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Research Article

Effect of methanolic extract of *Amaranthus viridis* leaves on reproductive functions in wistar female rats

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ABSTRACT

Objective : The aim of this study is to determine the pharmacological effects and the estrogenic properties of *Amaranthus viridis* leaves on the reproductive function of animal model (female rat).

Methods : Vaginal smears performed 9 days before treatment allowed to select female rats having alternated on two cycles a regularity. Thereafter, the selected rats were administered by gavage daily for 28 days taking care of smear every morning at 7am from the first day of treatment follow the evolution of the cycle. For this study 20 nulliparous rats, 2 months old, weighing between 120-150 g. The first group (control) was administered with olive oil and the other three batches received respectively the doses 200, 400 and 600 mg/kg of the methanolic extract of *Amaranthus viridis*. At the end of the 28-day treatment, ovary and uterine horn were removed, histological and hormonal parameters were studied for determine pharmacological effects of methanolic extract of *Amaranthus viridis*.

Results : The extract caused a disturbances of the cycle according to the doses administered. Disturbances at doses 200 and 400 mg/kg PC are significant. The calculation of the total duration of the different phases of the cycle revealed very significant increases in the estrous phase ($P < 0.01$) by 22.79 % and 17.13 % at the respective doses of 200 and 400 mg/kg b.w compared to control. Non-significant difference was recorded on FSH, LH and estradiol level. On progesterone level, administration of the methanolic extract showed a significant difference at dose of 600 mg/kg b.w compared to control. On histological structure of the ovary, the presence of active and degenerate corpus luteum, secondary follicles depending on the dose administered were recored.

Conclusion : The results showed that the methanolic extract of *Amaranthus viridis* contain estrogenic substances or estrogen-like substances according to a dose-dependent mechanism, with high estrogenic potential at doses of 200 and 400 mg/kg b.w .

Keywords: vaginal smears, *Amaranthus viridis*, methanolic extract, histology

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INTRODUCTION

Reproduction is the set of processes by which a species is perpetuated, by generating news individuals and which contains the half of each parent's genetic material as part of sexual reproduction [1]. It characterizes the essence of life, in the sense that the transmission of genetic information and the knowledge pass from one generation to another. However, the joy of perpetuating life or give birth to another person does not unanimity. This incapacity to beget characterize the infertility. The infertility is incapacity of a couple to procreate after a year together during regular and unprotected sex [2]. According to Barillier, the causes of infertility can be feminine origin (30 % case), mal origin (20 % case), mixed (40 % case) or idiopatic origin (10 % case) [3]. Modern treatments for infertility (hormonal treatment,

MAP, etc.) are very expensive for most of the population, especially in Africa. To remedy this, populations turn almost exclusively towards traditional treatments by the use of medicinal plants. According to WHO, more than 80% of the african population who would use medicinal plants as health care [4]. Medicinal plants are widely used in the treatment of much pathology like malaria, diarrhea, high blood pressure and diabetes [5-7]. To treat infertility, several plants like *Sarcocephalus latifolius*, *Cnestis ferruginea* and *Passiflora foetida* have been studied and revealed an interesting estrogenic potential [8-10]. Some plants like *Amaranthus viridis*, very well known by traditional medicine in the treatment of infertility have not experienced any particular attention. *Amaranthus viridis* is a plant of the family Amaranthaceae and dicotyledonous class. This plant is

known for its antioxidant activities, anti-inflammatory properties and antifungal activity [11-13].

The aim of this study is to determine the pharmacological effects and the estrogenic properties of *Amaranthus viridis* leaves extract on the reproductive tract of female rats

MATERIAL AND METHODS

Plant material

The plant of *Amaranthus viridis* were collected in the Abobo commune of the District of Abidjan (Ivory Coast) among herbalist. A sample of this plant has been authenticated at the National Center for Floristics (CNF) by Prof. Boraud N'tapké Maxime from Felix Houphouët Boigny University (Abidjan, Ivory Coast).

