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Attenuating effects of *Mangifera indica* leaves ethanolic extract against acetamiprid induced reproductive toxicity in male guinea pigs

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Abstract

Acetamiprid (ACP) belonging to the neonicotinoid family used against wide array of pests in agriculture and domestic purposes. In this study, we evaluated the attenuating effects of ethanolic extract of *Mangifera indica* leaves (EEMI) in averting reproductive toxicity caused by ACP in male guinea pigs. Thirty male guinea pigs were randomly assigned to five treatment groups (n = 6). Group 1 (T0) received distilled water orally; group 2 (T0-) was given 80 mg kg^{-1} of ACP and groups 3, 4 and 5 were treated, respectively, with EEMI at doses of 50, 100 and 200 mg kg^{-1} plus ACP. After 90 days, the reaction time, sexual organ weights, sperm count, motility and anomalies, spermatozoa with entire plasma membrane, testicular histology, serum testosterone concentration, testicular malondialdehyde (MDA) level, reduced glutathione (GSH) concentration, testicular superoxide dismutase (SOD) and catalase (CAT) activities were assessed. Co-administration of EEMI significantly reduced the reaction time, sperm anomalies and testicular MDA, SOD and CAT levels compared to the T0- group. Co-treatment of EEMI significantly alleviated sperm count and motility, percentage of spermatozoa with the normal plasma membrane, serum testosterone concentration, accessory sex gland weights, and testicular GSH concentrations. The ACP treatment induced cell membrane degradation in the testis and this effect was prevented with the addition of EEMI. In conclusion, ACP negatively affected the animal reproductive function and induced oxidative stress. The addition of EEMI alleviated the toxic effects of ACP on the reproductive function of male guinea pigs.

Introduction

The use of pesticides in farms and animal husbandry has been reported to threaten animal health leading to productivity restrictions. This usually occurs either from direct exposure or indirectly from contaminated feeds or water by such chemicals.[1] The toxic action of pesticides may be due to their ability to induce free radicals generation and accumulation in the body leading to oxidative stress,[2] a situation resulting from an imbalance between the free radicals generation and the system scavenging capacity of antioxidants,[3,4] in favor of the former. Oxidative stress can interfere with male reproductive system including spermatogenesis alteration, sperm DNA damaged cell membrane structure and fluidity disturbance, with consequences as sperm functions reduction.[5,6] This is because sperm cells, thanks to their richness in poly-unsaturated fatty acids, are more sensitive to oxidative damage.[7] In male reproductive function, acetamiprid (ACP) has ability to induce oxidative stress.[8,9]

Herbalism is an alternative practice referring to the use of a plant's parts or their extracts and essential oils for medicinal purposes. It has increased recently and attracted as well tremendous attention of many types of research all over the world. Therefore, natural plant products have been used to enhance male fertility and management of some physiological disorders.[10] This is because, they are rich in many natural antioxidants like phenols, flavonoids, terpenoids, and xanthons. Also, they are easily accessible, cheap and relatively safe.[11]

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Mangifera indica L. (Anacardiaceae) is a popular tropical fruit-bearing tree in the world.[12] The leaves of *M. indica* are used medicinally to treat ailments such as asthma, cough, diarrhea, dysentery, pains, malaria and diabetes.[13-15] Studies have shown that mango leaves extracts are rich in phenolic compounds with strong antioxidant power, particularly mangiferin, commonly named as super-antioxidant.[16-18] The extract of *M. indica* has shown protective effects against lipid peroxidation,[17] anti-fungal activity,[19] anti-ulcerogenic,[20] and aphrodisiac property.[21] However, effects of *M. indica* leaves against ACP-induced reproductive toxicity in male have not been reported so far. Hence, this study was undertaken to investigate the effects of ethanolic extract of *M. indica* leaves (EEMI) on ACP- induced reproductive toxicity in male guinea pigs.

Materials and Methods

Animals and lodging. Thirty adult male guinea pigs reared at Teaching and Research Farm of University of Dschang, Dschang, Cameroon were used. They were four months old and weighed 410.89 ± 39.15 g at the start of the trial. The animals were housed in well-controlled conditions at room temperature (25.00 ± 2.00 ˚C) and 12 hr light/dark cycle. Experimental protocols used in this study were approved by the Ethical Committee of the Department of Animal Science of the University of Dschang (ECDAS-UDs 23/02/2015/UDs/FASA/DSAES) and was in conformity 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.

