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Effects of Oxidative Stress Induced by Antouka Super®(Insecticide) on Some Reproductive Parameters of Male Japanese Quail (*Coturnixcoturnix japonica)* and Mitigation Strategies Using Aqueous Leaves Extract of *Persea americana*

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Abstract: In this study, the effect of oxidative stress induced by Antouka Super® on some reproductive parameters of male Japanese quail (*Coturnixcoturnix japonica*) and mitigation strategies using aqueous leaves extract of *Persea americana* were studied.Forty immature male Japanese quails (28 days old), were divided into five groups of 8 animals each subjected to the following treatments: Group 1, birds receivingdistilled water(negative control group: CO^-); Group 2, birds receiving 75 mg of Antouka Super®/kg b.w(positive control group: CO^+); while groups 3, 4 and 5 were administered 50, 100 and 200 mg/ kg b.w of aqueous leaves extract of *Persea americana* (AEPA) respectively together with antouka super at 75 mg/kg. All the test solutions were orally administered once a day for 60 days usingendogastriccanule. Dissection of the *vas deferens* was performed to obtain sperm cells. The protective effects of AEPA on the organ weight, oxidative stress biomarkers, serum hormones and sperm characteristics were evaluated. The results revealed that exposure to Antouka Super® significantly ($p<0.05$) decreased organ weight (testis, epididymis and vas deferens), levels of testicular proteins and serum hormones (LH, FSH and Testosterone). This insecticide also significantly ($p<0.05$) decreased sperms mobility, viability and density and increasedsperm abnormalities (minor and major). In addition, the activities of superoxide dismutase (SOD), total peroxidase (POD) and catalase (CAT) significantly decreased ($p<0.05$) in the testis; while malondialdehyde (MDA) significantly increased ($p<0.05$) compared to the values recorded in birds of negative control group. Administration of AEPA to treated-animals alleviates the reproductive toxicity and testicular oxidative damage induced by Antouka Super®. Inconclusion, exposure ofmale Japanese quail to Antouka Super® induce oxidative stress and deteriorate reproductive parameters; effects that can be alleviated using aqueousleaves extract of *Persea americana*.

Key words: Antouka Super · Reproductive toxicity · Oxidative stress · *Persea americana* extract · Japanese quail

INTRODUCTION

The increased human and animal population, especially in the last few decades, has led to the compelling need to increase food production. The development of high-yielding crop varieties and the formulation of more potent pesticides to aid in the elimination of pests that destroy crops are therefore imperative [1]. Animals and humans are potentially exposed to pesticides either directly through occupational exposure or indirectly via food and water consumption.

Antouka Super® is a broad-spectrum insecticide widely used in agriculture and crop's storage in many countries including Cameroon. It is made up of two insecticides: (Pirimiphos-methyl 16% and Permethrin 3%). Pirimiphos-methyl is a broad-spectrum organophosphate

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insecticide that distresses the nervous system by inhibiting acetyl cholinesterase activity [2]. It is used in agriculture to control insects and mites that infest cereals, fruits, stored grains and cotton. Ngoula *et al*. [3] reported that treatment of adult male rats with pirimiphos-methyl at the doses of (62.5-125) mg/kg b.w for 90 consecutive days alters sperm characteristics due to testicular damages. Permethrin, a pyrethroid insecticide class, is an axonic poison that affects nerve fibers by binding to a protein that regulates the voltage-gated sodium channel [4]. It is used in agriculture to control insects and mites that infest cereals, fruits, stored grains and cotton. Zhang *et al*. [5] showed that permethrin dramatically reduces testosterone levels and sperm counts in adult male mice. Induction of oxidative stress is one of the mechanisms implicated in pesticide induced-toxicity [6-8]. In our previous study, Antouka Super® increased the production of reactive oxygen species which in turn increased lipid peroxidation and decreased the levels of oxidative stress biomarkers, such as superoxide dismutase (SOD), catalase (CAT) and total peroxidase (POD). This insecticide also decreased sperm mobility, viability and density as well as the level of serum testosteroneand increased minor and major sperm anomalies (personal communication). Oxidative stress which results from imbalance in the body's oxidants and antioxidants in favor of the first, is known to induce cellular damage [9]. Under normal circumstances, the body is endowed with effective antioxidant systems, maintaining the antioxidant/pro-oxidant balance at the physiological level. However, in extreme oxidative challenge, such as those observed in pesticide poisoning [10], the body's antioxidant machineries are overwhelmed. Under these conditions therefore, there is need to supply the body with exogenous antioxidants.