Preparation of extract

The fresh leaves of *Amaranthus viridis* were washed then dried in ambient air in a room, away from the sun. Thereafter, the leaves are crushed using an electric blinder, brand RETSCH GM 300 to obtain a powder. 50 g of leaf powder were macerated in 1 L of methanol (95 °C) using a magnetic stirrer at 350 rpm for 48 hours. The macerate is filtered 4 times on poplin fabric and then on Wattman No. 1 paper. The filtrate obtained is concentrated at 40 °C in a rotary evaporator. A pasty extract is obtained [14].

Animals

The animal model used consisted of nulliparous female rats weighing between 120 and 150 g, from the vivarium of ENS (Ecole Normale Supérieure) of Abidjan. They were raised at ambient temperature of 22 ± 3 °C with 40 to 60% moisture and a photoperiod of 12 hours light and 12 hours darkness. The animals were fed with diet of fish, bread, corn and water *ad libitum*.

Vaginal smear technique

Smears are performed according to a method which consists to collect vaginal cells, then to color these cells and finally to examine them.

Collect vaginal cells consists to moisten a cotton swab with NaCl (0.9 %) and gently introduce it into the rats' vaginas without stressing them, then turn the cotton swab in the same direction chosen several times until the appearance of a slight resistance. The cotton is then removed and spread on a microscope slide. The collected cells are stained with methylene blue (basic dye). A drop of methylene blue concentrated at 2 % and diluted to 1/20 is deposited on the slide which the vaginal sample is spread. The colored slides are examined. The examination is done under an optical microscope counting a proportion of 200 cells from one end of the blade to the other [15]. There are three types of cells to know: eosinophilic or keratinized cells, basophilic cells and leukocytes. The proportion of different types of cells makes it possible to determine the different phases of the estrous cycle based on the following criteria :

- >proestrus: clean smear, 40 to 50% eosinophilic cells and low presence of leukocytes ;
- >estrus: clean smear, 60 à 90 % eosinophilic cells, no leukocytes;
- >diestrus I ou metestrus: dirty smear, 20 à 40 % eosinophilic cells, leukocytes quite numerous;
- >diestrus II ou diestrus: very dirty smear, 10 % eosinophilic cells, very many leukocytes.

Study of the effect of the extract on the estrous cycle

Vaginal smears performed 9 days before treatment allowed to select female rats having alternated on two cycles a regularity. Thereafter, the selected rats was administered by gavage daily for 28 days taking care of smear every morning at 7am from the first day of treatment follow the evolution of the cycle. The percentage of eosinophilic cells and leukocytes was calculated and represented on the same graph according to the doses of the methanolic extract of *Amaranthus viridis* leaves. For this study 20 nulliparous rats and 2 months old were used and distributed as follows :

- Lot 1 : Olive oil (control)
- Lot 2 : 200 mg/kg b.w.
- Lot 3 : 400 mg/kg b.w.
- Lot 4 : 600 mg/kg b.w.

Measurement of fresh and dry weight of ovary and uterine horn

The 29th day, the animals were sacrificed by rapid decapitation, the left ovaries and left uterine horns were removed and immediately weighed to note the fresh weight. The dry weight was obtained after staying the left uterine horn and the left ovary in an oven at 100 °C for 24 hours.

Study of the effect of the methanolic extract of *Amaranthus viridis* on hormone levels

At the end of the 28-day treatment, the blood of female rats, obtained after decapitation, was taken and centrifuged to obtain a serum. The sera obtained allowed to measure hormones such as FSH, LH, Estradiol and Progesterone. Hormonal assays were performed using an automatic analyzer Architect®.

Histological examination of the ovary and the uterine horn

Right ovary and right uterine horn removed and fixed in 10% concentrated formalin have undergone increasing baths of ethanol (80, 90, 100 °C) and toluene in order to be respectively dehydrated and lighten. thereafter, organs embedded in two liquid paraffin baths in an oven at 60 °C for 2 and 3h for each bath. The sections are made using a microtome (LEICA-RM2125-RTS) and deposited on microscope slides. Microscope slides have passed to the oven, then in toluene baths to be dewaxed. This microscope slides are rehydrated in descending ethanol baths then rinsed with distilled water. Finally, the slides are soaked in aqueous dyes such as hematoxylin and eosin before being observed using a microscope equipped with a camera [14,16].

