by Antouka Super® (AS) in male Japanese quail. Sixty male quails of 28 days old and weighing 106-119g were randomly distributed into 5 groups of 12 animals each and daily received for 60 consecutive days one of the treatments: group 1 and 2 received respectively 10 ml of distilled water and 75mg/kg of b.w of AS while groups 3, 4 and 5 in addition to 75mg/kg of b.w of AS, they received respectively 50, 100 and 200 mg/kg of b.w of AEPAL. The sexual behavior characteristics were evaluated during the trial period. At the end of the study, birds were humanly sacrificed, their blood collected for testosterone analysis and the testes removed for teste structure evaluation. Results revealed that exposure to AS significantly decreased testosterone level, time of shouting and appearance of foam, quantity of foam product, the volume and area of the cloacal gland, frequency of mount and frequency of successful mount, as well as degradation of the testes histology. The administration of AEPAL increased these parameters in a dose-dependent manner. Thus, these results demonstrate that AS had a toxic effect on secondary sexual traits and testis histology in male quail which could be alleviated by AEPAL administration at 200mg/kg of b.w.

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## Introduction

Pesticides are chemicals used in agriculture to eliminate pests (insects and weeds) in order to improve the quality and yield of agricultural production (Grasiela et al., 2014). However, because of their toxicological properties, ubiquity, persistence, presence and concentration in the food chain, they are responsible for genotoxicity (Benbrook, 2016), fertility problems such as inhibition of spermatogenesis, decreased motility and viability of spermatozoa, oestral cycle disruption (Andersen et al., 2000; Ngoula et al., 2017) related to the alteration of cells function through the production of free radicals which are responsible for oxidative stress (Banerjee et al., 2001; Amin and Hashem, 2012).

Antouka super® (Pirimiphos-Methyl 16% + Permethrin 3%) is an insecticide commonly used for the preservation of stored foodstuffs (corn, millet, soya, etc.) used as feeds for several animal species such as guinea pigs, rabbits, poultry, etc. These animals are therefore likely to be exposed to pesticide residues through their diet. In fact, in our previous studies (Ngoula et al., 2017), this insecticide has been reported to impair the level of oxidative stress biomarkers such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), sperm motility, viability and increase minor and major sperm anomalies in Japanese quails.

To address these issues, medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. They have an important role as antioxidant agents against the hazard of food contaminants and are solicited because of their availability and low toxicity as well as their variability in bioactive compounds which have antioxidant potential against oxidative stress (Molla et al., 2012; Vijayakumar et al., 2012; Ikpeme et al., 2014).

*Persea americana* is a fruit tree of the family of *Lauraceae* available in tropical regions and contains some of the bioactive compounds such as flavonoids, phenolic compounds, carotenoids, vitamins C and E (Owolabi et al., 2010; Vinha et al., 2013). The extracts of the leaves of this plant have been reported to be efficient against hepatic toxicity, inflammation (Ekor et al., 2006; Ojewole et al., 2006; Anaka et al., 2009; Imafidon and Amaechina, 2010; Noumi et al., 2011) and reproductive toxicity in Japanese quails exposed to Antouka super® (Ngoumtsop et al., 2017a, Ngoumtsop et al., 2017b). Because of its composition, this plant could be used to protect animals from the toxic effects of Antouka super® on sexual behavior and testis histology.

The secondary sexual traits are the set of behavioral and physiological manifestations that indicate the sexual maturity of the animals. They have been reported to be altered or delayed by pesticides in quails (Khan et al., 2008; Ahmad et al., 2012).

Several authors have reported on the protective effects of plant extracts against pesticides on secondary sexual characteristics and testis histology in mammal species (Wankeu et al., 2014, Vemo et al., 2018), but information related to birds remain rare.

This work aims, therefore, to contribute to a better knowledge of the outcome of oxidative stress induced by Antouka super® on sexual behavior and testis structure in Japanese quail as well as its alleviation using aqueous extract of *Persea americana* leaves.

