



The edible plant *Amaranthus hybridus* (Amaranthaceae) prevents the biochemical, histopathological and fertility impairments in colibri®-treated female rats

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ABSTRACT

Colibri® a commercial formulation of Imidacloprid severely impairs the reproductive function. This study aimed at evaluating the preventive effects of *Amaranthus hybridus* on the reproductive toxicity of colibri® in female rats. Eighty rats (n = 10/group) were orally treated with colibri® (22.5 mg/kg) and co-administered with either aqueous or methanolic extracts of *A. hybridus* (55 or 110 mg/kg) within four weeks. Control animals received either distilled water (10 ml/kg), clomiphene citrate or vitamin E. Starting from day 18 of treatment till the end, half of animals in each group (n = 5) was used for the fertility test whereas the remaining rats were kept under treatment until sacrifice. Blood, ovaries, uterus and vagina were collected after sacrifice for measurement of sexual hormones, oxidative stress markers and histological assessment. Exposure of female rats to colibri® was followed by a significant reduction (p < 0.05) in the ovarian and uterine weights, LH, FSH, estradiol and progesterone levels as well as ovarian superoxide dismutase, catalase and peroxidase activities. Moreover, alteration of ovaries, uteri and vagina histology, increase in MDA concentration, decrease in fertility and parturition indices and, pup's viability were recorded. Co-administration of colibri® and plant extracts significantly (p < 0.05–0.001) prevented the above-mentioned damages through biochemical parameter regulations. These results suggest that *A. hybridus* exerts a preventive effect against colibri®-induced female reproductive toxicity.

1. Introduction

Pesticides are a large group of heterogeneous chemicals widely used in agriculture as seed preservatives (corn, soybeans and beans), soil and leaf treatment in a variety of crops and orchards, and to control sucking insects [1,2]. The growing power of the agricultural sector not only has positive effects, it rather greatly harms the environment and affects human health [3,4]. At the environmental level, pesticides cause pollution of underground water sources and are a serious threat to aquatic life [5,6]. Humans can be intoxicated via direct or indirect contact through consumption of contaminated water or foods cultured with pesticides [7,8]. During the recent decade, imidacloprid (IMI) has been classified as the most used neonicotinoid insecticides despite several reports highlighting its toxicity toward non-target organisms

including humans [9].

Colibri®, a commercial formulation of IMI, is a widely used insecticide because of its broad spectrum of action [9]. Exposition to IMI causes severe toxicity on the female reproductive tract, marked by follicular damages leading to infertility [8,10]. Several physiological pathways have been proposed to explain the genesis of infertility caused by colibri®. IMI, acts centrally by binding to nicotinic $\alpha 7$ receptors ($\alpha 7nAchr$), leading to hyperpolarization of the hypothalamic neurons, altering GnRH and therefore the secretion of gonadotropins [11]. In the ovary, the intoxication to IMI leads to overproduction of reactive oxygen species and the resulting oxidative stress damages the germinal cells through lipid peroxidation and weakening of the antioxidant enzyme's activities [12]. These central and gonadic effects dysregulate the hypothalamic-pituitary-gonadic axis leading to fertility impairment.

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Modern treatments of infertility involve the use of antioxidants (i.e. selenium and vitamin E) and ovulation inducers (i.e. clomiphene citrate), although they are usually associated with several side effects such as teratogenicity for clomiphene or nausea for vitamin E [13]. Phytotherapy is nowadays encouraged in the management of reproductive dysfunctions, due to its fewer side effects, accessibility and diversity of its active components [14]. Previous studies have reported the effectiveness of some medicinal plants such as *Urtica urens* in preventing IMI-induced reproductive toxicity in female rats [15].

Amaranthus hybridus is a green leafy vegetable from the Amaranthaceae family. The maceration of its leaves and seeds is used in Cameroonian traditional medicine as fertility stimulant. Phytochemical screening of *A. hybridus* revealed the presence of phytochemicals such as alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, saponins, steroids, tannins and triterpenes [14]. Some studies have reported the antimicrobial [14], antidiabetic [16], anticancer [17] and antioxidant [18] properties of *A. hybridus*. In a preliminary study, we showed that *A. hybridus* (55 and 110 mg/kg) prevented the ovarian toxicity due to cyclophosphamide (a cytotoxic molecule) (*In Press*). With the hypothesis that the antioxidant properties of *A. hybridus* could protect the ovarian functions against the adverse effects of IMI, this work was therefore carried out to investigate the protective effects of the aqueous and methanol extracts of *A. hybridus* against IMI-induced ovarian toxicity in female rat.

2. Materials and methods

2.1. Chemicals

Colibri® (Sun Valley Hall Limited-Hong Kong), a commercial formulation of IMI (30 g/l), is a widely used insecticide because of its broad spectrum of action. One liter of this compound (Bach N° SVH161104) was purchased from the Dschang market and the working solution prepared in distilled water as described by [19]. Assay kits for estradiol, progesterone, LH and FSH (TAIWAN Mouse/Rat ELISA) were used according to the manufacturer's instructions. All other chemicals and reagents were of analytical grade and purchased from local suppliers.

2.2. Plant collection and extracts preparation

Leaves and seeds of *A. hybridus* were harvested in February 2018 in Dschang, West Region of Cameroon. Botanical authentication was done at the Cameroon National Herbarium, Yaoundé, with comparison to the sample registered under voucher specimen N° 42324/HNC. Plant material of both leaves and seed was shade-dried at room temperature and reduced into powder using an electric grinder.

The aqueous extract was prepared by macerating 19 g of powder in 250 ml of distilled water for 48 h and occasionally stirred. The macerate was filtered, freeze-dried (0 °C, under reduced pressure) and 3.2 g of a brownish residue were obtained (extraction yield: 1.28%). To obtain the methanol extract, the powder of *A. hybridus* (19 g) was macerated in 250 ml of methanol for 72 h and filtered. The resulting filtrate was freeze-dried (between –20 and –80 °C) to obtain 10.8 g of a brownish residue (extraction yield: 4.32%). Aqueous and methanolic extracts were used for a comparative approach.

2.3. Animals

Adult female Wistar rats (10 weeks old, and 170–200 g body weight) used in this study were maintained under natural conditions and, had free access to food and tap water. Male rats were used for fertility assessment. The project was presented and validated by the scientific committee of the Department of Animal Biology, which follows the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Economic Community

guidelines; EEC. 2010 Council Directive 2010/63/EU of 22 November 2010 [20].