Analysis Statistical

All data are expressed on average \pm SEM. Graphical representation and data processing were performed using Graph Pad Prism and EXCEL software. The statistical analysis was performed by analyzing the variances (ANOVA One-Way). The differences between the averages were determined according to the Newman-Keuls test. $P < 0.05$ is considered significant, $P < 0.01$ very significant and $P < 0.001$ highly significant.

RESULTS

Effect on the estrous cycle

The extract caused a disturbances of the cycle according to the doses administered. Daily evolution of eosinophilic cells and leucocytes obtained from the different vaginal smears performed, allowed to appreciate these disturbances of the cycle (Figure 1). The rate of variation of the cells involved in

normal proportions in the control reflecting the regularity of their estrous cycle.

600 mg/kg dose slightly disrupted the cycle with a slight increase in eosinophilic cell peaks. Disturbances at doses 200 and 400 mg/kg PC are significant. It is more pronounced at the dose of 200 mg/kg, resulting in an abundance of eosinophilic cell peaks and a decrease or even absence of leukocyte cells from the 6 th day of treatment for 10 days.

This abundance of eosinophilic cells translating estrus is followed by decrease and then again by a recovery.

The 400 mg/kg dose induced an abundance of eosinophilic cells from the 4 th day of treatment. This abundance is followed by a decrease then moderate disturbances with an abundance of eosinophilic cells peaks until the end of treatment.