Animal feeding, pesticide and plant material. Animals were fed a basal ration of elephant grass and a supplement of compound feed. The pesticide used was 20.00%. ACP commercialized under the name of OPTIMAL 20 SP, purchased from the Louis Dreyfus Commodities Society, industrial zone Bonaberi-Douala, Cameroon. Fresh and mature leaves of *M. indica* (Anacardiaceae) were collected around the campus of the University of Dschang, Dschang, Cameroon in February 2017. The plant was identified by the National Herbarium of Cameroon with a specimen number of 18646/SRFCAM. The leaves were washed thoroughly with tap water, shade-dried and crushed to a fine powder. The dried powdered leaves of *M. indica* (1000 g) were extracted in ethanol (5.00 L) and allowed to stand at room temperature for about 72 hr. The percolate was collected and dried in a rotary evaporator at 60 ˚C to obtain EEMI.

Animal treatment. Thirty animals were divided into five groups of six animals each including group 1 (T0) received distilled water orally; group 2 (T0-) treated with 80 mg kg^{-1} of ACP (dose having the most adverse effects in all studied parameters from our previous study[22]) and groups 3, 4 and 5 received both ACP (80 mg kg^{-1}) and 50, 100 and 200 mg kg^{-1} of EEMI, respectively. The animals were treated for 90 days (to ensure a complete cycle of spermatogenesis and seminal epithelial cycle). The doses of the insecticide and ethanolic extract (doses of the plant extract were chosen from earlier works[23]) were read-justed weekly according to the weight of guinea pigs. Table 1 shows the bioactive compounds detected using phytochemical tests according to Harborne.[24]

Table 1. Phytochemical screening of ethanolic extract of *Mangifera indica* leaves.

Compounds	Ethanolic extract
Alkaloids	-
Flavonoids	+
Phenolic compounds	++
Saponins	++
Steroids	++
Tannins	++
Triterpens	+

- : Absent; + : Present; ++ : Highly present.

Reaction time. A week prior to sacrifice, each animal was housed with an adult female and the chronometer was set off. This latter was stopped as soon as the reactions including pursuit of the female, descriptive curves around the female, smelling of the ano-genital tract and attempt to mount were observed from the male and time was noted. The maximum observation time for any possible reaction of the male in the presence of female was 5 min.

Sexual organ weights. Ninety days after the treatment, the animals were sacrificed and organs including testes, epididymides, vas deferens, and accessory sex glands were removed and weighed.

Serum testosterone concentration. Blood was collected by cardiac puncture using a syringe and used for serum testosterone level quantification using ELISA method based on the instructions of the kit (Omega Diagnostics Ltd., Alloa, Scotland).

Sperm characteristics. Immediately after dissection, the epididymal tails were minced using surgical scissors in 5.00 mL of 0.90% NaCl solution (at 37.00 ˚C) for sperm concentration, motility, cell membrane integrity, and morphology evaluations. For sperm motility, a drop of the homogenous suspension was placed on a pre-warmed slide, covered with a coverslip and evaluated under the light microscope at 400× magnification. The motility score was attributed according to Baril *et al*. using a scale from 0 to 5.[25] The sperm count was evaluated using Thoma hemocytometer (Thermo Scientific, Waltham, USA), while the percentages of sperm with small head, big head and coiled tails and spermatozoa plasma membrane integrity were evaluated using an eosin-nigrosin solution and the hypo-osmotic test, respectively.

Oxidative stress parameters. A 15.00% (w/v) homogenate was prepared using the right testis. Each testis was crushed in a porcelain mortar containing 0.90%

NaCl and centrifuged (D-78532; Hettich Zentrifugen, Tuttlingen, Germany) at 3,000 rpm for 30 min and the supernatant was used for biochemical analyses. The malondialdehyde (MDA) and reduced glutathione (GSH) concentrations were determined by the thiobarbituric acid method of Nilsson *et al*. and potassium iodate method of Habbu *et al*., respectively.[26,27] Meanwhile, the superoxide dismutase (SOD) and catalase (CAT) activities were assessed according to Misra and Fridovich and the chromic acetate method as described by Sinha, respectively.[28,29]

Testicular histology. The left testes were fixed in 10.00% formalin, then washed, dehydrated with ascending grades of alcohol bath, clarified in xylene immersion, embedded in paraffin, cut at 5.00 µm thickness and stained with Hematoxylin and Eosin. The sections were observed under a light microscope (400× magnification).

Statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS (version 20.0; SPSS Inc., Chicago, USA) software followed by post-hoc Duncan's test. Results were expressed as the mean ± standard deviation. The $p < 0.05$ indicates a statistically significant difference between groups.