2.4. Animal treatment and sample collection

Eighty female rats were randomly divided into 8 groups (n = 10). Control groups received either distilled water (10 ml/kg), clomiphene citrate [21] or vitamin E [22]. Experimental groups were orally co-administered with colibri® (22.5 mg/kg) [19] and aqueous or methanolic extracts of *A. hybridus* (55 or 110 mg/kg) within four weeks. Doses of plant extracts were selected from our previous study in which the doses of 55 and 110 mg/kg showed the highest preventive effects against cyclophosphamide-induced ovarian toxicity (*In Press*). Animals were daily weighed and the gavage volume adjusted. All animals were treated during four weeks. Using half of animals in each group, the fertility test was performed starting from day 18.

Twenty-four hours following the last gavage, the remaining animals (five rats/group) were sacrificed under diazepam/ketamine anesthesia. Blood was collected through the abdominal artery and centrifuged for 15 min at 3000 rpm. The plasma was thereafter gently pipetted and kept in sealed tubes at –20 °C for sexual hormones (LH, FSH, estradiol and progesterone) measurements. Ovaries, uterus and vagina were also collected, weighed and fixed in formaldehyde for histological analysis. Oxidative stress markers (lipid peroxidation, superoxide dismutase, catalase, total peroxidases) were evaluated in the ovary.

2.5. Fertility test

On the 18th day of treatment, 5 females were randomly selected in each group and mated with males of proven fertility (Ratio 1:1). For each mated female, the vaginal smear was daily prepared and microscopically examined for sperm detection. Each female with positive vaginal smear was followed until delivery (approximately between day 21–24) and the mean litter size, parturition index and pup viability were recorded according to Ratnasooriya and Dharmasiri (2000) [23].

2.6. Sexual hormone measurements

Plasma concentration of LH, FSH, estradiol and progesterone were measured by ELISA method according to the manufacturer's instructions (TAIWAN Mouse/Rat ELISA). Briefly, after preparation of reagents and samples, the mixture was placed in the Lalisystem Multiskam RC ELISA reader at 450 nm absorbance. The absorbance of samples was read within 5 s and the standard curve helped to determine the corresponding concentration [25].

2.7. Measurement of oxidative stress markers

Ovaries were crushed in a mortar containing Tris buffer solution (pH 7.4) so as to obtain 10% homogenate. The supernatant collected after cold centrifugation (5 °C, 3000 rpm for 10 min) was used for protein, malondialdehyde (MDA), superoxide dismutase (SOD), catalase and total peroxidase analysis. Proteins were measured using a commercial kit (Roche Diagnostics Cobas c-1111) and the procedure followed the manufacturer's instructions. MDA content was measured using thio-barbituric acid reaction [24]. The tissue SOD and catalase activities were evaluated as described by Dimo et al. (2006) [25]. Total peroxidase activities were measured using the potassium iodate method [26].

2.8. Histological analysis

The fixed ovaries, uterus and vagina were dehydrated in ascending grades of alcohol, embedded in paraffin and sectioned at 5 µm thick sections. Tissue sections were stained with Haematoxylin and Eosin (H&E). Uterine and vaginal epithelial heights were microscopically measured (200X) using light microscope (OLYMPUS: Tokyo, Japan).

Ovarian folliculogenesis and follicular count were done as described by Hamzeh et al. (2018) [27].

2.9. Statistical analysis

Results are presented as mean \pm standard error of mean (SEM) and analyzed using Graph Pad Prism, 5.03. One-way analysis of variance (ANOVA) and Tukey-HSD post-hoc test were used to determine statistical differences. A probability of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of treatments on body weight

A significant body weight loss ($p < 0.001$) was recorded in colibri®-treated rats compared with the normal animals. Groups co-administered with colibri® and vitamin E, clomiphene citrate or plant extracts showed a significant increase ($p < 0.5–0.001$) in the body weight compared with colibri®+distilled water group (Table 1).

3.2. Effects of treatments on sexual organ weights

According to Table 2, a significant decrease ($p < 0.001$) was noted in the ovarian and uterine weights of animals in the colibri® plus distilled water group compared with those of group 1 (normal). On the contrary, in female rats co-treated with colibri® and either clomiphene citrate (relative weight), vitamin E (absolute and relative weights), aqueous or methanol extracts of *A. hybridus*, there was an increase weight ($p < 0.05–0.001$) in these sexual organs.

3.3. Effects of treatments on plasma concentration of sexual hormones

Animals in the colibri® untreated group (group 2) showed a significant decrease ($p < 0.01–0.001$) in the plasmatic FSH, LH, estradiol and progesterone when compared with group 1 animals (Table 3). Vitamin E, clomiphene citrate and *A. hybridus* extracts significantly ($p < 0.5–0.01$) prevented this decrease (Table 3).

3.4. Effects of treatments on the ovarian total proteins and MDA

Colibri® provoked a drop in the ovarian total proteins and an increase in MDA concentration ($p \leq 0.001$) in animals receiving distilled water whereas in all co-treatment groups (groups 3–8), there was a clear prevention of this negative effect of colibri® through an increase

Table 1

Effects of treatments on body weight in colibri®-exposed rats during 4 weeks.

Group number	Treatments	Initial weight (g)	Final weight (g)	Variation (%)
Group 1	Normal	178.8 \pm 14.2	187.0 \pm 22.8	7.66 \pm 1.4
Group 2	Colibri®+DW	179.8 \pm 19.3	166.6 \pm 16.9	-12.2 \pm 1.3***
Group 3	Colibri®+Vit E	176.6 \pm 15.0	172.2 \pm 13.4	-3.6 \pm 0.4###
Group 4	Colibri®+CC	181.4 \pm 11.5	169.6 \pm 14.2	-10.6 \pm 1.8#
Group 5	Colibri®+AE 55	189.4 \pm 5.6	183.16 \pm 14.5	-5.3 \pm 0.4###
Group 6	Colibri®+AE 110	173.2 \pm 19.1	164.8 \pm 16.1	-7.6 \pm 1.7##
Group 7	Colibri®+ME 55	180.8 \pm 11.9	176.2 \pm 17.2	-5.2 \pm 1.7###
Group 8	Colibri®+ME 110	177.0 \pm 15.2	171.4 \pm 15.8	-5.7 \pm 1.5###

All values are expressed as mean \pm SEM; Number of rats per group = 5; * ** : $p < 0.001$ significantly different compared with normal (group 1); #: $p < 0.05$; ## $p < 0.01$ ###: $p < 0.001$ significantly different compared with Colibri®+DW (group 2). DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

($p \leq 0.05–0.001$) in total proteins and a decrease ($p \leq 0.05–0.001$) in MDA concentrations.