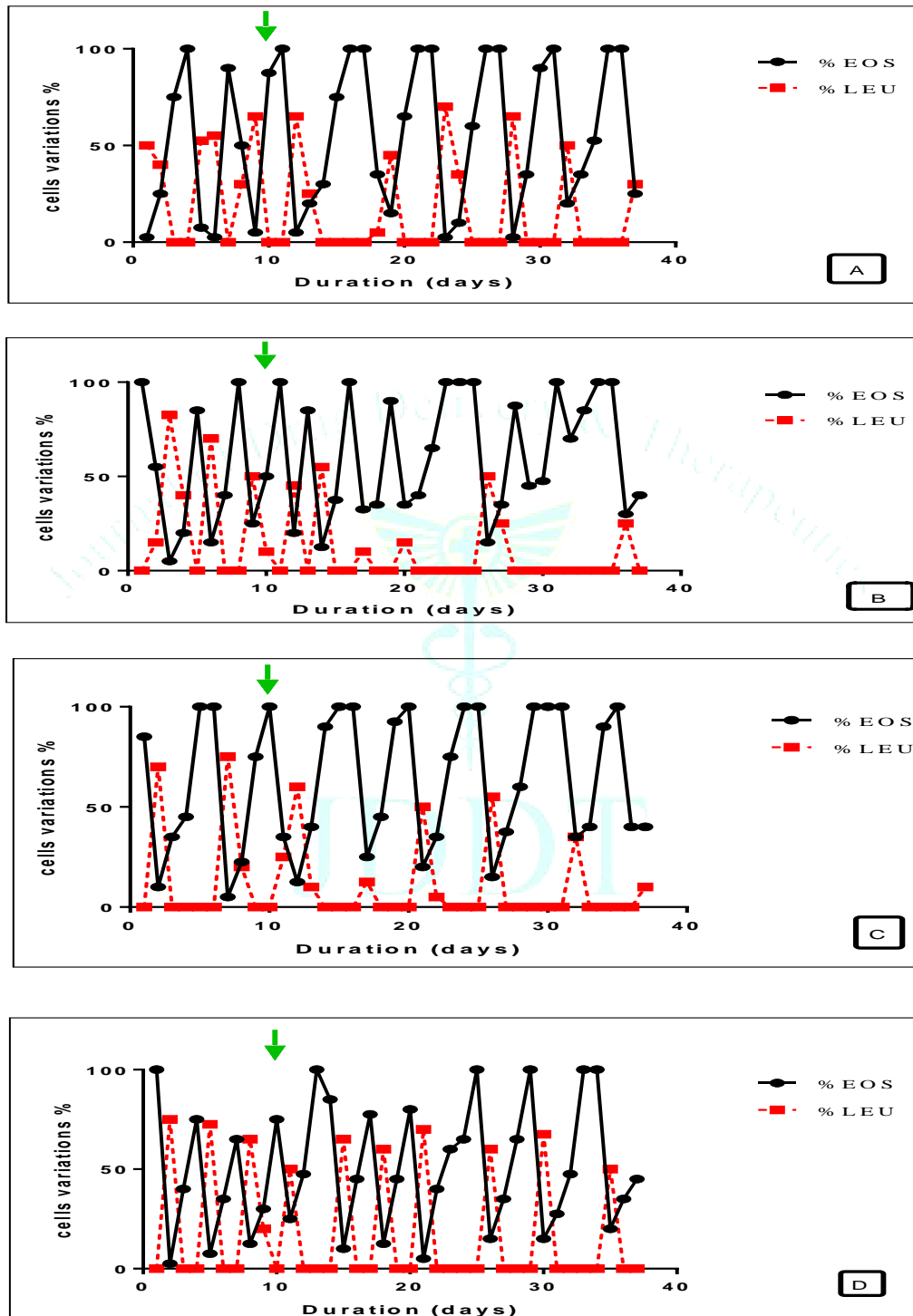


Figure 1 : Evolution of estrus cycle of treated females rats with methanolic extract of de *A. viridis*

A : Control (olive oil)

B : Female rats treated with 200 mg/kg b.w. methanolic extract of *Amaranthus viridis*

C : Female rats treated with 400 mg/kg b.w. methanolic extract of *Amaranthus viridis*

D : Female rats treated with 600 mg/kg b.w. methanolic extract of *Amaranthus viridis*

The arrow (↓) indicates the start of treatment

The calculation of the total duration of the different phases of the cycle revealed very significant increases in the estrous phase (P<0.01) by 22.79 % and 17.13 % at the respective doses of 200 and 400 mg/kg b.w compared to control. The total duration of the diestrus, has significantly decreased (P<0.001) by 72.72 %, 59.08 % and 45.45 % at the respective doses of 200, 400 and 600 mg/kg b.w compared to control (Figure 2). The total duration of metestrus experienced slight non-significant fluctuations (P>0.05). The total duration of the proestrus phase increased by 60 % (P<0.01), 33,34 % (P<0.05) and 86,66 % (P<0.001) at the respective doses of 200, 400 and 600 mg/kg b.w compared to control.

Effect on the relative weight of the ovary and uterine horn

Table I summarizes the relative weights of different estrogen-dependent organs collected in female rats after 28 days of treatment according to the administered dose.

On the ovary, the results showed non-significant change (P>0.05) on fresh and dry weight whatever the dose methanolic extract of *Amaranthus viridis* administrated compared to control. However, an increase in fresh ovarian weight of 11.94% is noted at a dose of 200 mg/kg compared to control. On the uterine horn, the results showed an increase in fresh weight of 10.49 %, 31,90 % (P <0.01) and 10.15 % at the respective doses of 200, 400 and 600 mg/kg

b.w compared to control. The dry weight of the uterine horn has also increased very significantly (P<0.001), following the administration of the different doses of the methanolic extract compared to control group. These increases translate into 42.23 % ; 82.10 % et 70.11 % at the respective doses of 200, 400 and 600 mg/kg b.w compared to control.

Effect of methanolic extract of *Amaranthus viridis* on hormone levels

Table II summarizes the results for hormone levels such as FSH, LH, estradiol and progesterone. The effect of the administration of the methanolic extract on the serum FSH level, revealed that the values obtained with the different doses are lower than the value of the detection threshold. The rate of LH showed non-significant variation between controls and treated animals. On the estradiol level, administration of the methanolic extract of *Amaranthus viridis*, showed non-significant difference between the treated and the control. However, increases of 12.5% and 43.75% were recorded at the respective doses of 200 and 400 mg/kg compared to the control group. On progesterone level, administration of the methanolic extract showed a significant difference (P<0.05), resulting in an increase of 34.85% at a dose of 600 mg/kg compared to control. Doses 200 and 400 mg/kg showed no significant difference compared to control group.

