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Research Article

Bioactivity of the aqueous extract of *Flueggea virosa* (Roxb. ex Willd.) Royle, leaves on fertility parameters in rats

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Abstract

Female infertility is becoming a major public health issue. This study aims to evaluate the effects of the aqueous extract of *Flueggea leaves virus* (*F. virosa*) on fertility in female Wistar rats , in comparison with clomiphene citrate (Col 50). Three groups of rats with regular cycles were formed: one group treated by gavage with *F. virosa* (100 mg/kg), another with Col 50 (5 mg/kg) and a third untreated control, for thirty days. The estrogenic index (OI) was determined by daily vaginal smear. Ovarian follicles were counted by histological sections and estradiol and progesterone levels were measured. The rats treated with *F. virosa* presented the highest OI (86.66 ± 8.62%), followed by those treated with Col 50 (68.33 ± 3.05%) and the control (62.66 ± 2.51%). The number of follicles was also higher with *F. virosa* (14.67 ± 1.53) than with Col 50 (13 ± 1.0) and the control (8.67 ± 0.57). Finally, the highest hormonal levels were observed with *F. virosa* : estradiol (85.80 ± 6.79 pg / mL) and progesterone (48.58 ± 1.53 pg / mL). These data show that *F. virosa* could be used in the treatment of female infertility.

Keywords: Fertility, *Flueggea virosa* , ovarian follicles, hormones, rat

INTRODUCTION

Infertility is a condition affecting the male or female reproductive system, resulting in the inability of a couple to conceive after 12 months of unprotected intercourse [1]. The global prevalence of infertility is estimated at nearly 17.8%. Regionally, Africa has the highest rates of subfertility, estimated at 13% to 19%, compared to 15% to 20% in Europe and 13.4% in the United States [2,3]. Subfertility results from various factors such as age, hormonal imbalances, bacterial and viral infections, and vaginal yeast infections. Aging affects the hypothalamic-pituitary -gonadal axis, disrupting hormone production and the quality of reproductive cells [4] . Furthermore, infections can impair fertility either by affecting the reproductive organs or by obstructing the reproductive ducts [5]. To treat infertility, modern medicine uses three approaches: medical, surgical and ART [6]. Medical treatment is based on fertility inducers such as clomiphene citrate, as well as antibiotics and antifungals for sexually transmitted infections [7]. Despite the advances of modern medicine in the treatment of infertility, many couples still face major obstacles. The high cost of treatments, the shortage of qualified personnel and the lack of suitable health infrastructure make access to specialized care difficult or impossible [8]. Faced with these obstacles, populations in developing countries are turning to traditional medicine and medicinal plants to alleviate reproductive disorders [9,10] . It is in this context that certain plant species, such as *F. virosa* (Roxb . ex Willd .) Royle , are used in the north of Ivory Coast to improve female fertility. Ethnobotanical studies have highlighted the use of *F. virosa* in the treatment of couple infertility, suggesting the presence of bioactive compounds capable of interacting with the hormonal mechanisms regulating the ovarian cycle [11,12]. Thus, this study aims to evaluate the bioactive effects of the aqueous extract of *F. virosa* leaves on the estrous cycle and the production of ovarian hormones in female albino rats of the Wistar strain. Its aim is to contribute to the pharmacological valorization of this plant in the management of female infertility.

MATERIALS AND METHODS

1. Plant material

Plant material consists of the leaves of *F. virosa* harvested in Korhogo (Northern Ivory Coast) on morning of February 23, 2023 using a knife. After harvesting, the plant was authenticated on September 8, 2023 at the National Floristics Center of the Felix HOUPHOUËT-BOIGNY University in Abidjan (Côte d'Ivoire) where it is preserved under the herbarium number UCJ006375. Then, leaves were dried at room temperature (22 °C) for 30 days in a room at the Peleforo GON COULIBALY University in Korhogo (Ivory Coast). After drying, they were pulverized with an electric grinder (Nasco, BL2048B-CB; China) to obtain a fine powder, used for preparation of extracts.