3.5. Effects on SOD, Catalase and total peroxidase activities

Table 4 shows the toxic effect of IMI in the colibri® untreated animals (group 2). Thus, a significant drop was noticed in the activities of SOD, catalase and total peroxidases. After vitamin E and clomiphene citrate gavage concomitantly with IMI, there was a significant ($p \leq 0.05–0.001$) preservation of these enzymatic activities compared to the untreated colibri® rats (group 2). The aqueous and methanol extracts of *A. hybridus* dose-dependently increased these enzymatic activities and the group receiving colibri and the dose 110 mg/kg of the methanolic extract of *A. hybridus* was the most efficient.

3.6. Effects of treatments on uterine histology

Uterine sections of rats co-treated with distilled water and colibri® showed a significant decrease ($p < 0.001$) in the epithelium height compared with normal rats (group 1) (Fig. 1). In contrast, vitamin E, clomiphene citrate and *A. hybridus* significantly ($p < 0.01–0.001$) prevented this abnormal uterine epithelium decrease due to colibri®.

3.7. Effects of treatments on vagina histology

Fig. 2 shows the effects of treatments on the vaginal epithelium of rats after four weeks. In the normal group (group 1), it was unaltered and the germ (G), granular/intermediate (Gr) and corneum (C) layers from the lumen (Lu) were not disrupted. Colibri® exposition (group 2) significantly altered ($p < 0.01$) the height of the vaginal epithelium which was made of a single unstratified epithelial layer. Vitamin E, clomiphene citrate and *A. hybridus* significantly ($p < 0.05$) protected the vaginal epithelia height denaturation and preserved the presence of vaginal layers, compared with the negative control (group 2) (Fig. 2).

3.8. Effects of treatments on ovarian histology

Histological sections of the ovaries of normal rats (group 1) showed numerous follicles at different stages of development and multiple corpus luteum (Fig. 3). Colibri® altered the architecture of the ovaries after 4 weeks (group 2). This was characterized by the presence of fibrosis (tissue clumps) in the medulla area, abundance of cystic and atretic follicles and few corpus luteum (Fig. 3). Vitamin E, clomiphene citrate and *A. hybridus* extracts prevented these ovary damages. Hence, a reduction in the number of cystic follicles and an increase in the number of corpus luteum were observed.

3.9. Effects of treatments on follicular dynamics

In rats co-administered with colibri® and distilled water (group 2), a significant decrease ($p < 0.001$) in the number of secondary and tertiary follicles, De Graaf follicles and corpus luteum and, a predominance of cystic and atretic follicles was observed (Table 5). On the contrary, animals given vitamin E (group 3), clomiphene citrate (group 4) or plant extracts (groups 5–8) showed a significant increase ($p < 0.05–0.001$) of the secondary follicles after four weeks of continuous treatment. Moreover, the number of cystic follicles was significantly reduced when compared with the negative control group (group 2).3.10. Effect of treatments on fertility of rats exposed to colibri® during 4 weeks.

In all untreated colibri® rats (group 2), a decrease was recorded in parturition index, litter size and pup viability when compared with the fertility performance of normal females (group 1). Colibri® animals receiving either vitamin E (group 3), clomiphene citrate (group 4) or *A. hybridus* (groups 5–8) prevented all these fertility impairments (Table 6).

Table 2
Effects of *Amaranthus hybridus* on sexual organ weights of colibri®-exposed female rats during 4 weeks.

Group number	Treatments	Ovary		Uterus	
		AW (mg)	RW (mg/100 g bw)	AW (mg)	RW (mg/100 g bw)
Group 1	Normal	0.18 ± 0.00	37.30 ± 2.3	0.61 ± 0.05	93.73 ± 3.47
Group 2	Colibri®+DW	0.07 ± 0.00 * **	16.11 ± 3.6 * **	0.08 ± 0.0 * **	35.16 ± 3.28 * **
Group 3	Colibri® + Vit E	0.15 ± 0.13###	24.15 ± 1.6##	0.31 ± 0.03###	45.21 ± 3.05#
Group 4	Colibri® + CC	0.09 ± 0.00	22.73 ± 1.79##	0.21 ± 0.06##	39.25 ± 2.87
Group 5	Colibri® + AE 55	0.12 ± 0.00###	22.09 ± 1.76##	0.34 ± 0.02###	43.26 ± 3.45#
Group 6	Colibri® + AE 110	0.11 ± 0.01###	22.44 ± 5.72##	0.47 ± 0.02###	52.70 ± 4.14##
Group 7	Colibri® + ME 55	0.14 ± 0.01###	26.85 ± 1.41###	0.46 ± 0.05###	56.07 ± 5.1##
Group 8	Colibri® + ME 110	0.16 ± 0.00###	31.88 ± 0.98###	0.58 ± 0.01###	53.42 ± 2.70##

All values are expressed as mean ± SEM; Number of rats per group = 5; * ** : p < 0.001 significantly different compared with the normal (group 1); #: p < 0.05; ##: p < 0.01; ###: p < 0.001 significantly different compared with Colibri®+DW (group 2). DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg. AW: absolute weight. RW: relative weight.

Table 3
Effects of different treatments on plasma sexual hormones in colibri®-exposed female rats during 4 weeks.

Group number	Treatments	FSH (ng/ml)	LH (ng/ml)	Estradiol (ng/ml)	Progesterone (ng/ml)
Group 1	Normal	5.28 ± 1.11	6.40 ± 0.20	505.00 ± 20.98	86.65 ± 1.14
Group 2	Colibri®+DW	2.86 ± 0.03 * *	1.97 ± 0.13 * **	213.75 ± 45.24 * **	46.71 ± 1.76 * **
Group 3	Colibri® + Vit E	4.86 ± 0.10#	4.18 ± 0.30###	426.50 ± 20.23##	69.39 ± 2.44##
Group 4	Colibri® + CC	4.34 ± 0.10#	4.17 ± 0.16###	532.50 ± 20.23###	74.56 ± 4.13###
Group 5	Colibri® + AE 55	3.53 ± 0.05#	3.17 ± 0.22###	448.50 ± 108.98##	65.73 ± 9.19##
Group 6	Colibri® + AE110	3.89 ± 0.12#	4.41 ± 0.02###	369.82 ± 56.24	67.56 ± 4.13##
Group 7	Colibri® + ME55	4.03 ± 0.13#	3.25 ± 0.32###	373.50 ± 8.42	63.82 ± 2.24##
Group 8	Colibri® + ME110	4.83 ± 0.13#	4.97 ± 0.32###	483.50 ± 8.42##	79.82 ± 2.24###

All values are expressed as mean ± SEM; Number of rats per group = 5; * ** : p < 0.001 significantly different compared with normal (group 1); #: p < 0.05; ##: p < 0.01; ###: p < 0.001 significantly different compared with Colibri®+DW (group 2). DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

Table 4
Effects of *A. hybridus* on ovarian oxidative stress markers in colibri®-exposed female rats during 4 weeks.