In recent years, extensive research works has been focused on the use of natural compounds with antioxidant potential against toxic oxidative materials to reduce their damaging effects. *Persea americana* is a fruit tree of the family of *Lauraceae*, native of South America and growing in the tropics. All parts (roots, fruits, leaves, etc.) are used in traditional medicine for treatment of diseases such as gastrointestinal and respiratory disorders, diabetes, cancer [11, 12] and fertility problems [12, 13]. *Persea americana* contains phenols, flavonoids, carotenoids, steroids, vitamins (A, C and E) [14, 15] and amino acids [16]. Considerable data on the protective effects of plant extracts against pesticides on some reproductive performances in mammal species are documented, but information related to birds are rare. The objective of this study was therefore to contribute to a better knowledge of the consequences of oxidative stress induced by Antouka Super® on the reproductive performances of Japanese quails and to explore some mitigative options by using aqueous leavesextract of *Persea americana*, in order to establish strategies for sustainable management. Specifically, the study aimed to evaluate the effects of oxidative stress induced by Antouka Super® on relative weight of sexual organs, oxidative stress parameters, sexual hormones and characteristics of vas deferens spermatozoa and the remedial effects of aqueous leaves extractof *Persea americana* on these reproductive parameters.

MATERIALS AND METHODS

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 19 g/kg and Permethrin (1*RS*, 3RS; 1*RS*, 3*SR*) - 3- (2, 2- Dichlorovinyl)- 2, 2- dimethylcyclopropane-1- carboxylate (3- phenoxyphenyl)) concentrated at 3 g/kg.

PlantHarvesting and ExtractPreparation: *Persea americana*leaves were collected in October 2015 inDschang, West Region of Cameroon and authenticated at the Cameroon National Herbarium under the voucher number 18604/Sfr/Cam. The plant material was washed, dried in the shade and ground to obtain fine powder. A kilogram of this powder was macerated into 10 liters of distilled water and the mixture was stirred twice a day. After 72 hours, this mixture was filtered with a Whatman N°3 filterpaper, froze for 24 hours and dried using freeze-dryer. The extraction yield was 19,53%. 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.

Phytochemistry of *Persea americana* Aqueous Leaves Extract: Chemical screening of the AEPA revealed the presence of alkaloids, tannins, phytosterols, triterpenes, anthraquinones, phenols, saponins, flavonoids, glycosides andcoumarin.

Experimental Birds: Healthy 28 days male Japanese quails weighing 109 -118 g were used in this study. These birds were kept in specialized wire cages, twelve per cage, in a centralized animal care facility maintained at 22 to 25°C with a relative humidity of 76 ± 5%. Birds were kept in a 12 h light-dark cycle,with free access to water and a laboratory diet.

Ethical Consideration: Experimental protocols used in this study were strictly conformed with 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 [17].

Experimental Design: Birds were randomly divided into five groups of 8 quails each and treated as follows: Group 1, birds receivingdistilled water (negative control group: CO^-); Group 2, birds receiving 75 mg of Antouka Super®/kg b.w (positive control group: CO^+); while groups 3, 4 and 5 were administered 50, 100 and 200 mg/ kg b.w of aqueous leaves extract of *Persea americana* (AEPA) respectively together with antouka super at 75 mg/kg. All the test solutions were administered *per os*once a day for 60 days using an endogastriccanule. The doses of Antouka Super® used in the study were selected from a pilot study and represent 1/15 of LD_{50} value obtained in quails (1125 mg/kg b.w) (personal communication). During the treatment, body weight was weekly measured.