2. Animal material

Animal material consists of 27 albino rats (*Rattus norvegicus*) of Wistar strain , with an average weight of 121 ± 0.98 g and 98 days old. These rats were raised in cages made for this purpose at the National Laboratory for Agricultural Development Support (LANADA) in Korhogo with good ventilation and a 12-hour/12-hour photoperiod. Animals were fed a mixture of 70% corn and 30% growth feed from IVOGRAIN (Côte d(Ivoire).

3. Preparation of plant extracts

The aqueous extract of *F. virosa* leaves was prepared according to the method described by Ouattara et *al*. (2012) [13]. Indeed, 100 g of powder from the plant leaves were macerated in 1 liter of distilled water. The whole was homogenized in a blender (Nasco, BL1008A-CB; China) and homogenate obtained was wrung out in a white percale cloth and double filtered on hydrophilic cotton. The filtrate obtained was concentrated in an oven (45 °C) until the solvent had completely evaporated in order to obtain a dry aqueous extract.

4. Treatment of animals

Effects of aqueous extract of *F. virosa* on female reproductive parameters were evaluated following method described by Zougrou (2017) [14]. Indeed, 3 batches of 9 rats each were made and treated as follows:

- batch 1 (control): distilled water;

- batch 2: 1 mL of aqueous extract of *F. virosa* at 100 mg/Kg of body mass per animal.

- batch 3: 1 mL of '' Clomiphene citrate '' at 5 mg /kg of body mass per animal; clomiphene is an ovulation inducer available in pharmacies .

The animals were treated by gavage daily for 30 days.

5. Evaluation of *F. virosa* aqueous extract effects on fertility parameters

5.1. Determination of estrogenic index of rats

Estrogen index corresponds to the percentage of superficial eosinophil cells and allows to determine different phases of oestrous cycle in rats. It was determined by performing a vaginal smear per animal in each batch according to the method described by Zougrou (2017) [14]. Indeed, vaginal samples were taken using a cotton swab soaked in saline solution (NaCl, 9 ‰), gently inserted into vagina, then withdrawn after slight resistance. Then, sample was spread on a microscope slide, fixed in an alcohol-ether mixture (50:50, v/v), and stained using the Harris- Shorr technique. The Observation under an optical microscope (× 40) of preparation allowed counting of leukocytes and vaginal cells for calculation of the estrogen index, using the following formula:

$$\textit{Estrogenic index (\%)} = \frac{\text{Number of keratinized surface cells}}{\text{Total number of cells counted}} X\ 100$$

From the calculated indices, different phases of the ovarian cycle were determined: diestrus or anestrus (10% eosinophilic cells and several leukocytes); metestrus (20 to 40% eosinophilic cells and quite numerous leukocytes); proestrus (40 to 50% eosinophilic cells and a very low number of leukocytes) and estrus (60 to 90% eosinophilic cells and an absence of leukocytes).

5.2. Serum determination of ovarian hormones in rats

Progesterone and estradiol assays were performed using Vidas automaton (BioMérieux, Franch) by fluorescence immunoenzymology assay (ELFA) technique. Before initiation of treatment (day 0), as well as at days 15 and 30, 3 rats were randomly selected from each group, then anesthetized using ether-soaked cotton, placed in a hermetically sealed jar for 45 seconds. After anesthesia, 2 mL of blood was collected from each rat at the retro-orbital sinus using a Pasteur pipette, in dry tubes. Collected blood was centrifuged at 3000 rpm for 5 min using a centrifuge (Thermo Scientific ™ ST16R, USA), and the serum was collected for ovarian hormone assay. After calibration, a volume of 100 µL of serum was collected and introduced directly into the machine for each hormone. Doses were read on screen and printed via connected interface [15].