Results

Reaction time. The reaction time decreased significantly ($p < 0.05$) in groups co-treated with 100 or 200 mg kg^{-1} of EEMI compared to T0- group (Fig. 1A).

Reproductive parameters. The accessory sex glands weights significantly increased ($p < 0.05$) in the control animals and group treated with both ACP and 100 mg kg^{-1} of EEMI compared to the group received ACP alone. The epididymides and vas deferens weights increased in T0 group and animals treated with ACP and EEMI compared to guinea pigs given only ACP, no significant ($p > 0.05$) difference was observed (Table 2).

The sperm motility, spermatozoa count and the percentage of spermatozoa with entire plasma membrane augmented significantly ($p < 0.05$) in controls and groups co-receiving EEMI compared to T0- group. Meanwhile, the percentage of sperm anomalies declined significantly ($p < 0.05$) in animals given ACP and 50 or 100 mg kg^{-1} of EEMI with reference to T0- group (Table 2). Moreover, guinea pigs co-exposed to different doses of EEMI showed higher concentration of testosterone compared to T0- group (Fig. 1B).

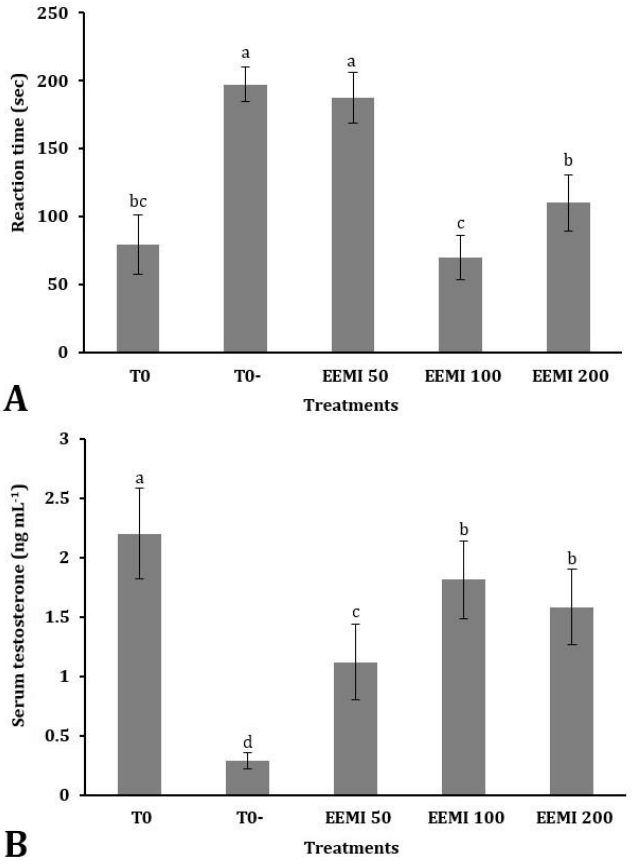

Fig. 1. Effects of different concentration of ethanolic extract of *Mangifera indica* leaves (EEMI) on male guinea pigs exposed to acetamiprid (ACP). **A)** Animal reaction time and **B)** Animal serum testosterone levels.

Table 2. Effects of ethanolic extract of *Mangifera indica* leaves on reproductive parameters in male guinea pigs (n = 6) exposed to acetamiprid (ACP).

Reproductive parameters	Treatments*				
	T0	T0-	EEMI 50	EEMI 100	EEMI 200
Organ weights (g per 100 g of body weight)					
Testes	0.23 ± 0.03^{a}	0.16 ± 0.04^{b}	0.18 ± 0.04^{ab}	0.18 ± 0.04^{ab}	0.19 ± 0.04^{ab}
Epididymides	0.10 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.10 ± 0.03	0.08 ± 0.03
Vas deferens	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.01
Accessory glands	0.24 ± 0.06^{a}	0.15 ± 0.04^{b}	0.20 ± 0.05^{ab}	0.25 ± 0.07^{a}	0.21 ± 0.07^{ab}
Epididymal spermatozoa characteristics					
Motility (%)	90.00 ± 7.56^{a}	62.50 ± 12.58^{b}	85.71 ± 20.70^{a}	86.00 ± 8.94^{a}	88.33 ± 7.53^{a}
Count per g of tail (×10^{6})	39.80 ± 6.03^{a}	18.92 ± 4.30^{c}	38.55 ± 6.37^{a}	27.80 ± 3.14^{b}	38.59 ± 4.72^{a}
Spermatozoa with Integral plasma membrane (%)	74.89 ± 12.20^{a}	38.00 ± 9.61^{b}	63.29 ± 9.46^{a}	64.40 ± 11.67^{a}	64.00 ± 9.30^{a}
Micro and macro cephalies (%)	4.22 ± 1.09^{bc}	12.83 ± 1.47^{a}	4.57 ± 0.79^{b}	3.20 ± 0.84^{c}	1.33 ± 0.52^{d}
Coiled tails	1.00 ± 0.50^{b}	4.17 ± 0.98^{a}	1.29 ± 0.49^{b}	1.60 ± 1.34^{b}	3.83 ± 1.17^{a}