## Material and Methods

### *Birds*

Sixty (60) healthy and 28-days-old Japanese male quails weighing between 106 and 119 g were used in this study. They were obtained from the farm of the Faculty of Agronomy and Agricultural Sciences, University of Dschang, Cameroon. They were housed in specialized wire cages, six per cage, in a centralized birds care facility maintained at 22 to 25°C with a relative humidity of 76 ± 5%, for 8 weeks. Birds were kept in a 12 h light-dark cycle and provided with *ad libitum* water and diet. The experiments were approved by the Ethical Committee of the Department of Animal Science of the University of Dschang (ECDAS-UDs23/02/2015/UDs/FASA/DSAES) and were performed under the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Community guidelines; EEC Directive 86/609/EEC, of the 24th November 1986.

### *Chemical*

Antouka Super® (SYNGENTA, United Kingdom) is a combined insecticide whose active principles are pirimiphos-methyl (0, 2-diethylamino-6-methylpirimidin-4-yl O, O-dimethyl phosphorothioate) concentrated at 19g/kg and permethrin (1*RS*, 3RS; 1*RS*, 3*SR*) - 3- (2, 2-Dichlorovinyl)- 2, 2- dimethylcyclopropane-1- carboxylate (3- phenoxyphenyl)) concentrated at 3g/kg. This insecticide was purchased at the local market of Dschang, Cameroon.

### *Plant Material Collection and Preparation of Extracts*

The plant material was harvested between 8-10 a.m in Dschang, West Region of Cameroon and authenticated at the Cameroon National Herbarium (CNH) under the voucher number 1860/SFR/Cam. Fresh leaves of *Persea americana* were harvested and cut into small pieces, dried and grounded by a blender (Moulinex) into a fine powder. One thousand grams (1000 g) of the crushed leaves were dissolved in 10 L of distilled water, kept for 72 h at 25°C and occasionally stirred. Thereafter, it was filtered through a Whatman N° 3 filter paper and then concentrated in an oven (45°C) for 100 hours to obtain the extraction yield. Then, the aqueous extract was frozen than dried using freeze-dryer. The aqueous extract stock solutions (50, 100 and 200 mg/5mL) were freshly prepared for each set of experiments and stored at 4°C for up to 5 days.

### *Phytochemical Screening*

The qualitative phytochemical evaluation performed on *Persea americana* aqueous extract according to Ramde-Tiendrebeogo et al. (2012) method revealed the presence of phenols, saponins, flavonoids, glucosides, coumarins, alkaloids, tannins, sterols, triterpene, and anthraquinones.

### *Experimental Design*

After an acclimatization period of 1 week, birds were equally divided into five groups repeated twice (6 quails in each) and treated as follows: those of group 1 (negative control) and group 2 (positive control) received respectively 10 ml of distilled water and 75 mg/kg of body weight (b.w) of Antouka Super®. Those of groups 3, 4 and 5 (respectively T1, T2, and T3) in addition to 75 mg/kg of b.w of Antouka Super® received doses 50, 100 and 200 mg/kg of b.w respectively of AEPAL. Antouka Super® and AEPAL were administered orally by gavage for 60 days. The dose of Antouka Super® used in the study was selected from a pilot study and represents 1/15 of $LD_{50}$ value obtained in quails (1125 mg/kg b.w) (Ngoula et al., 2017).

### *Sexual Behavior*

At the end of the experiment, the sexual behavior of male quail was determined. Each male quail was placed in the observation cage chambers for 5 minutes to acclimatize with the cage environment. Sexually receptive egg-laying female quail was then dropped silently from one side of the chamber as a stimulus for 10 minutes. The observations for sexual behavior such as mount latency, mount frequency, and successful mount frequency were recorded. Mount latency was calculated as the time between the introduction of females to the occurrence of the first mount; mount frequency was observed as a total number of mounts within 10 min; successful mount frequency was observed as a total number of mounts followed with ejaculation.

### *Crowing and Foam Production*

Time of shouts and foam occurrence was observed every week during this study for 2 minutes/group and classified according to the method described by Ahmad et al. (2012). The quantity of foam production was assessed by manual pressure on the cloacal gland and ranked according to Marin and Satterlee's (2004) scale ranging from no foam to the maximum quantity of foam produced.