5.3. Histological analysis of rat ovaries

Counting of follicular structures was done according to the method described by Zougrou *et al*. (2018) [16]. Indeed, anesthetized rats were sacrificed and autopsied according to veterinary medicine procedure adopted at the National Laboratory for Agricultural Development Support in Korhogo. After autopsy, the ovaries fixed in a 10% formalin solution supplemented with 9 ‰ NaCl were subjected to progressive dehydration in ethanol, clarified with toluene, and then embedded in paraffin. Serial sections of 5 microns in thickness were made using a rotary microtome (Leica RM2125 RTS, Germany). Deparaffinized sections were then systematically stained with hematoxylin and eosin. The observation of the sections was carried out using a tri-ocular light microscope (Olympus CK41SF, Philippines) connected to a computer. Imaging and morphometric analysis were performed using Videomet software version 8.0.9 (France).

6. Statistical analysis of data

Statistical tests were carried out using SPSS software (statistics 26) and the graphs were produced using Excel 2016 software. The significance of differences observed between the different test groups is assessed by analysis of variances (ANOVA) associated with Turkey's multiple comparison test at 5% threshold.

RESULTS

1. Effect of *F.virosa* extract on estrogenic index of rats and estrous cycle

Estrogen index (IO) values obtained after administration of the different treatments are represented in **Figure 1.**

Before any treatment (D_0), the estrogen indices of the different batches of rats were between 47.33 ± 7.09% and 51.33 ± 2.51%. Statistical analysis revealed no significant difference between treated and control females ($P < 0.05$). On the 5th day of treatment, females treated with *F. virosa* moved from proestrus stage to estrus stage with an index of 62.66 ± 3.78%. In contrast, females in control group and those in the group treated with clomiphene citrate (Col 50 mg) remained in the proestrus stage, with an OI ranging from 49.66 ± 1.15% to 55.33 ± 7.09%. Statistical analysis revealed that there is a significant difference between OI of animals in the control group and that of females treated with *F. virosa* ($P < 0.05$).