Group number	Treatments	Proteins (g/g tissue)	MDA (M/g ovary)	SOD (U/Min/g protein)	Catalase (U/ min/g protein)	Total peroxydases (M/Min/g PTT)
Group 1	Normal	3.64 ± 0.05	17.41 ± 3.10	2.91 ± 0.46	6.93 ± 0.05	1.00 ± 0.00
Group 2	Colibri®+DW	1.93 ± 0.85 * **	36.67 ± 7.11 * **	0.57 ± 0.06 * **	3.99 ± 0.19 * **	0.60 ± 0.19 * **
Group 3	Colibri®+Vit E	3.46 ± 0.22##	19.82 ± 5.17##	2.10 ± 0.12###	5.63 ± 1.06#	0.96 ± 0.01##
Group 4	Colibri®+CC	3.26 ± 0.42##	23.15 ± 4.09#	1.26 ± 0.03##	4.95 ± 0.80#	0.82 ± 0.00#
Group 5	Colibri®+AE55	3.21 ± 0.15##	20.15 ± 3.06##	1.29 ± 0.09##	5.63 ± 0.96#	0.83 ± 0.01#
Group 6	Colibri®+AE110	3.76 ± 0.1###	20.23 ± 4.05##	1.98 ± 0.06##	5.84 ± 0.43#	0.80 ± 0.00#
Group 7	Colibri®+ME55	3.36 ± 0.34##	19.92 ± 5.07##	2.07 ± 0.2###	5.84 ± 0.44#	0.80 ± 0.02#
Group 8	Colibri® +ME110	3.69 ± 0.12###	19.79 ± 0.06##	2.12 ± 0.10###	6.02 ± 0.20##	0.93 ± 0.01##

All values are expressed as mean ± SEM; Number of rats per group = 5; * ** : p < 0.001 significantly different compared with normal (group 1); #: p < 0.05; ##: p < 0.01; ###: p < 0.001 significantly different compared with Colibri®+DW (group 2). DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

4. Discussion

Although they are important in boosting agricultural yields, pesticides such as colibri® with imidacloprid (IMI) as active ingredient are incriminated in many pollution-derived pathologies including infertility [28]. Toxicants that interfere with ovarian function can directly affect the ovary or indirectly by acting at the level of hypothalamus or pituitary gland or both [29,30]. The aim of the present study was to evaluate the preventive effects of *Amaranthus hybridus* on the reproductive toxicity of colibri® in female rats. Colibri® caused severe damages in the female reproductive tract, marked by follicular damages with subsequent weight, hormonal and fertility potential decrease. In the present study, we showed that colibri® significantly decreased the body, ovarian and uterine weights. Similar results were reported in rats treated with IMI and methidathion [29]. These decreases could be attributed to the presence of IMI among the components of colibri®. The drop in body weight gain could be due to the anorexic potentials of IMI [31] while that of ovaries and uterus weights could be considered as a direct consequence of the decrease in estradiol and protein contents. It is well

established that estradiol is the primary sex hormone with potent anabolic properties in various animal systems. In female, estradiol plays a key role in the development of sexual organs and secondary sex characters such as muscles and bone growth [32]. IMI, the active principle of colibri®, is a nicotinic receptor agonist which could also inhibit steroidogenesis. Kasson et al. found that nicotine and its agonists exert an inhibitory effect on the 17-alpha hydroxylase, therefore preventing the conversion of pregnenolone and progesterone into androstenedione, thereby inhibiting ovarian estradiol synthesis [33]. This detrimental effect of IMI on estradiol production has been previously reported [15, 34]. Results from vitamin E, clomiphene citrate and plant extracts groups may arise from their capacity to counter the oxidative stress, thereby preventing ovarian cells toxicity [18]. It is well known that insecticides affect pituitary homeostasis by inhibiting gonadotropin releasing hormone (GnRH) secretion, which alters the secretion of LH and FSH and thus ovarian hormone synthesis [35]. In normal rat, estradiol is synthesized in the granulosa cells from androgens under the influence of LH and FSH, while progesterone is secreted by the corpus luteum [36]. These hormones are responsible of the growth of sexual