*T0: Neutral control; T0-: ACP-only (80 mg kg^{-1} of ACP); EEMI: Ethanolic extract of *Mangifera indica* leaves.
^{ab} Same letters indicate no significant differences in each row ($p > 0.05$).

Testicular histopathology. In the T0 group, the arrangement of cells from the basal membrane to the lumen was normal with the presence of morphologically mature spermatozoa in the lumen (Fig. 2A). This arrangement was disturbed in T0- group (Fig. 2B) and the group received 50.00 mg kg^{-1} of EEMI (Fig. 2C) with few immature germinal cells observed in the lumen and the basal membrane of the seminiferous tubules was degraded in T0- group. Testes of guinea pigs treated with higher doses (100 or 200 mg kg^{-1}) of EEMI showed almost normal structure (Figs. 2D and 2E).

Oxidative stress indicators. Generally, the testicular MDA concentration and SOD and CAT activities (Table 3) significantly decreased ($p < 0.05$) in ACP+EEMI groups compared to the T0- group. Contrarily, the testicular levels of GSH significantly increased ($p < 0.05$) in guinea pigs receiving ACP and 100 or 200 mg kg^{-1} of EEMI compared to T0- group.

Discussion

In the present study, the phytochemical screening of plant extract revealed the presence of flavonoids, phenols, saponins, steroids, tannins, and triterpenes. Hence, due to these bioactive molecules with possible antioxidant and androgenic activities, the EEMI was administered to male guinea pigs in order to alleviate reproductive toxicity caused by ACP. The weight of reproductive organs increased in guinea pigs given both ACP and EEMI compared to the negative control. The positive effects of EEMI on the reproductive organs could be due to the presence of androgenic activities of bioactive constituents (steroids and saponins) in this plant. Indeed, reproductive organs development and function are under androgenic control,[30] hence, the presence of these compounds helped in maintaining the structure of reproductive organs. Also, antioxidant compounds (i.e., flavonoids and phenols) of EEMI could prevent testicular tissues oxidation in animals treated with ACP, enabling better development of germ cells. The increase in testosterone production in this study might have led to increased libido by decreasing the reaction time. This could be due not only to the antioxidant compounds present in the *M. indica* leaves but also to the androgenic properties of molecules such as steroids, triterpenes and saponins. In fact, androgens have been reported to be important modulators of male sexual behavior including libido.[31] Also, the antioxidant molecules might have protected the structure and function of the testes leading to the increase of androgens and thus their effects on the libido. These observations are in agreement with results reported previously recording a significant increase in the serum testosterone level in guinea pigs co-treated with cypermethrin and *Bersama engleriana* extract.[32] The administration of *M. indica* leaves markedly protected against the adverse effects of ACP by normalizing the sperm count, motility and plasma membrane integrity and decreasing the percentage of abnormal spermatozoa. These effects might be due to the presence of antioxidant compounds found in the EEMI being able to counteract the effects of free radicals by preventing lipid peroxidation. As a matter of fact, mango polyphenols like