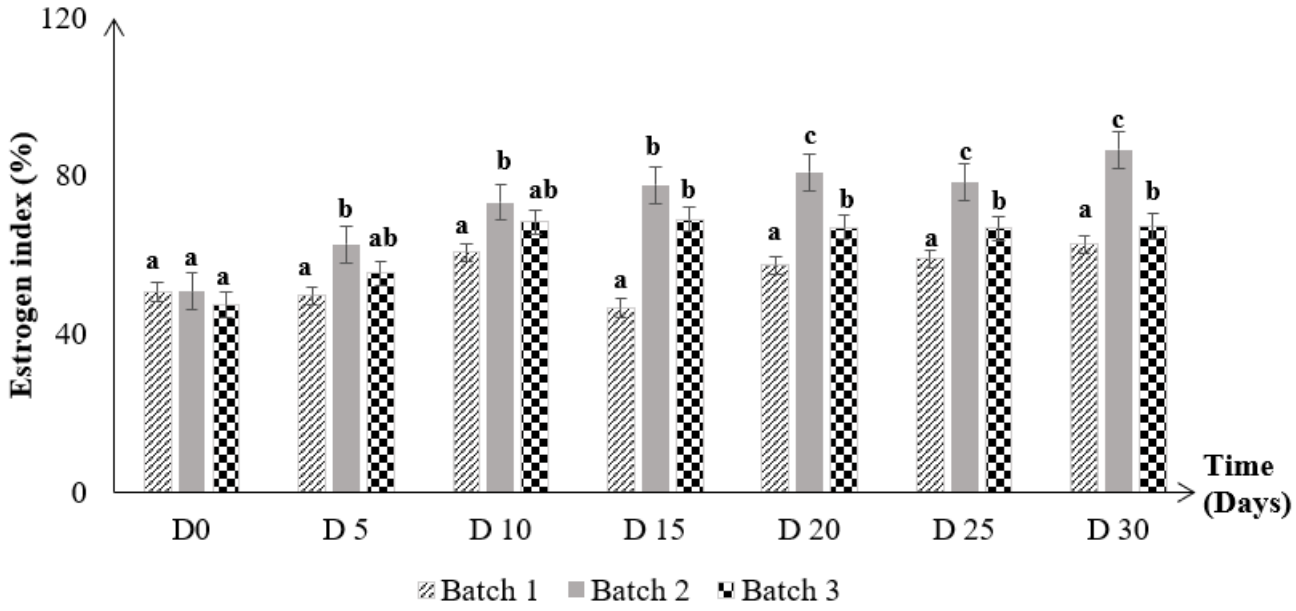


Figure 1: Estrogenic index of different batches of rats

Batch 1: control; batch 2: rats treated with aqueous extract of F. virosa ; batch 3: rats treated with clomiphene citrate 50 mg. Estrogen indices were compared independently per day between the different treatments ; bands with the same letter did not show a significant difference (P > 0.05)

From the 10th day of treatment until the end of the experiment, vaginal smears revealed an OI greater than 60% in the treated groups, respectively from 68.33 ± 3.05% to 67.33 ± 2.51% (Col 50 mg) and from 73.33 ± 2.08% to 86.66 ± 8.62% (*F. virosa*) with a significant difference ($p < 0.05$) between these groups . These OIs indicate a permanence of estrus in these different females. On the other hand, estrus was observed only on the 10th day (60.66 ± 4.04%) and the 30th day (62.66 ± 2.51%) in the females of the control group.

2. Effect of *F.virosa* extract on ovarian hormones of rats

2.1. Estradiol levels

The effects of treating rats with *F. virosa aqueous extract* and clomiphene citrate (Col, 50 mg) for 15 and 30 days on serum estradiol concentration are shown in the graph in **Figure 2.** Analysis of this graph shows that estradiol levels increased significantly in all groups of rats after 15 days of treatment. Indeed, *F. virosa aqueous extract* induced a significant ($P < 0.05$) increase in estradiol levels from 18.83 ± 1.54 to 64.95 ± 8.47 pg / mL. This increase was higher than that observed in untreated animals (19.17 ± 1.93 to 37.33 ± 2.52 pg / mL) and those treated with Col 50 mg (18.67 ± 1.70 to 54.66 ± 3.05 pg / mL). Statistical analysis also revealed a significant difference between untreated group and one treated with Col 50 mg. A similar trend was observed after 30 days of treatment. Highest rate was recorded in rats treated with aqueous extract of *F. virosa* (85.80 ± 6.79 pg / mL), followed by the group treated with clomiphene citrate (69.66 ± 0.57 pg / mL), while control group presented the lowest rate (50.58 ± 10.05 pg / mL).