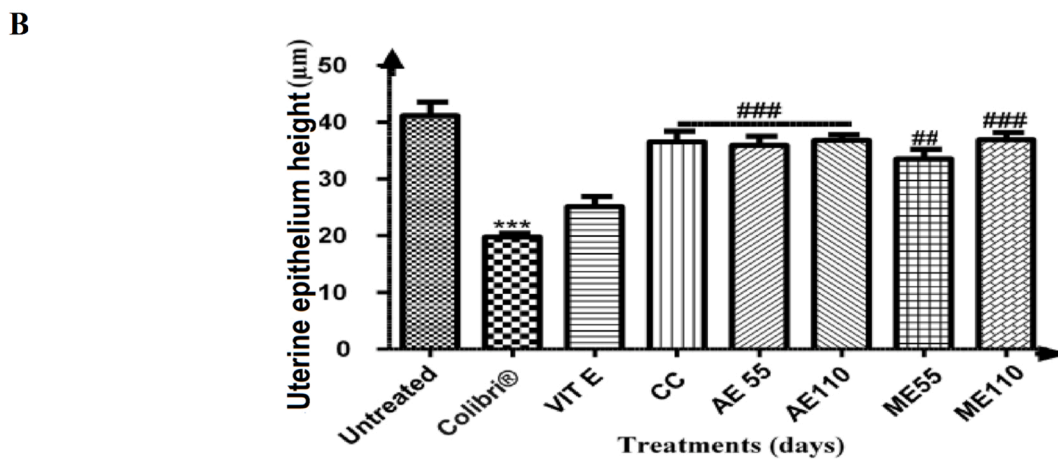
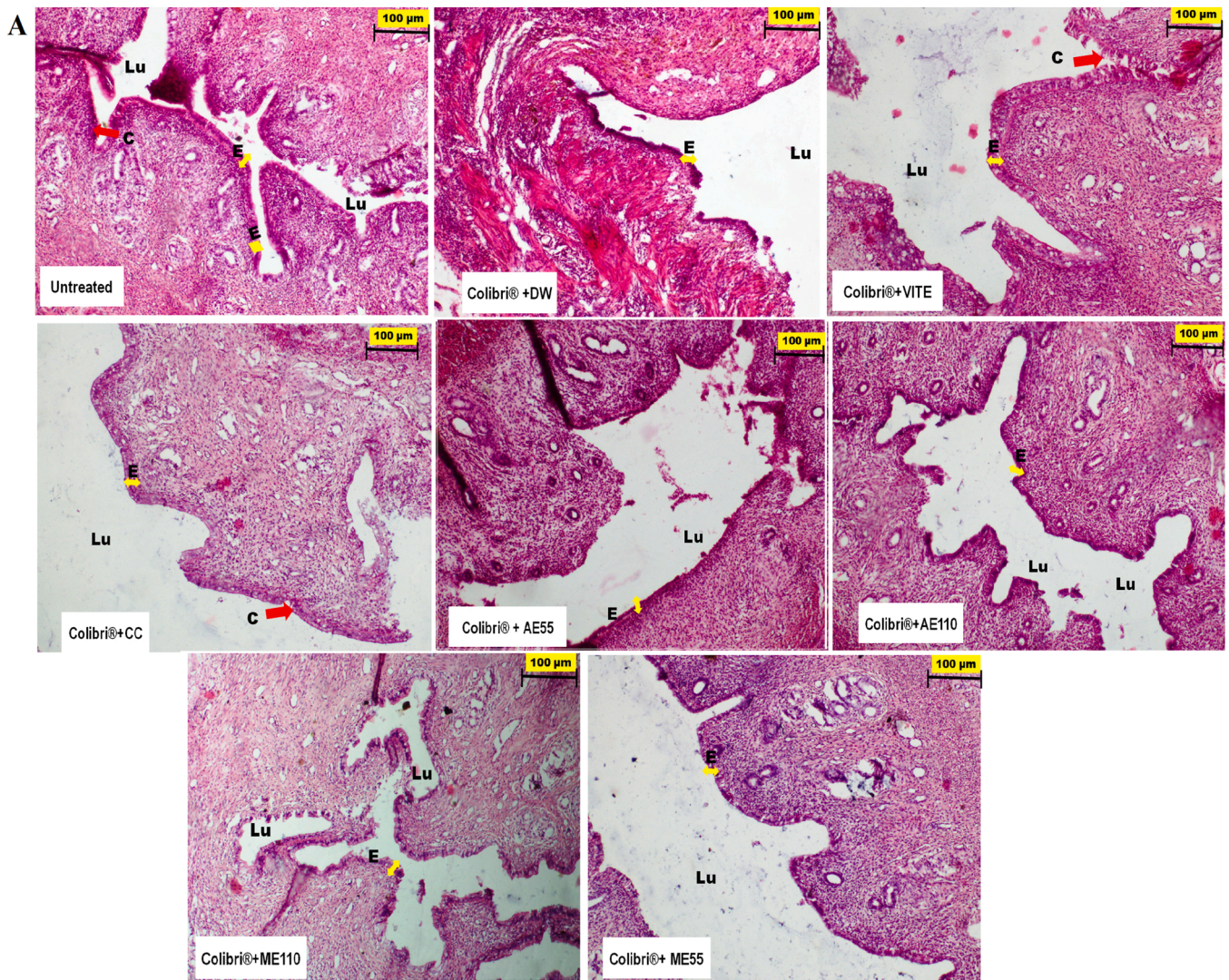


Fig. 1. A) Uterine histological images and B) Graph of uterine epithelium of rats exposed to Colibri® during 4 weeks. Magnification x200, HE. Calibration bar = 100 µm.

All values are expressed as mean ± SEM; Number of rats per group = 5; ***: $p < 0.001$ significantly different compared with normal (group 1); ##: $p < 0.01$; ###: $p < 0.001$ significantly different compared with untreated group. DW: distilled water; CC: Colibri® + clomiphene citrate; Vit E: Colibri® + vitamin E; AE 55: Colibri® + aqueous extract 55 mg/kg; AE 110: Colibri® + aqueous extract 110 mg/kg; ME 55: Colibri® + methanol extract 55 mg/kg; ME 110: Colibri® + methanol extract 110 mg/kg. E; epithelial cell; Lu: lumen; C: large cubic cells.