Blood and Organ Collections: At the end of treatments (60th day), blood was collected after sectioning the jugular vein ofeach bird. Serum was prepared and stored at -20°C for subsequent analysis. After blood collection, birds were rapidly killed by decapitation and testis, epididymis and *vas deferens* were carefully removed, freed of adipose tissue, blotted dry and weighed separately. The left testis of each bird was then homogenized at 20% (weight/volume) in cold 0.9% NaCl solution and aliquots of supernatant were kept at - 20°C for biochemical analysis.

Evaluation of Sperm Characteristics: Immediately after the sacrificeof each bird, vas deferens were carefully removed, minced in 10 ml of 0.9% NaCl (40°C) and used to evaluate sperm motility, concentration, viability and morphology. The sperm motility was estimated on scale basis as described by Mamun *et al*. [18]. The sperm viability and morphology were determined using hypo-osmotic swelling test [19] and eosine/nigrosine test respectively [3]. Sperm viability was expressed as percentage of swelled spermatozoa and the morphology expressed as percentage of abnormal sharp sperm. The morphological defects of head, middle-piece, tail and the proportions of cells affected were evaluated. For each of the both parameters (viability and morphology), a total of 200 spermatozoa were counted in at least five different microscope fields according to the protocol descried by Revell and Mrod [20]. The sperm density was determined using Thomas hemocytometer.

Biochemical Analysis: The testis proteins level was determined using CHRONOLAB kit following the manufacturer's protocol. Serum LH, testosterone and FSH levels were determined using commercial kit (ELISA AccuDiag™, Diagnostic Automation Inc, 23963 Craftsman Rd Suites: D/E/F Calabas, ca 91305 USA and ELISA EIA, Gmrh, DRG, 1288 Germany). Total peroxidase (POD) and superoxide dismutase (SOD) activities, malondialdehyde (MDA) level and catalase activity (CAT) were measured in testicular homogenates using spectrophotometer (GENESYS 20.0) according to the methods described respectivelybyHabbu *et al*. [21] and Dimo *et al*. [22] and Kodjo *et al*. [23] and Sajeeth *et al*. [24].

Statistical Analysis: Values are presented as Mean ± SE. One-wayANOVA was performed for comparison with post-hoc Duncan test. At value of $p<0.05$ was considered statistically significant. Statistical analyses were performed with the aid of SPSS for Windows software program (Release 20.0).

RESULTS

Relative Weights of Reproductive Organs: The relative weight of testis, epididymis and vas deferens of birds in group CO+ was significantly lower compare to groups T1, T2, T3 and CO-. Furthermore, the relative weight of testis, epididymis and vas deferens of birdsthat received AEPA,regardless of the doses, showed no significant differences with group CO- (Table 1).

Oxidative Stress Biomarker: As reported in Table 2, oral administration of Antouka Super® (75 mg/kg b.w for 60 consecutive days) led toa significant decrease in the levels of testicular proteins and in the activities of SOD, CAT and POD, as compared to the negative control (CO-). Co-administration of AEPA, in a dose-dependent manner, with 75 mg AS/kg b.w increased the values of testicular proteins, SOD, CAT and POD and decreased the level of MDA (Table 2).

Serum Levels of Reproductive Hormones: The levels of FSH, LH and Testosterone in male Japanese quails exposed to Antouka Super and treated with AEPA are reported in Table 3. Oral administration of AS at 75mg/kg b.w induced a significant ($p<0.05$) decrease in serum

Table 1: Effects of aqueous leaves extract of *Persea americana* on body and reproductive organs weight of male Japanese quails exposed to Antouka Super®