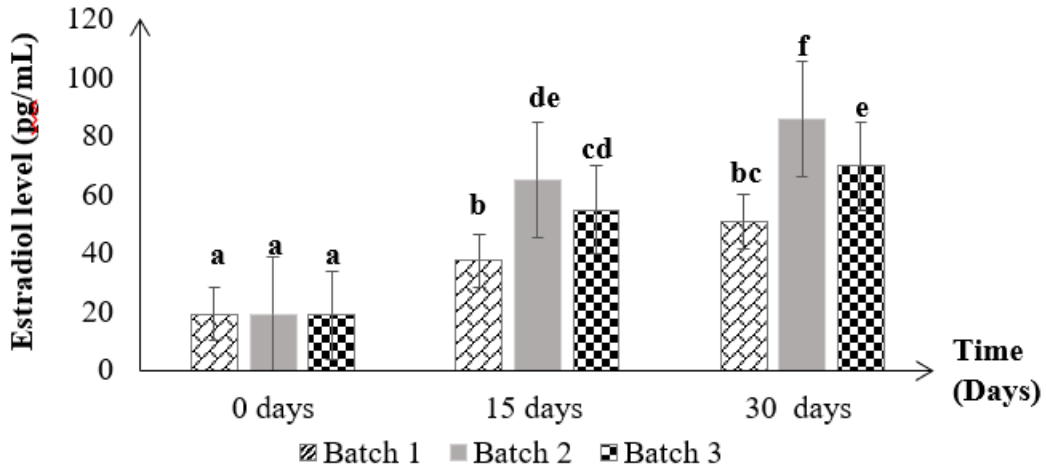


Figure 2: Effect of *F. virosa* extract on estradiol levels after treatments

Batch 1: control; batch 2: rats treated with aqueous extract of F. virosa ; batch 3: rats treated with clomiphene citrate 50 mg. Hormone levels are compared between the different treatments according to the treatment period; bands with the same letter do not show a significant difference (P >0.05).

2.2. Progesterone levels

Graph in **Figure 3** presented evolution of progesterone level according to treatments with aqueous extract of *F. virosa* and Col 50 mg. Analysis of the graph showed a significant increase (P < 0.05) in progesterone level in all batches throughout the treatment. After 15 days, level of this hormone increased respectively from 15.16 ± 4.05 to 25.58 ± 0.53 pg / mL (*F. virosa*) and from 15.33 ± 4.28 to 24.84 ± 1.85 pg / mL (Col 50 mg), compared to 14.67 ± 2.67 to 21.66 ± 4.33 pg / mL in the control batch. The increase in this hormone continued until day 30, with a very significant increase in the group treated with *F. virosa* (48.58 ± 1.58 pg / mL). This increase was significantly higher than that observed with Col 50 mg (38.67 ± 1.04 pg / mL) and in the control group (30.47 ± 3.14 pg / mL). Statistical analysis also revealed a significant difference between Col 50 mg and the control.

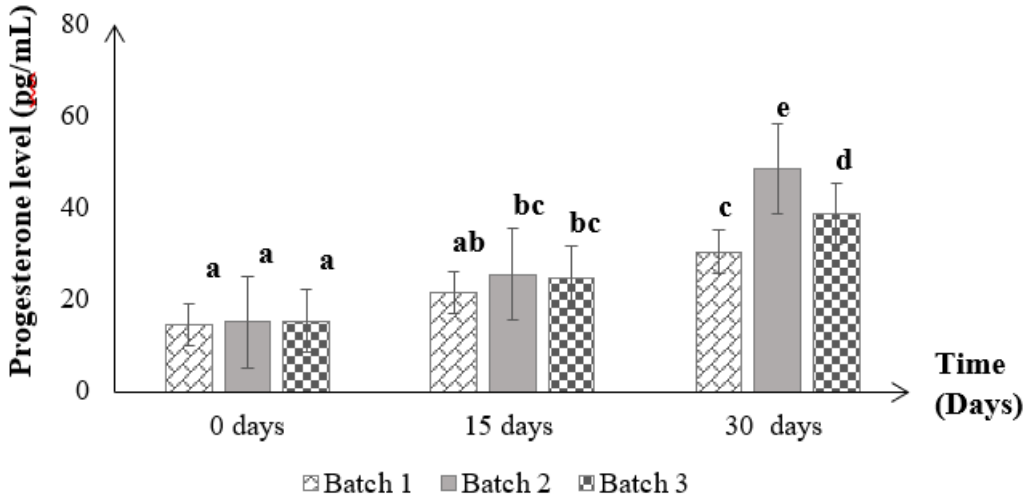


Figure 3: Effect of *F.virosa* extract on progesterone levels after treatments

Batch 1: control; batch 2: rats treated with aqueous extract of F. virosa ; batch 3: rats treated with clomiphene citrate 50 mg. Hormone levels are compared between the different treatments according to the treatment period; bands with the same letter do not show a significant difference (P >0.05).