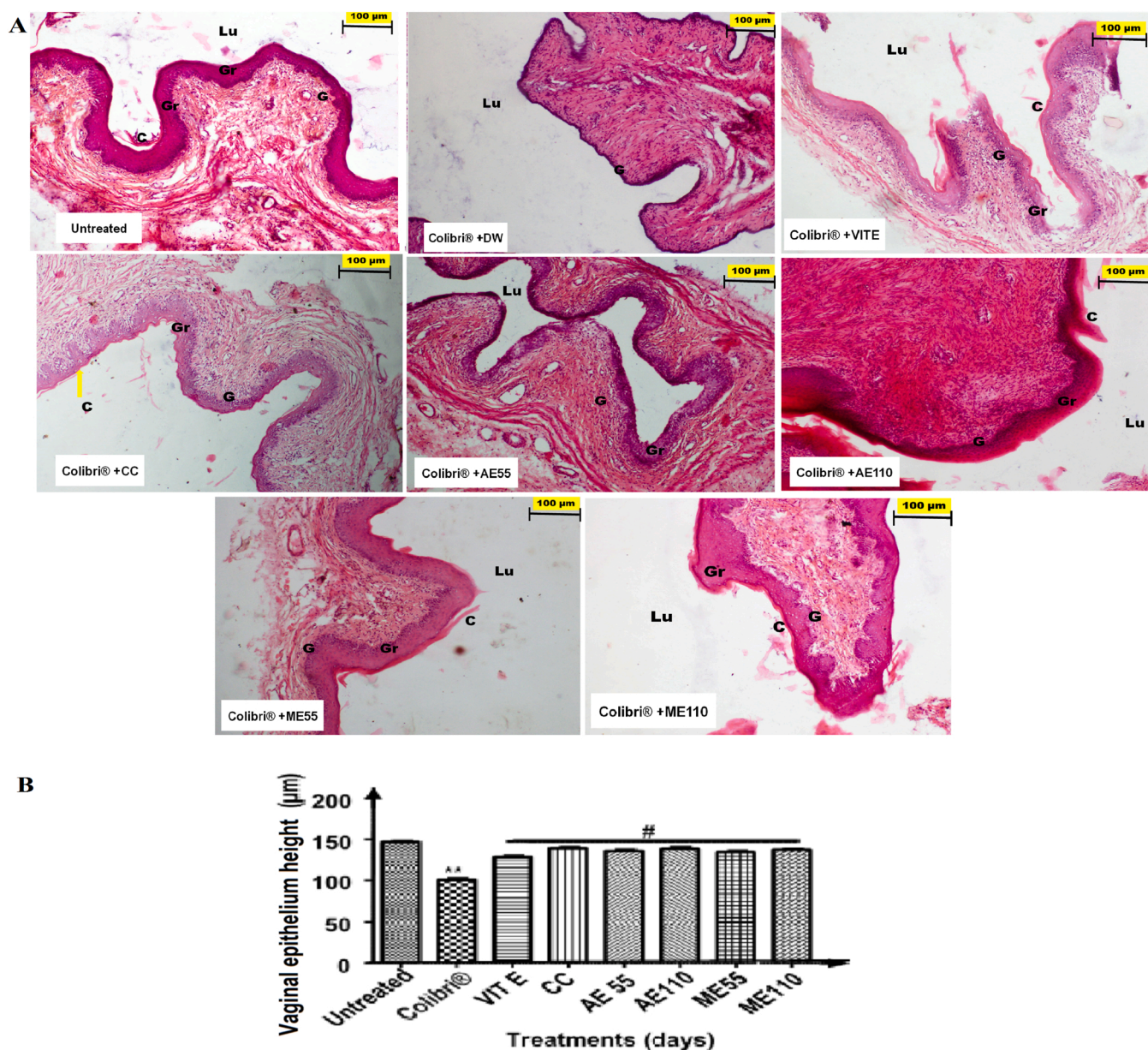


Fig. 2. A) Vaginal histological images and B) Graph of vaginal epithelium of rats exposed to Colibri® during 4 weeks. Magnification x200, HE. Calibration bar = 100 µm. All values SEM. Number of rats per group = 5, ** : p < 0.001 significantly different compared with untreated group (group 2); #: p < 0.05 significantly different compared with Colibri® + DW. DW: distilled water; CC: Colibri® + clomiphene citrate; Vit E: Colibri® + vitamin E; AE 55: Colibri® + aqueous extract 55 mg/kg; AE 110: Colibri® + aqueous extract 110 mg/kg; ME 55: Colibri® + methanol extract 55 mg/kg; ME 110: Colibri® + methanol extract 110 mg/kg. C: corneum stratum; G: germ stratum; Gr: granular stratum are expressed as mean ± SEM.

organs and also possess anabolic effect on body tissues [37]. The decrease in body and sexual organ weights was associated with that of plasma concentration of LH, FSH, progesterone and estradiol in colibri®-treated rats. However, this decline was significantly prevented by clomiphene citrate as well as aqueous and methanol extracts of *A. hybridus*. Clomiphene citrate is a selective estrogen receptor modulator used in the management of anovulatory infertility [38]. Clomiphene citrate blocks estrogen receptors, resulting in a high synthesis of gonadotropin-releasing hormone (GnRH), which in turn elevates the secretion of LH, leading to ovulation [38]. Results obtained with *A. hybridus* suggest that the aqueous and methanol extracts are capable of promoting sexual hormone synthesis, but these properties are yet to be proven with adequate protocols. Moreover, the effects of the plant extracts are consistent with our previous report (*In Press*) in which we found that *A. hybridus* was capable to prevent the ovarian toxicity due to

cyclophosphamide in rats by preventing the disruption of the estrus cyclicity, the decrease in ovary and uterus weights, the drop in estradiol level and, by preserving the ovarian architecture.

Insecticide toxicity is usually associated with reactive oxygen species (ROS) over-production which leads to oxidative stress [39]. Oxidative stress disrupts cellular redox circuits, resulting in disturbances of redox-regulated cellular processes [9]. ROS react with cellular lipids, proteins, and nucleic acid leading to tissue structural damages and steroidogenesis inhibition in ovarian cells. The decrease in ovarian and pituitary hormone concentrations observed in colibri®-treated rats may also be due to the oxidative stress. It has been shown that oxidative stress is commonly reported in animal models exposed to IMI [9]. In the present study, the reduction observed in SOD, CAT and peroxidases activities and the high MDA contents are indicative of oxidative stress and lipid peroxidation respectively. These findings are similar to those of