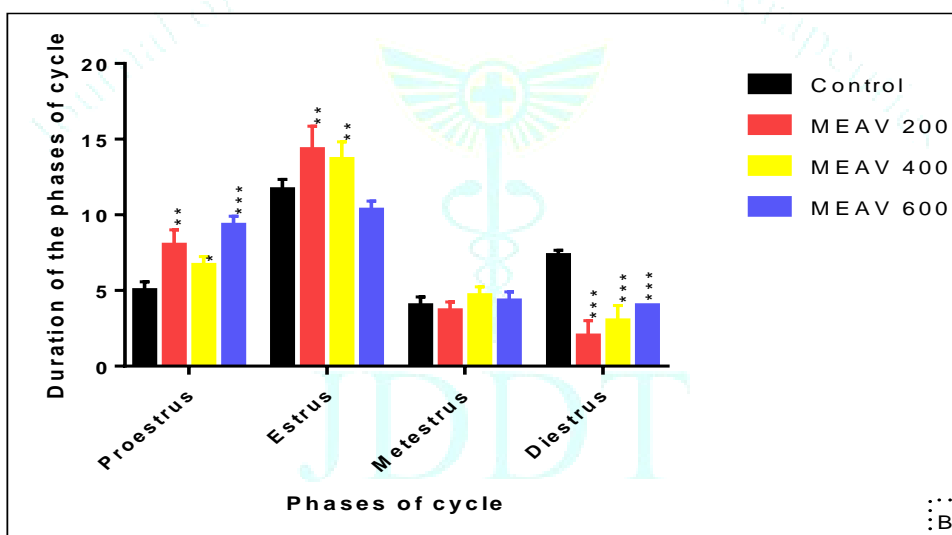


Figure 2 : Effects of methanolic extract *Amaranthus viridis* on the total duration of the different phases of the estrus cycle in rats after 28 days of treatment

*: P<0.05; **: P<0.01 ; ***: P<0.001 ; significant difference compared to control MEAV : Methanolic extract of *Amaranthus viridis*

Table I : Effects of the methanolic extract of *Amaranthus viridis* on the weight of estrogen-dependent organs after 28 days of treatment

Organs mg/100g	State	Méthanolic extract			
		Control	MEAV 200	MEAV 400	MEAV 600
Ovary	Frais	19,5±1,088	21,83±1,077	19,92±1,103	16,81±1,106
	Sec	6,247±0,1824	6,156±0,1921	6,358±0,1616	6,409±0,1876
Uterin horns	Frais	55,73±2,5	61,58±2,85	73,51±2,789**	61,39±2,505
	Sec	11,01±0,1631	15,66±0,1834***	20,05±0,1413***	18,73±0,1298***

** : P<0.01 ; *** : P<0.001 ; significant difference compared to control

MEAV : Methanolic extract of *Amaranthus viridis*

Table II: Effects of the methanolic extract of *Amaranthus viridis* on hormone levels after 28 days of treatment

Parameters	Methanolic extract			
	Control	MEAV 200	MEAV 400	MEAV 600
FSH mIU/ml	0,055	<0,05	<0,05	<0,05
LH mIU/ml	0,025±0,015	0,025±0,005	0,02±0,01	0,03±0,01
ESTRADIOL pg/ml	16±1	18±1	23±2	12,5±1,5
PROGESTERONE ng/ml	12,05±0,95	10,1±0,8	8,85±0,95	16,25±0,75*

* : P<0.05 ; ** : P<0.01 ; *** : P<0.001 ; significant difference compared to control

MEAV : Methanolic extract of *Amaranthus viridis*

Histology of the ovary and uterine horn

Administration of the methanolic extract on the histological structure of the ovary revealed the presence of active and degenerate corpus luteum, secondary follicles depending on the dose administered (**Figure 3**). The administration of the 200 mg / kg of b.w revealed the presence of newly formed corpus luteum, or active corpus luteum. The dose of 400 mg / kg of b.w showed degenerating corpus luteum and the dose of 600 mg / kg of b.w revealed the presence of secondary and atretic follicles.