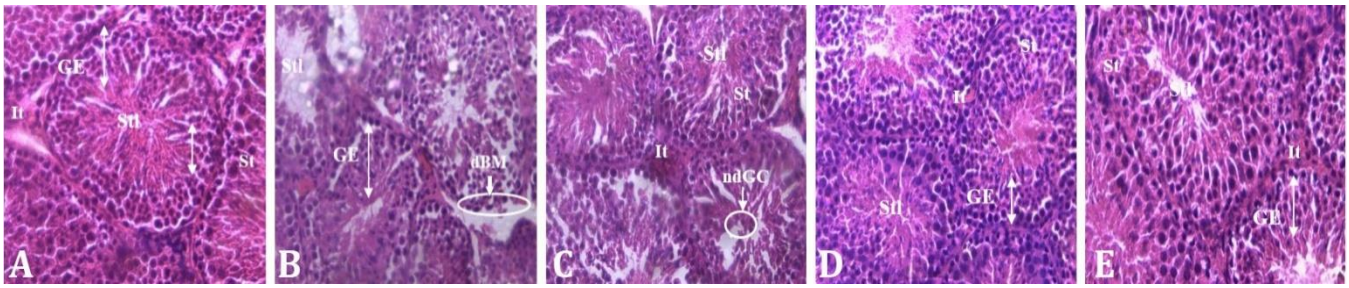

Fig. 2. Cross-sections of testes in guinea pigs exposed to acetamiprid (ACP) and treated with ethanolic extract of *Mangifera indica* leaves (EEMI). **A)** Control; **B)** ACP-only (80 mg kg^{-1} of ACP); **C)** ACP+EEMI 50; **D)** ACP+EEMI 100; **E)** ACP+EEMI 200. dBM = Degraded basal membrane; ndGC = Non-differentiated germinal cells; Is= Interstitial space; Stl = Seminiferous tubule lumen; It = Interstitial tissue; St = Seminiferous tubule; GE = Germinal epithelium., (H&E; 400×).

Table 3. Effects of ethanolic extract of *Mangifera indica* leaves on the oxidative stress indicators in male guinea pigs' testis (n = 6) exposed to acetamiprid (ACP).

Oxidative stress indicators	Treatments*				
	T0	T0-	EEMI 50	EEMI 100	EEMI 200
MDA (µM mg^{-1})	36.29 ± 4.10^{b}	50.27 ± 7.83^{a}	47.38 ± 3.75^{a}	36.42 ± 5.92^{b}	37.71 ± 6.67^{b}
GSH (µM g^{-1})	205.3 ± 28.43^{a}	122.2 ± 38.78^{c}	146.47 ± 39.33^{bc}	178.53 ± 31.38^{ab}	182.17 ± 41.81^{ab}
CAT (µmol min^{-1})	13.73 ± 1.42^{b}	21.19 ± 2.85^{a}	15.63 ± 1.71^{b}	14.92 ± 0.93^{b}	14.07 ± 1.19^{b}
SOD (U mg^{-1})	7.91 ± 1.34^{c}	15.19 ± 3.62^{a}	8.63 ± 2.27^{bc}	11.59 ± 2.51^{b}	7.55 ± 2.00^{c}

MDA: Malondialdehyde; GSH: Reduced glutathione; CAT: Catalase; SOD: Superoxide dismutase

*T0: Neutral control; T0-: ACP-only (80 mg kg^{-1} of ACP); EEMI: Ethanolic extract of *Mangifera indica* leaves.

^{abc} Same letters indicate no significant differences in each row ($p > 0.05$).

other polyphenolic compounds, mainly work as antioxidants protecting cells against damage due to oxidative stress leading to lipid peroxidation.[16] Since the plasma membrane of spermatozoa is rich in polyunsaturated fatty acids, sperm cells are susceptible to lipid peroxidation. Thus, lipid peroxidation increase can impair sperm motility, increase morphological changes in sperm and decrease sperm count.[33] The antioxidant compounds present in plants could, therefore, protect spermatozoa DNA against free radicals and improve sperm characteristics.[34] The basal membrane re-arrangement of the seminiferous tubules in groups given ACP and EEMI might be due to the antioxidant bioactive molecules (i.e., tannins, flavonoids, and phenols) protecting the membrane from oxidation. The decreases in testicular concentration of MDA and SOD and CAT activities in guinea pigs exposed to ACP and EEMI in the present study are similar with the former observations in guinea pigs exposed to 137.50 mg kg^{-1} of cypermethrin and 100 or 200 mg kg^{-1} of *Bersama engleriana* extract.[32] This might be as a result of the action of antioxidant compounds contained in the ethanolic extract of mango leaves. The antioxidant molecules could have neutralized free radicals by transferring protons[35] or inhibiting enzymes responsible of their production such as aldose reductase, lipoxygenase and phospholipase[36] resulting in cytoprotection against ACP-induced oxidative stress. Indeed, phenolic compounds found in *M. indica* directly contribute to antioxidative actions as they are regarded to be the most important antioxidative components.[17] Such molecules and their actions can prevent the lipid peroxidation of lipid membranes, thereby reducing the MDA concentration and antioxidant enzymes (SOD and CAT) activities. The decrease in the concentration of MDA might explain the concomitant increase in GSH level which is implicated in the degradation of this latter.

It can be concluded that EEMI is capable of mitigating the toxic effects of ACP on male guinea pigs reproductive system. This plant can, therefore, be a source of bioactive compounds with anti-toxicity potential against pesticide exposure.

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Conflict of interest

The authors declare that there is no conflict of interest.

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