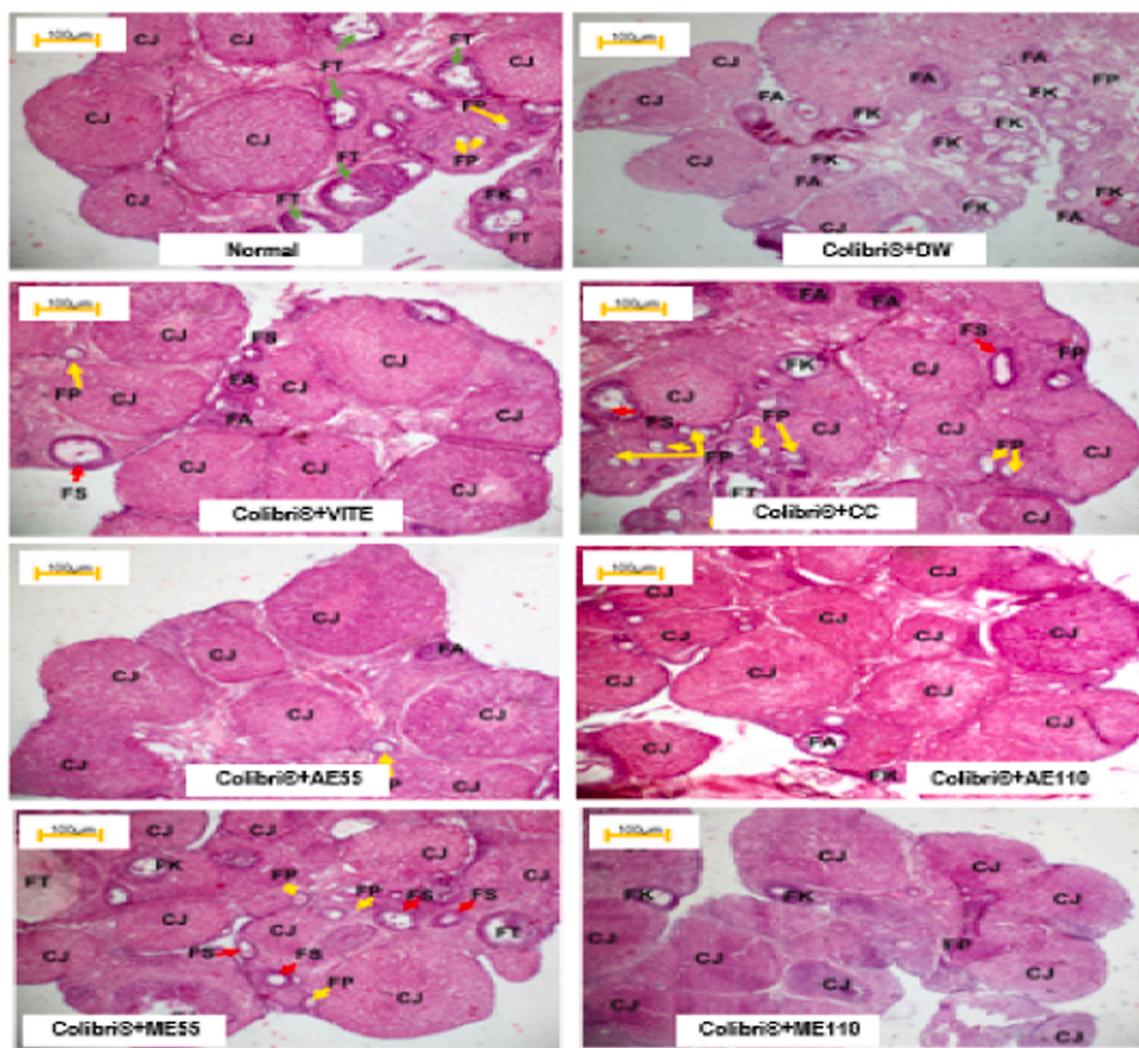


Fig. 3. Effects of *A. hybridus* on ovarian histology in rats orally exposed to colibri® during 4 weeks. Magnification x200, HE. Calibration bar = 100 µm. CJ: corpus luteum; FP: primary follicles; FS: secondary follicles; FT: tertiary follicles; FK: cystic follicles; FA: atretic follicles. DW, distilled water; clomiphene citrate, Vit E Vitamin E, AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

Table 5
Effects of treatments on follicle count of rats exposed to colibri® during 4 weeks.

Group number	Treatments	Primary follicles	Pre-antral follicles	Antral follicles	Corpus luteum	Atretic follicles	Cystic follicles
Group 1	Normal	39.33 ± 1.15	26.00 ± 0.57	18.66 ± 0.33	21.00 ± 0.57	2.83 ± 0.43	0.33 ± 2.18
Group 2	Colibri® + DW	49.00 ± 13.10	10.00 ± 1.00***	3.84 ± 0.66***	4.94 ± 0.30***	23.34 ± 0.01***	16.33 ± 2.96***
Group 3	Colibri® + Vit E	38.82 ± 1.15	27.00 ± 1.15###	11.00 ± 0.57##	14.56 ± 0.33###	4.8 ± 1.52###	2.36 ± 5.76###
Group 4	Colibri® + CC	42.33 ± 0.66	31.66 ± 0.33###	21.33 ± 6.33###	29.33 ± 0.33###	3.15 ± 0.66###	5.11 ± 2.02###
Group 5	Colibri®+AE 55	35.09 ± 2.84	27.66 ± 1.33###	12.76 ± 0.34##	11.66 ± 0.66###	3.90 ± 0.10###	1.34 ± 2.16###
Group 6	Colibri®+AE110	34.04 ± 0.57	26.10 ± 9.10##	14.45 ± 0.66###	9.00 ± 1.15##	2.00 ± 2.15###	3.43 ± 1.76###
Group 7	Colibri®+ME55	39.08 ± 9.00	23.66 ± 1.15##	11.00 ± 0.57##	11.11 ± 0.39###	2.16 ± 0.12###	2.07 ± 0.02###
Group 8	Colibri®+ME110	37.00 ± 11.10	29.43 ± 9.36###	14.83 ± 2.03###	13.56 ± 4.93###	1.21 ± 0.17###	2.51 ± 0.79###

All values are expressed as mean ± SEM. * ** : p < 0.001 significantly different compared with untreated group (group 2); ##: p < 0.01; ###: p < 0.001 significantly different compared to Colibri® + DW. DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

Tetsatsi et al. (2019) [19] who reported an imbalance of oxidative stress markers in colibri®-treated male rats. Similar to vitamin E, *A. hybridus* extracts significantly decreased MDA level and increased CAT, SOD and peroxidase activities. Vitamin E has long been used in various pathological conditions as an antioxidant agent [12]. It normalizes the oxidant status by potentiating the antioxidant enzymes activity and neutralizing the superoxide anion [9], thereby protecting ovarian cells from peroxidation, [12]. Therefore, the alleviating effects of *A. hybridus* could be

attributed to its proven antioxidant properties [14]. These effects are positively correlated with the sexual organ weights and hormones decrease prevention.

Recent studies revealed severe damages of ovarian tissues in colibri®-treated rats [9,15,40]. In the current work, the histological examination of the ovarian sections of colibri®-treated (group 2) rats showed severe impairments of ovarian morphology and folliculogenesis, which could be attributed to the damaging effects of colibri®. Colibri®

Table 6
Effects of treatments on fertility of rats exposed to colibri® during 4 weeks.

Group number	Treatments	Mated females	Positive vaginal smear females	Pregnant females	Parturition index	Litter size	Viability (%)
Group 1	Normal	5	5	5	100.00	9.64 ± 0.76	100.00
Group 2	Colibri® + DW	5	2	1	50.00	5.00 * *	60.00
Group 3	Colibri®+Vit E	5	4	4	100.00	8.09 ± 0.43##	100.00
Group 4	Colibri®+CC	5	4	4	100.00	7.32 ± 0.54##	100.00
Group 5	Colibri®+AE55	5	4	4	100.00	8.00 ± 0.49##	100.00
Group 6	Colibri®+AE110	5	4	4	100.00	8.67 ± 0.65##	100.00
Group 7	Colibri®+ME55	5	5	5	100.00	7.51 ± 0.40##	100.00
Group 8	Colibri®+ME110	5	4	4	100.00	8.37 ± 0.11##	100.00