On uterine horn, administration of methanolic extract of *Amaranthus viridis* showed a dilation of the uterine light at different doses administered compared to control.

Effects on the height of endometrial epithelial cells and the diameter of the uterine glands

Table III summarizes height of endometrial epithelial cells and the diameter of the uterine glands. Results relating to the height of endometrial epithelial cells showed non-significant difference (P>0.05) between control and treaties. On the diameter of the uterine glands, the results revealed very significant differences (P<0.001), resulting by increase of 32.40 % and 36.37 % at the respective doses 200 and 400 mg/kg b.w of methanolic extract of *Amaranthus viridis* compared to control.

Table III: Effects of methanolic extract of *Amaranthus viridis* leaves on height endometrial epithelial cells and diameter of uterine glands

Measured parameter	Methanolic extract			
	Control	MEAV 200	MEAV 400	MEAV 600
Height endometrial epithelial cells (µm)	24,688±1,38	29,767±0,46	23,308±0,92	27,905±1,32
Diameter of uterine glands (µm)	80,387±0,23	106,434±0,31***	109,624±0,18***	81,592±0,27

* : P<0.05 ; ** : P<0.01 ; *** : P<0.001 ; significant difference compared to control

MEAV : Methanolic extract of *Amaranthus viridis*

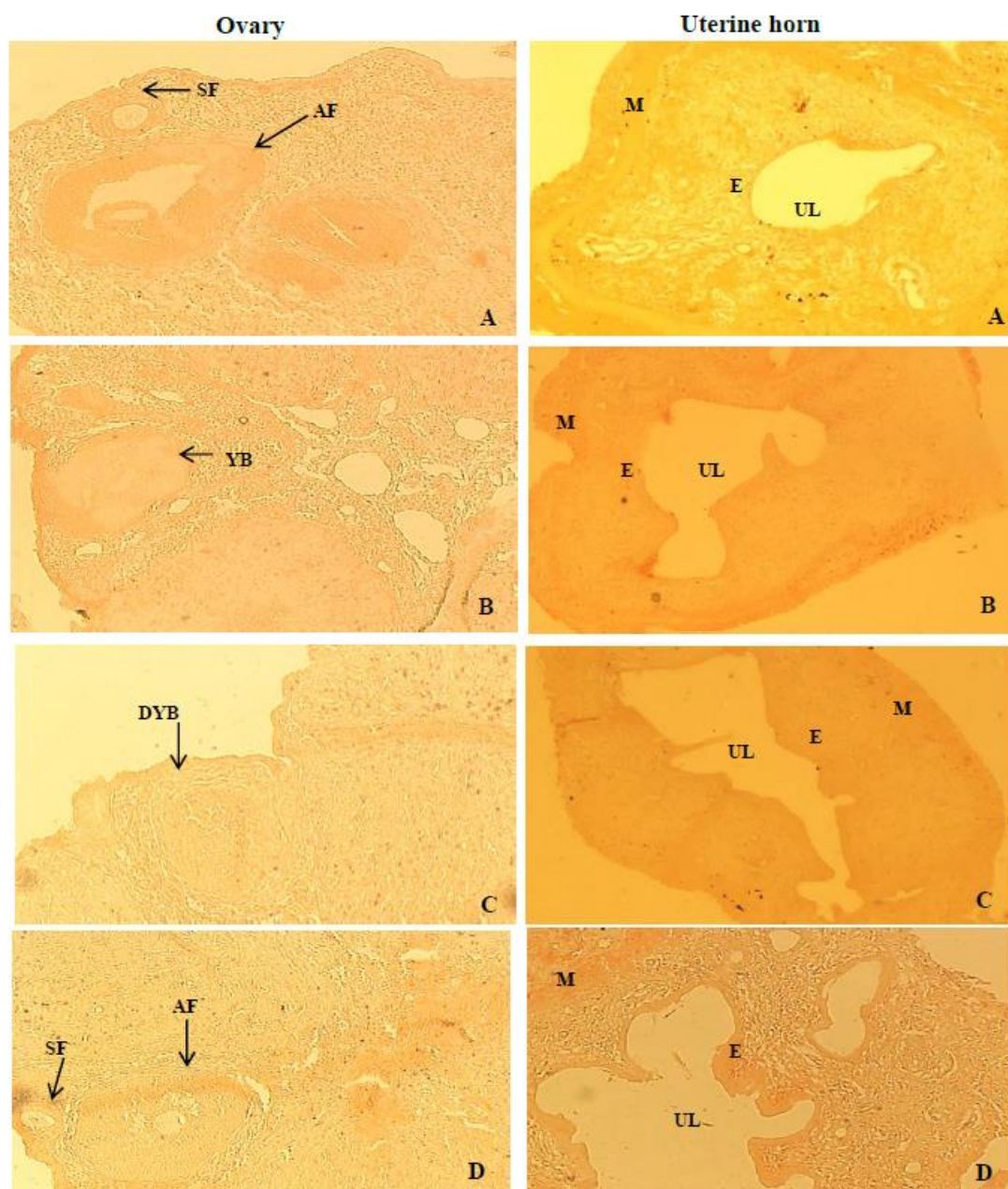


Figure 3: Photomicrograph of sections of the ovary and uterine horn of female rats after 28 days treatment with methanolic extract of *Amaranthus viridis*

100X magnificant

A: Control; **B :** Traited with 200 mg/Kg; **C:** Traited with 400 mg/Kg; **D:** Traited with à 600 mg/Kg

AF : Atretic Follicle; **CL:** Corpus luteum **DCL:** Degenerated corpus luteum ; **E :** Endometrial ; **M :** myometrium ; **SF :** Secondary Follicles ; **UL :** uterine light

DISCUSSION

Determination of the effect of the methanolic extract on the estrous cycle is based on observation of different cell types of the vaginal epithelium (basophilic, eosinophilic and leukocyte cells), which, according to their proportion, reflect the stage of the cycle [17-18].

The different vaginal smears performed daily on female rats, at different doses of the methanolic extract of *A. viridis* leaves (MEAV), allowed notice disturbances of the estrous cycle compared to control. These disturbances are more important at doses 200 et 400 mg/kg b.w. They result in increases in the number of eosinophilic cell peaks compared to leukocyte cells, which reflects a state of estrus. These observed effects