### Cloacal Gland Measurements

Before killing the birds, cloacal gland size measurements (length and width) were taken using a digital caliper. The cloacal gland area and volume were calculated from these measurements according to the formula proposed by Siopes and Wilson (1975) respectively for the area (length of the gland × width) and Chaturvedi et al. (1993) ($4/3 \times 3.5414 \times a \times b^2$, where a=0.5 × long axis and b = 0.5 × short axis) for the volume.

### Testosterone Analysis

At the end of treatment, the birds were humanely sacrificed and their blood collected after sectioning the jugular vein. Serum was prepared and stored at -20°C for serum testosterone analysis which was determined using a commercially available kit (ELISA AccuDiag™, Diagnostic Automation Inc).

### Testis Structure

The right testis of each quail was fixed in Bouin's fluid for 1 week, embedded in paraffin, cut at 5 µm and stained with Harris hematoxylin and eosin. The tissue sections were observed under a light microscope (Leica DM 750, X10 and X40) for morphology and cellular integrity.

### Statistical Analysis

Values are presented as Mean ± SD. ANOVA was performed for comparison with the posthoc Tukey test to compare the level of significance between the control and experimental groups. A value of $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed using XL STAT for Windows 10 Software.

## Results

### Serum Testosterone, Cloacal Gland

Significant ($P<0.05$) decrease in testosterone level, cloacal gland area, and volume was observed in the male quails treated with Antouka Super® (mg/kg) compared to the control (Table 1). However, increasing the dose of the AEPAL induced an increasein these parameters in a dose-dependent manner without reaching the negative control record.

### Crowing and Foam Production

Crowing and foam appearance occurred earlier (in two weeks) in the negative control group compared to the groups treated with Antouka Super® (75 mg/kg) (Figure 1). However, in Japanese quails that were given Antouka Super® (75 mg/kg) with AEPAL, these parameters generally appeared earlier when compared to the positive control group. Besides, the highest quantity of foam was recorded in the negative control group (Note 5) as compared to groups treated with Antouka Super®, while AEPAL administration increased these parameters in dose dependant manner when compared to the positive group (Figure 2).

Table 1. Effects of AEPAL on testosterone level, cloacal gland area and volume of male Japanese quails exposed to Antouka Super®

| Parameters | Doses of AEPAL (mg/kg b.w) | | | | | Probability |
|---|---|---|---|---|---|---|
| | CO⁻ (n=8) | CO⁺ (n=8) | T1 (n=8) | T2 (n=8) | T3 (n=8) | |
| Testosterone | 1.90±0.16a | 0.45±0.00d | 0.49±0.01cd | 0.56±0.01c | 0.87±0.04b | <0.001 |
| CGA ($mm^2$) | 359.43±49.62$^a$ | 201.85±29.04$^c$ | 218.17±25.14$^c$ | 213.30±28.82$^c$ | 264.32±37.34$^b$ | <0.001 |
| CGV ($mm^3$) | 3499.42±669.38$^a$ | 1462.44±314.39$^c$ | 1751.41±404.01$^c$ | 1860.28±335.53$^{bc}$ | 2357.61±529.87$^b$ | <0.001 |

CGA: cloacal gland area; CGV: cloacal gland volume; $CO^-$: Negative control; $CO^+$: 75 mg AS/kg b.w; T1: 75 mg AS+ 50 mg AEPAL/kg b.w; T2: 75 mg AS+100 mg AEPAL/kg b.w; T3: 75 mg AS+200 mg AEPAL/kg b.w. AEPAL: aqueous extract of *Persea americana* leaves, n=number of animal, each value represents mean ±standard deviation; (a,b,c,d) means bearing different letters in a row differ significantly at P≤ 0.05

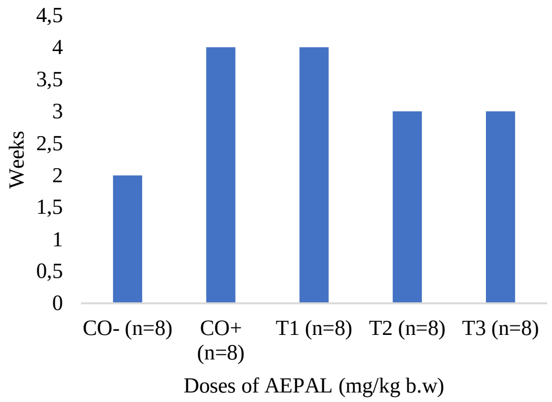


Figure 1. Effects of AEPAL on crowing and foam production of male Japanese quails exposed to Antouka Super®