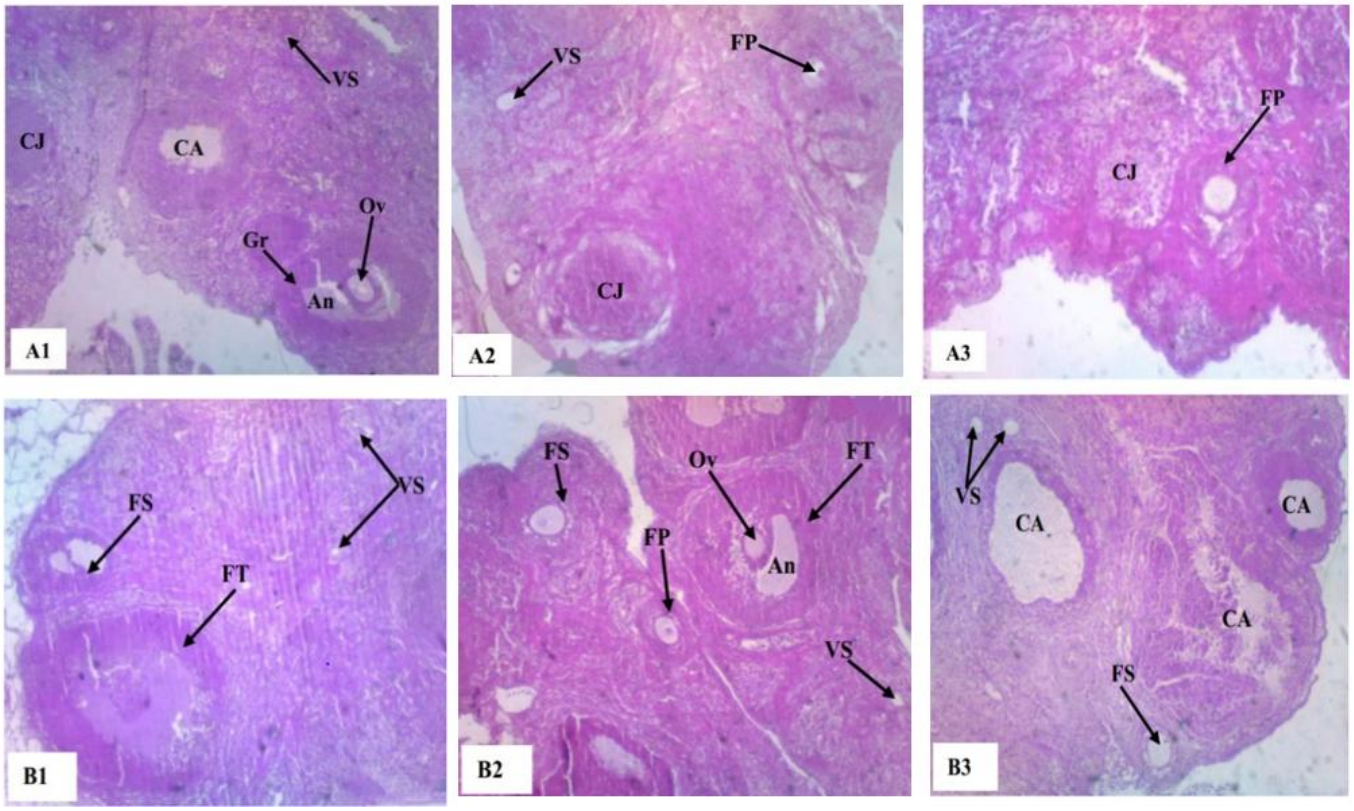
3. Effect of *F. virosa* extract on follicular structures of rats

Histological micrographs of the ovaries of female rats after 15 and 30 days of treatment with aqueous extract of *F. virosa* and clomiphene citrate (Col 50 mg) are shown in **Figure 4.** Analysis of histological sections revealed a significant increase (P < 0.05) in follicular structures in all groups of rats throughout treatment, quantities of which are recorded in **Table 1**.