are similar to those obtained by **Bleu**, which showed that the administration of aqueous, methanolic and hexane extracts of *Passiflora foetida* leaves, causes an increase in the duration of estrus, as well as a blockage at this phase in all treated female rats from the fourth week of treatment [14]. The increase in the duration of the estrous phase could be explained by the presence in methanolic extract, estrogenic or estrogen-like substances. the works of **Affy et al.** and **Ashok et al.** on phytochemical screening of methanolic extract of *Amaranthus viridis* revealed the presence of Polyphenols, Flavonoids, Tannins, Saponins, Alkaloids, Glycosides, Sterols-Polyterpenes and Leucoanthocyanines [19-20]. The presence of flavonoids, tannins and saponins in this extract could explain these disturbances of the estrous cycle and the increase of the duration of the estrous phase.

Indeed, the works of **Mustapha et al.** showed that the presence of tannins, saponosides and flavonoids have induced an increase in the duration of proestrus and oestrus in treated female rats with ethanolic extract of *Rhynchosia sublobata* [21]. Flavonoids are well known for their estrogenic activity; they characterize the group of phytoestrogens [22]. Phytoestrogens are non-steroidal chemicals naturally produced by plants, which because of the similarity of their molecular structure with estradiol (17 β - α estradiol) have the ability to bind to estrogen receptors, by producing estrogenic or anti-estrogenic effects [23-24]. Estrogen is a steroid hormone ovarian, produced by granulosa cells and promotes follicle growth until ovulation as well as the thickening of the vaginal and uterine mucosa during proestrus and estrus [25]. On ovary, the administration of the methanolic extract showed non-significant change in fresh and dry weight at all doses of treatment. A slight increase in this organ is noted at the dose 200 mg/kg compared to control. These results seem to show that ovarian activity is not or is weakly stimulated following the administration of the extract at the doses studied.