Values for the litter size are expressed as mean ± SEM; * ** : p < 0.01 significantly different compared with normal (group 1); ##: p < 0.01 significantly different compared with Colibri®+DW. DW: distilled water; CC: clomiphene citrate; Vit E: vitamin E; AE 55: aqueous extract 55 mg/kg; AE 110: aqueous extract 110 mg/kg; ME 55: methanol extract 55 mg/kg; ME 110: methanol extract 110 mg/kg.

has been proven to inhibit follicle development and estradiol synthesis in rats [15]. Other reports revealed that this chemical compound significantly decreased primary, pre-antral and antral follicles and corpus luteum but increases atretic and cystic follicles contents [41,42]. The recorded large number of corpus luteum in the untreated colibri® females (group 2) probably indicates a mixture of mature and atretic corpus luteum. Indeed, the vaginal smear of rats at diestrus stage (which lasts for about 59 h) for instance, usually presents a mixture of new and passed follicles which may lead to more corpus luteum. Co-treated rats with *A. hybridus* extracts and colibri® showed a normal ovarian architecture with the presence of follicles at different stages of development. Phytocomponents found in the extracts of *A. hybridus* [14] may be responsible of these ovarian features. Many authors have already demonstrated that the compounds found in *A. hybridus* possess antioxidant [14,43] and antidiabetic [16] properties.

As a consequence of the effect of IMI on sexual hormone synthesis and ovarian dynamic, a significant drop (p < 0.01) in the parturition index and litter size (viability) was noticed in colibri®-treated rats (group 2). These findings are consistent with those of Andrew et al. (2018) [12] who highlighted the relationship between pesticide exposure, infertility and congenital malformations occurring in agroworkers.

A significant prevention of these damages was observed in rats treated with vitamin E, clomiphene citrate or *A. hybridus* extracts.

Two main mechanisms, including inhibition of sexual hormones synthesis and genesis of oxidative stress sustain the harmful effects of colibri® on the reproductive function. Prevention of fertility disruption by the plant extracts is indicative of normal functioning of the ovaries. This could arise from the action of the plant components on the hypothalamic-pituitary axis to prevent LH and FSH inhibition by IMI. Extracts may also have prevented a free radical release and boosted the ovarian antioxidant systems since their antioxidant properties have been already reported [14]. Although the methanol extract (110 mg/kg) was more effective in sexual organ weights and hormones increase, the aqueous extract showed the highest effect in boosting the fertility parameters and could therefore be considered as the most effective dose in the present study. The overall effects of *A. hybridus* are summarized in Fig. 4.

5. Conclusion

Present results showed that colibri® intoxication lead to ovarian toxicity marked by a decrease in ovarian and uterus weights, a decline in

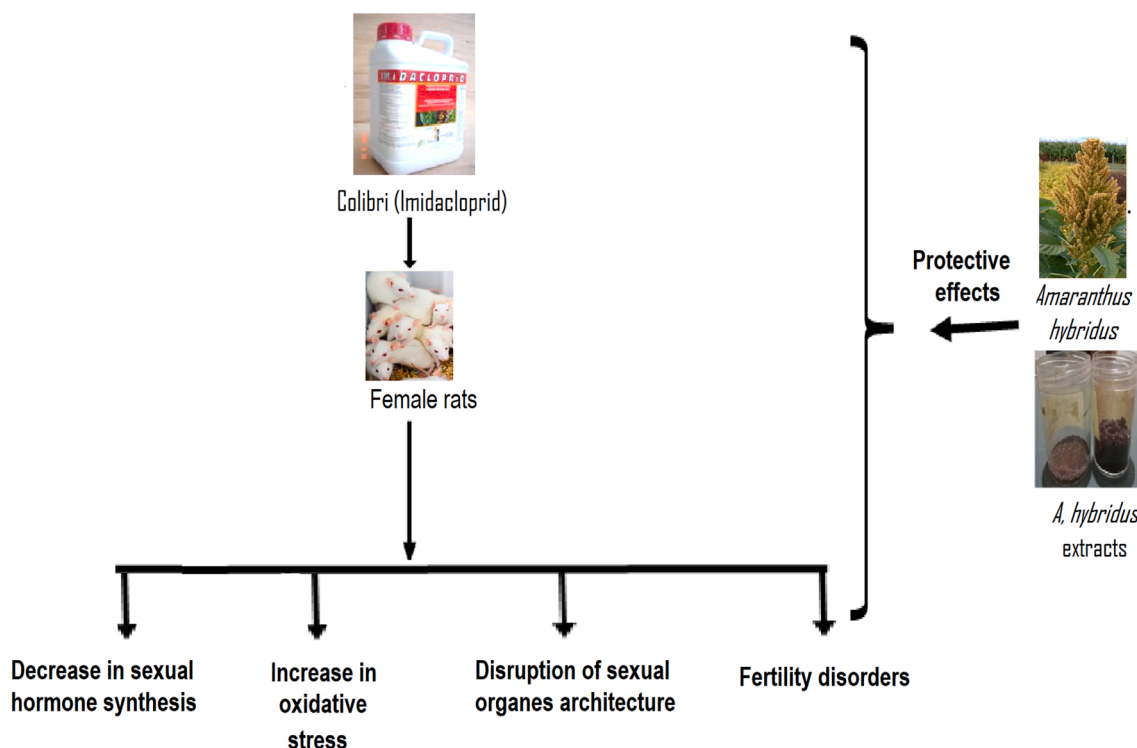


Fig. 4. Summary of *A. hybridus* effects in colibri®-exposed female rats.

sexual hormone's levels and ovarian structural damages leading to fertility disruption. Aqueous and methanol extracts of *A. hybridus* effectively prevented these damages by promoting sexual hormones synthesis and antioxidant defense. These plant effects are in accordance with the previous reports and further support the traditional use of *A. hybridus* as fertility booster. *A. hybridus* could therefore be proposed as a solution to tackle the harmful effects of environmental hazards on female fertility.

Ethical approval

The study protocol was approved by the Scientific Committee of the Department of Animal Biology, University of Dschang, Cameroon, which follows the internationally accepted standards of ethical guidelines for laboratory animal use and care.

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CRedit authorship contribution statement

Pierre Watcho, Prechmy Carole Nguoyong Nsamou, Aimé Césaire Tetsatsi Momo and Yannick Baudouin Petnga Tchatat participated in the study design. François Xavier Kemka, Georges Romeo Bonsou Fozin and Esther Ngadjui collected the data and carried out the statistical analysis. Pierre Watcho, Prechmy Carole Nsamou Nguoyong and Aimé Césaire Tetsatsi Momo drafted the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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