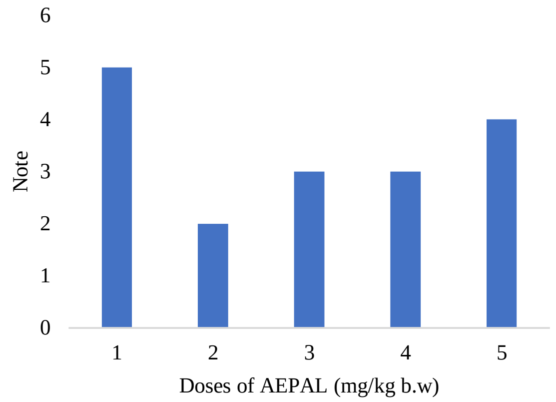


Figure 2. Effects of AEPAL on the quantity of foam product in male Japanese quails exposed to Antouka Super®

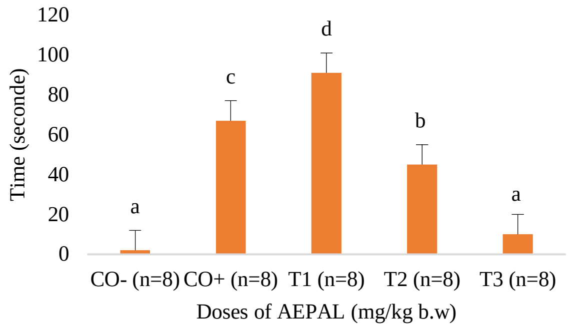


Figure 3. Effects of AEPAL on Mount latency in Japanese quails exposed to Antouka Super®

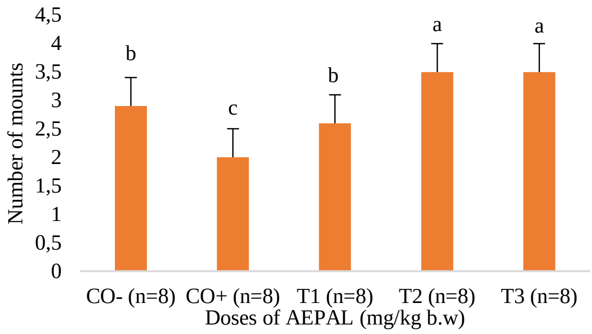


Figure 4. Effects of AEPAL on number of mounts in Japanese quails exposed to Antouka Super®

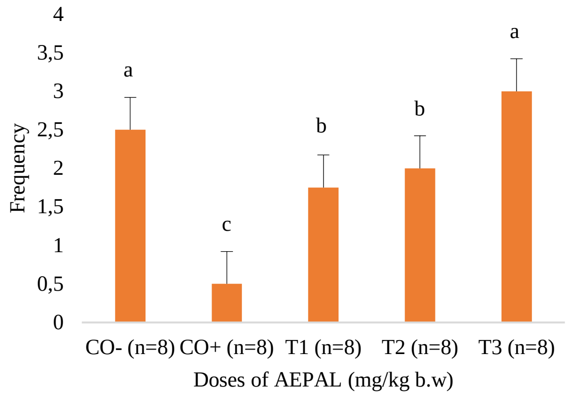


Figure 5. Effects of AEPAL on successful mount frequency in Japanese quails exposed to Antouka Super®

***Sexual Behavior***

The mount latency of male Japanese quails in the presence of females (Figure 3) decreased significantly ($P<0.05$) in the negative control and T3 groups when compared to the positive control, T1 and T2. The inverse trend was recorded for the number of mounts and the successful mount frequency (Figures 4 and 5).

***Testis Structure***

From figure 6, a typical testis structure was observed in Japanese quails from the negative control group. Testes contained normal seminiferous tubes where spermatogenesis was maintained and the germinal epithelium was well organized. Lumen in the same group contained normal flagellated spermatozoa. However, for Japanese male quails from the positive control and T1, more severe changes were observed such as a dramatic depletion in the germ layers of seminiferous tubules with degeneration of connective tissue between seminiferous tubules and increased intertubular space. Japanese male quails from T2 and T3 had a slight degeneration in the germ layers of seminiferous tubules and intertubular space but the lumen contained normal flagellated spermatozoa.