According to **Diel et al.**, the estrogenic activity of a plant is manifested on uterine weight, biochemical parameters and histology [26]. Administration of methanolic extract induced a significant increase in fresh uterine weight at dose 400 mg/kg and a very significant increase in uterine dry weight at all treatment doses compared to control. The increase in uterine weight following administration of a plant extract was observed by **Shaik et al.** in the study of *Artemisia vulgaris* leaves [27]. MEAV could contain phytoestrogens or estrogen-like substances. Indeed, Estradiol stimulates, in particular, growth of uterine epithelial cells according to a dose-dependent effect. This proliferative action is relayed by the synthesis of growth factors (EGF : *epithelial growth factor*, IGF : *insulin-like growth factor*, PDGF : *platelet derived growth factor* ...) on cells, which results in increased of uterine weight. Moreover, increase in the dry weight of the uterus revealed that there is protein synthesis, so an anabolic effect.

On LH and estradiol, the analysis of the results showed non-significant difference between the treaties and control. However, increases in 12.5 % et 43.75 % compared to control are recorded at the respective doses of 200 et 400 mg/kg b.w. These effects are similar to those obtained by **Bleu** which showed a very significant increase in estradiol in non-ovariectomized female adult rats, in the study of *P. foetida* [14]. On the other side, at 600 mg/kg of MEAV, a decrease 21.87 % estradiol level is recorded compared to control. **El-Mahady et al.** observed a reduction of estradiol in the study of the pathological and hormonal effects of aflatoxins on the reproductive system of female rats [28]. Estradiol is produced by the granulosa cells and at a certain concentration by internal theca cells [29]. These results seem to show that the estrogenic or fertilizing potential of MEAV could have a dose-dependent action. On progesterone, non-significant difference between control and treated animals at doses 200 et 400 mg/kg b.w. On the other side, a significant increase is recorded at 600 mg/kg dose b.w. Slight reductions in progesterone, could explain by increasing the production of estradiol recorded at these same doses, characterizing the follicular phase and ovulation. Indeed, during the follicular phase and ovulation, the concentration of progesterone is relatively low. The elevation of progesterone prevents any positive feedback of the necessary estradiol to trigger the peak of LH and therefore ovulation [30]. The increase of progesterone in the blood leads to physiological changes. It is translated at the level of the uterus by pseudo-gestation state, therefore could have an

anti-fertilizing or contraceptive action. Thus, these dose-dependent effects observed on the level of progesterone could contribute to a fertilizing effect.

Photomicrographs of ovarian histology, revealed the presence of newly formed corpus luteum and degenerating corpus luteum, at the respective doses 200 et 400 mg/kg b.w of methanolic extract. These results could translate that the methanolic extract of *Amaranthus viridis* induced follicle maturation and ovulation. Non-significant difference in height of endometrial epithelial cells was recorded between tcontrol and the treaties. The diameter of the uterine glands increased significantly at doses 200 and 400 mg/kg b.w. **Zougrou et al.** observed similar effects, in the study of aqueous extract of *Cnestis ferruginea* on the histological structure of the uterine horn and ovary of female rats [9]. These results on histology could confirm the dose-dependent estrogenic potential that contains methanolic extract of *Amaranthus viridis*.

CONCLUSION

The present study show that methanolic extract of *Amaranthus viridis* contain estrogenic substances or estrogen-like substances which justifies its use by traditional medicine. This estrogenic potential could act according to a dose-dependent mechanism, with high estrogenic potential at doses of 200 and 400 mg/kg b.w. However, additional studies are required in order to valorize again this plant.

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