Body and organs weight (g)	Doses of AEPA (mg/kg b.w) CO-(n=8)	CO+(n=8)	T1 (n=8)	T 2 (n=8)	T3 (n=8)
Initial body	113.83±4.45	113.83±4.95	114.33±3.93	113.17±4.53	115.17±2.48
Final body	228.17±2.85^{a}	187.17±16.15^{d}	192.83±12.36^{c}	209.17±8.25^{bc}	216.83±10.18^{ab}
Body gain	110.64±14.29^{a}	71.00±14.95^{cb}	78.50±13.48^{b}	96.00±9.79a^{b}	101.61±9.97^{ab}
Relative weight of reproductive organs (g/100g bw)					
Testis	1.54±0.14^{a}	0.54±0.19^{c}	1.06±0.19^{b}	1.42±0.30^{b}	1.53±0.18^{ab}
Epididymis	0.029±0.003^{ab}	0.023±0.008^{b}	0.032±0.007^{a}	0.027±0.008^{a}	0.027±0.008^{ab}
Vas deferens	0.037±0.008^{a}	0.025±0.007^{b}	0.046±0.008^{a}	0.041±0.008^{a}	0.044±0.006^{a}

n=number of animals, each value represents mean ±standard error

(a,b,c,d) means bearing different letters in a row differ significantly at p< 0.05.

AS: Antouka Super®; CO-: Negative control; CO+: 75 mg AS/kg b.w; T1: 75 mg AS+ 50 mg AEPA/kg b.w; T2: 75 mg AS+100 mg AEPA/kg b.w; T3: 75 mg AS+200 mg AEPA/kg b.w; AEPA: Aqueous leaves extract of *Persea americana*.

Table 2: Effects of aqueous leaves extract of *Persea americana* on oxidative stress biomarkers in the testis of male Japanese quails exposed to Antouka Super®

Oxidative stress Parameters in the testis	Doses of AEPA (mg/kg b.w) CO- (n=8)	CO+ (n=8)	T1 (n=8)	T 2 (n=8)	T3 (n=8)
Testicularprotein (mg/ml)	9.53±0.47^{a}	4.18±0.11^{e}	4.74±0.32^{d}	6.42±0.24^{c}	7.65±0.17^{b}
MDA (nmol/mg tissues)	11.39±1.94^{d}	23.87±1.47^{a}	24.19±1.05^{a}	17.4±1.25^{b}	15.4±1.9^{c}
SOD (UI/mg tissues)	22.47±1.11^{a}	10.66±0,51^{e}	11.48±1.05^{d}	14.37±1.06^{c}	16.96±2.31^{b}
CAT (UI/mg tissues)	6.52±0.32^{a}	4.75±0.12^{d}	4.97±0.08^{d}	5.28±0.06^{c}	5.54±0.1^{b}
POD (μM/mg tissues)	19.02±0.40^{a}	12,93±0,33^{d}	12.98±0.24^{d}	13.86±0.31^{c}	16.65±0.49^{b}

n=number of animals, each value represents mean ±standard error

(a,b,c,d,e) means bearing different letters in a row differ significantly at p<0.05.

MDA: Malondialdehyde;SOD:Superoxide dismutase; CAT:Catalase and POD:total peroxidase

AS:AntoukaSuper®; CO-: Negative control; CO+: 75 mg AS/kg b.w; T1: 75 mg AS+ 50 mg AEPA/kg b.w; T2: 75 mg AS+100 mg AEPA/kg b.w; T3: 75 mg AS+200 mg AEPA/kg b.w;AEPA: Aqueous leaves extract of *Persea americana*.

Table 3: Effects of aqueous leaves extracts of *Persea americana*on serum hormones of male Japanese quails exposed to Antouka Super®

Hormones characteristics (ng/ml)	Doses of AEPA (mg/kg b.w) CO- (n=8)	CO+ (n=8)	T1 (n=8)	T 2 (n=8)	T3 (n=8)
FSH	4.08±0.37^{a}	1.35±0.18^{c}	1.47±0.10^{c}	1.83±0.68^{c}	2.98±0.32^{b}
LH	3.46±0.13^{a}	0.7±0.14^{d}	0.7±0.12^{d}	1.58±0.07^{c}	1.88±0.07^{b}
Testosterone	1.91±0.16^{a}	0.46±0.01^{d}	0.49±0.01^{cd}	0.56±0.01^{c}	0.87±0.05^{b}

n=number of animals, each value represents mean ±standard error

(a,b,c,d) means bearing different letters in a row differ significantly at p<0.05.

FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone

AS: Antouka Super®; CO-: Negative control; CO+: 75 mg AS/kg b.w; T1: 75 mg AS+ 50 mg AEPA/kg b.w; T2: 75 mg AS+100 mg AEPA/kg b.w; T3: 75 mg AS+200 mg AEPA/kg b.w; AEPA: Aqueous leaves extract of *Persea americana*.

Table 4: Effects of aqueous leaves extract of *Persea americana* on some sperm characteristics of male japanese quails exposed to Antouka Super®

Spermcharacteristics	Doses of AEPA (mg/kg b.w) CO- (n=8)	CO+ (n=8)	T1 (n=8)	T 2 (n=8)	T3 (n=8)
Mobility (%)	4.16±0.51^{a}	2.41±0.66^{c}	2.16±0.40^{c}	3,08±0.20^{b}	3.25±0.52^{b}
Viability (%)	75.67±5.37^{a}	36.17±3.76^{d}	37.50±4.13^{d}	44.33±6.74^{c}	62.83±6.43^{b}
Number/vas deferens (10^{9})	2.32±0.12^{a}	0.67±0.052^{d}	0.74±0.056^{d}	0.92±0.05^{c}	1.35±0.08^{b}
Semen morphology (%)					
Major anomalies	5.83±2.13^{c}	19.83±1.47^{a}	12.83±3.71^{b}	13.83±2.13^{b}	8.17±2.13^{c}
Minor anomalies	8.83±2.04^{c}	22.50±3.27^{a}	21.67±2.58^{a}	16.83±3.12^{b}	16.17±2.63^{b}

n=number of animals, each value represents mean ±standard error

(a,b,c,d) means bearing different letters in a row differ significantly at p<0.05.

AS: Antouka Super®; CO-: Negative control; CO+: 75 mg AS/kg b.w; T1: 75 mg AS+ 50 mg AEPA/kg b.w; T2: 75 mg AS+100 mg AEPA/kg b.w; T3: 75 mg AS+200 mg AEPA/kg b.w; AEPA: Aqueous leaves extract of *Persea americana*.

FSH, LH and Testosterone concentrations. AEPA administration significantly increased levels of theses hormones as compared to the positive control (CO+) group.

Sperm Traits: Spermmotility, viability anddensity per vas deferens in treated groups significantly decreased ($p<0.05$) in AEPA treated birds compared to those of negative control group (CO-) (Table 4). Generally, birds co-treated with AS and AEPA demonstrated asignificant ($p<0.05$) increase in their sperm mobility, viability and density per vas deferens when compared to the positive control group (CO+) values. The inverse was recorded with major and minor anomalies.

DISCUSSION

The protective effects of the aqueous leaves extract of *Persea americana* on reproductive toxicity induced byAntouka Super® were investigated in male Japanese quail. The reduction in the relative weight of the testis, epididymis and vas deferens in the Antouka Super® treated quails indicates the toxic effects of Antouka Super® (AS) on these reproductive organs. This study corroborates the observations of Ngoula*et al*.[3] who reported that exposition of adult male rat toPirimiphos-methyl (62.5-125 mg/kg b.w for 90 consecutive days)decreased the weightof reproductive organs. Testicular weight is a valuable index of reproductive toxicity in male animals and the decrease in testicular weight was consistent with elimination of germ cells [25]. Co-administration of AS and aqueous leaves extract of *Persea americana* clearly restored the reproductive organ weight towards normal which may be due to its androgenic activity. The restoration of the weight of reproductive organs may be due to the presence of saponin and sterol revealed in this extract, which may belong to the steroidal family [26], which could possess an androgenic property[27]. The weight, size and secretory function of testes and epididymis are closely regulated by androgens [28]. In fact androgens, especially testosterone have anabolic properties which are characterized by an increased synthesis of proteins and therefore muscle mass. Androgens then contribute to the increase of the volume and the weight of testis and epididymis by stimulating protein synthesis [29, 30]. Sperm parameters such as density, motility, viability and morphology are key indices of male fertility, as these are the prime markers in testicular spermatogenesis. The decrease of the values of these parameters by AS treatment in our study corroborates with the findings of Ngoula *et al*. [3] who reported that treatment of adult male rat with Pirimiphos-methyl decreased sperm density and motility; and increased abnormal sperm morphologies. Low FSH and testosterone concentration may be responsible of the adverse effects of AS on sperm parameters, as high level of these hormones in testis is critically required for normal spermatogenesis, development / maintenance of sperm morphology and normal physiology of seminiferous tubules [31, 32].