## Discussion

Secondary sexual traits happen when animals, after reaching adulthood, get engaged in specific behavior that later on results in reproductive behavior when males and females get along (Berend et al., 2011). In this study, the protective effect of *Persea americana* aqueous leaves' extract on secondary sexual traits alteration induced by AS in Japanese quails was investigated.

Results revealed that the cloacal gland area and volume in treated birds with AS decreased significantly ($P<0.05$) when compared to the control. These results are similar to findings of Hanafy et al. (2016) in Japanese quails exposed to 1, 5, or 10 mg $kg^{-1}$ b.w of Bisphenol A and those of Krister et al. (1990) in Japanese quails exposed at 6, 19 and 57 ng/g egg of diethylstilbestrol. The decrease in the cloacal gland area and volume reported in this study should be attributed to the decrease in the testosterone level reported in this study. The development of the cloacal gland in quail is under androgen activity and it is considered as an indicator of testicular development, animal maturity and sexual activity (Siopes and Wilson 1975; Delville et al., 1984, Shit et al., 2010). Shit, et al. (2010) reported a direct relationship between cloacal gland size and testicular activity in quail and confirmed the use of cloacal gland area as an indicator straightforward selection marker during the breeding program for detecting sexually active males. Hence, the cloacal gland area and volume are good indicators of the androgen status in the male quails during sexual maturation (Ball and Balthazart, 2010). The volume and area of the cloacal gland were improved by the AEPAL administration ($P<0.05$). Similar results have been reported by Busso et al. (2010) in quails subjected to plasma corticosterone stress. This result could be explained by the presence of flavonoids, phytosterols, saponins, and polyphenols which would increase the production of testosterone by inhibiting the enzyme $P_{450}$ aromatase which catalyzes the conversion of estrogens from androgens (Saitoh et al., 2001). Several studies have reported these compounds to increase the level of testosterone, the main hormone that controls sexual behavior (Hodeck et al., 2002; Yoshitake et al., 2010).

Male of the Japanese quail are unique among birds to possess a well-developed cloacal gland. At their maturity stage, this gland produces a foamy substance that is introduced into the female during sexual intercourse, suggesting that the foam plays a key role in increasing male reproductive success (Adkins-Regan, 1999). The male quails receiving AS begin to crow and produce foam later compared to the positive control ($P<0.05$). Similar results were observed when Japanese quails were exposed to Benomyl at doses of 100, 400 and 1000 mg/kg of b.w. (Khan et al., 2008). However, treatment with the AEPAL improved these characteristics in a dose-dependent manner without reaching the control value.