After exposure of male quail to AS for 60 days, a decrease in serum level of FSH, LH and testosterone was recorded. The decrease of the levels of these hormones may be either due to the direct effect of AS on the androgen biosynthesis pathway in the testis or to its effect on brain hypothalamus/anterior pituitary gland and indirectly affecting testosterone biosynthesis in the quail testes. The decrease of testosterone levelmay also be induced by the stimulation of P_{450} aromatase (P_{450}arom), which catalyzes estrogen production from androgen; thereby decreasing androgen levels. Co-treatment of quails with AS and aqueous leaves extract of *Persea americana* attenuated spermatogenic damages induced by AS treatment. In addition, the increase of testosterone, LH and FSH levels after co-administration with AS and aqueous leaves extract of *Persea americana* might have stimulated the production of quantitatively and structurally normal spermatozoa. The restoration of sperm characteristics and hormones concentration in intoxicated birds treated with AEPA might be due to antioxidative properties of phenols and flavonoidsfound in the extract. Spermatogenesis requires LH and FSH for initiation and maintenance in male quails. LH stimulates Leydig cells to secrete testosterone.Normal testicular function is dependent of FSH and testosterone is absolutely required for normal spermatogenesis. The spermatozoa, in common with all cell types have developed an antioxidant defense system consisting of enzymes such as catalase (CAT), superoxide dismutase (SOD) and Total peroxidase (POD) that scavenge and regulate the production of ROS [33, 34]. Estimation of end products of lipid peroxidation such as malondialdehyde (MDA) is an index of the extent of oxidative damage to cellular structures [33]. In this study, Antouka Super® treated quails showed an increase in MDA level. This increased of MDA concentration might be linked to a decreased production of antioxidants in the Antouka Super® treated quails tissues thereby shifting the delicate balance in favor of ROS thus leading to a plethora of pathologic damages to sperm cells and concomitant loss of their function [35, 36]. SOD concentrations and the

activities of CAT and POD in the testis were observed to be significantly reduced in Antouka Super® (AS) treated quails. These results are comparable to those found by Gultekin *et al*. [6], Altuntas *et al*. [37] and Tuzmen *et al*. [38]. This decrease of antioxidant capacity (SOD, CAT and POD)was accompanied with a significantincrease of MDA. It has also been suggested that AS generate free radicals. These free radicals interfere with the antioxidative defense system in the testis and results in the tissue injury. Studies have also shown that levels of ROS correlate with sperm motility [39]. ROS appears to play a role in the apoptosis of spermatozoa and consequently a decrease of sperm count. Therefore, overproduction of free radicals and hence oxidative stress may account at least in part to the testicular toxicity associated with AS treatment. There was an inhibition of peroxidase seen by decrease in MDA level; increase of POD, CAT and SOD activities in male Japanese quail co-treated with AS and aqueous leaves extract of *Persea americana*. This result corroborates with the work of Hawazen and Lamfon [40] in male rat concomitantly treated with carbendazim andaqueous seed extract of fenugreek. This could be attributed to the antioxidative compounds (polyphenols and flavonoids) found in this extract that protect cell membrane from the deleterious action of oxidative attack and reduces lipid peroxidation.

CONCLUSION

In conclusion, oxidative stress induced by Antouka Super® (AS) significantly reduces the weight of reproductive organs, sperm characteristics and serum hormones levels manifested by induction of lipid peroxidation and depletion of antioxidative enzymes in testis of male Japanese quail. In contrast, treatmentof quails with aqueous leaves extract of *Persea americana* inhibit the reproductive toxicity and oxidative damage induced byAS. Thus, aqueous leaves extract of *Persea americana* could be used as an alternative to alleviate the effects of oxidative stress induced by(AS)in male reproductive system.

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