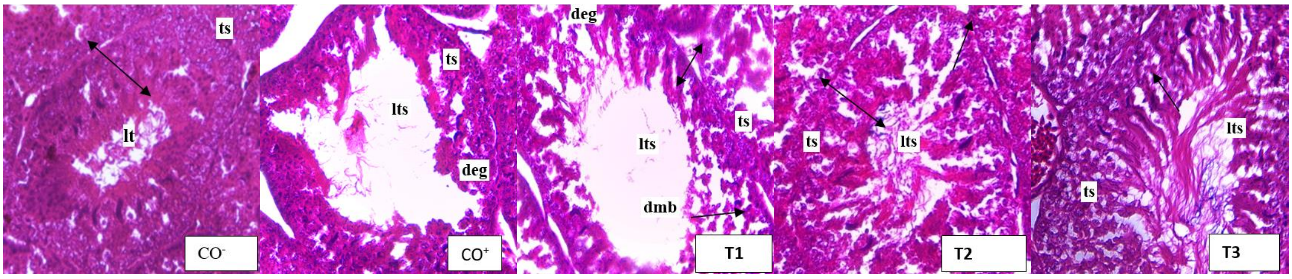


Figure 6: Effects of AEPAL on histological sections of testis of male Japanese quails exposed to Antouka Super® (H&E X 400), CO-: normal testis (negative control), showing normal structure with more spermatozoa in the lumen of seminiferous tubules (lts); CO+: showing dramatic depletion in the germ layers of seminiferous tubes (ts) and degeneration of connective tissue between germ layers (deg), seminiferous tubes are poor in spermatozoa (lts) and increased intertubular space; T1: showing severe degeneration (deg) and space formation in the germ layers of seminiferous tubes (ts) the lumen of these seminiferous tubes also present few spermatozoa (lts) and increased intertubular space; T2: exhibiting slight degeneration in the germs layers of seminiferous tubules (deg) and intertubular space, the lumen of the seminiferous tubes shows more spermatozoa (lts); T3: showing slight degeneration in the germs layers of seminiferous tubules (deg), the lumen of the seminiferous tubes present more spermatozoa.

It is well known that male quail sexual behavior is androgen-dependent and develops in response to circulating testosterone (Balthazart et al., 2003; Phillips-Farfan and Fernandez-Guasti, 2009). Hence, any disturbance on the rise of sexual hormones production by gonads at puberty can lead to adverse effects. The results of the present study show a drastic decrease in the level of this hormone in Japanese quails exposed to AS. These results are in agreement with those of Prakash et al. (2010), on mice exposed to Cypermethrin at a dose of 250mg / kg of b.w. for 28 days. However, the administration of AEPAL resulted in an increase in this parameter in a dose-dependent manner. These results agree with those of Saeid et al. (2011) in cocks treated with aqueous extract of *Zingiber officinale*. The increase in the level of this hormone could be attributed to the phytosterol compounds found in this extract. Gauthaman and Ganesan (2008) and Ahangarpour et al. (2013) reported that saponins are compounds of a steroidal nature that tend the stimulation of steroidogenesis. This compound would stimulate the hypothalamic-pituitary-testicular axis, resulting in the synthesis of testosterone. Steroidal saponins thus have the capacity not only to increase the level of testosterone produced by Leydig cells but also to bind either to the receptors of this hormone or to the enzymes involved in the synthesis to increase their functions (Gauthaman and Ganesan 2008, Ahangarpour et al., 2013). This increase in testosterone level should be associated with a change in sexual behavior (mounting and the frequency of successful mounting) once exposed to AS and treated with AEPAL. Similar results have been reported by Wankeu et al. (2014) in rats exposed to Streptozotocin and treated at 100 and 500 mg/kg b.w of *Dracaena arborea* aqueous leaves' extract. Improvement sexual behavior characteristics could be due to the flavonoids, phenols, and saponins which are reported to increase the level of testosterone (Yoshitake et al., 2010). These compounds induce changes in the level of neurotransmitters involved in erectile function, modulate the action of these neurotransmitters, and thus improve the sexual libido (Suresh et al., 2000).

The testicular histopathology was damaged in Japanese quails exposed to AS, agreeing with findings of Ngoula et al. (2017) in male Japanese quails exposed respectively to 37.5, 56.25 and 75 mg of AS/kg b.w for 49 days and Vemo et al. (2018) in male guinea pigs exposed to 92, 137.5 and 275 mg/kg b.w/day of Cypermethrin for 90 days. The improvement in the testicular structure in animals treated with AEPAL observed in this study might be a consequence of AEPAL bioactive compounds' activity. According to Oguntibeju et al. (2010), antioxidant molecules protect tissues against oxidation and degeneration of free radicals. Thereby, antioxidant compounds such as phenols, flavonoids, and saponins contained in the AEPAL could have prevented the oxidation of the testes tissues in animals exposed to AS, favoring in such a way better spermatogenesis and steroidogenesis.

## Conclusion

This study revealed that *Persea americana* aqueous leaves' extract alleviates secondary sexual traits and histopathological damages induced by Antouka Super® in male Japanese quails when administrated at 200 mg/kg b.w due to its bioactive compounds (phenols, saponins, flavonoids, glucosides, coumarins, alkaloids, tannins, sterols, triterpene, and anthraquinones) extracted from leaves. Hence, it can be used as an alternative to synthetic antioxidants and conditionally applied to alleviate reproduction disturbance in toxicological cases. However, as the values obtained were lower compared to the positive control, further studies are highly recommended with increased doses of this extract on the studied parameters.

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