After 15 days of treatment, aqueous extract of *F. virosa* administration induced a significant increase in the number of Graafian follicles from 2.33 ± 0.57 to 8.67 ± 1.53. This progression was significantly higher than that observed in the untreated group (2.67 ± 0.57 to 4.67 ± 1.00) as well as in the group treated with Col 50 mg (2.33 ± 1.53 to 6.00 ± 1.00). This trend was maintained at day 30, with respective values of 8.00 ± 1.53 for the aqueous extract, 6.67 ± 1.53 for Col 50 mg and 4.33 ± 1.15 for the control group.

Regarding corpora lutea, their number increased in all groups after 15 days of treatment. However, this increase was more significant in rats treated with aqueous extract of *F. virosa* (1.33 ± 0.57 to 12.67 ± 1.57) and with Col 50 mg (2.00 ± 1.00 to 10.33 ± 2.08), compared to the control group (1.67 ± 0.57 to 6.33 ± 1.15). This increase continued on day 30, with an intensification of the number of corpora lutea in all groups. Highest values were recorded in females treated with *F. virosa* (14.67 ± 1.53), followed by those treated with clomiphene citrate (13.00 ± 1.00).

Finally, the number of corpora albicans also increased significantly after 15 days of treatment, particularly in batch treated with *F. virosa* (0.33 ± 0.57 to 6.67 ± 1.15). This value was significantly higher than that observed in untreated batch (0.00 ± 0.00 to 2.33 ± 1.53) and in batch treated with Col 50 mg (0.33 ± 0.57 to 3.66 ± 1.15). Beyond 30 days, a slight increase was observed in the batches treated from 6.67 ± 1.15 to 7.67 ± 2.08 for *F. virosa* and from 3.66 ± 1.15 to 4.33 ± 1.15 for Col 50 mg. In contrast, value recorded in control batch decreased slightly from 2.33 ± 1.53 to 1.67 ± 1.15.



A1: Control (15 days); A2: clomiphene citrate 50 mg (15 days); A3: F. virosa (15 days); B1: Control (30 days); B2: clomiphene 50 mg (30 days); B3: F. virosa (30 days); An : An trum; CA : C orpus A lbucans ; CJ : Corpus L uaune ; FP : P rimordial Follicle ; FS : Secondary Follicle ; Gr : Granulosa

Figure 4: Micrograph of the ovary of control and treated females

Table I: Effect of *F. virosa* and the reference molecule on ovarian structures

Durations	Batches of rattes	Number of follicular structures		
		Degraaf's follicles	Yellow bodies	Corpus albicans
0 days	Batch 1	2.67 ± 0.57 ^{a}	1.67 ± 0.57 ^{a}	0.00 ± 0.00 ^{a}
	Batch 2	2.33 ± 0.57 ^{a}	1.33 ± 1.15 ^{a}	0.33 ± 0.57 ^{ab}
	Batch 3	2.33 ± 1.53 ^{a}	2.00 ± 1.00 ^{a}	0.33 ± 0.57 ^{ab}
15 days	Batch 1	4.67 ± 2.08 ^{ab}	6.33 ± 1.15 ^{b}	2.33 ± 1.53 ^{abc}
	Batch 2	8.67 ± 1.53 ^{c}	12.67 ± 1.57 ^{de}	6.67 ± 1.15 ^{cd}
	Batch 3	6.00 ± 1.00 ^{abc}	10.33 ± 2.08 ^{cd}	3.66 ± 1.15 ^{bcd}
30 days	Batch 1	4.33 ± 1.15 ^{ab}	8.67 ± 0.57 ^{bc}	1.67 ± 1.15 ^{ab}
	Batch 2	8.00 ± 1.53 ^{bc}	14.67 ± 1.53 ^{e}	7.67 ± 2.08 ^{d}
	Batch 3	6.67 ± 1.53 ^{bc}	13.00 ± 1.53 ^{de}	4.33 ± 1.15 ^{bcd}

Batch 1: control; batch 2: rats treated with aqueous extract of F. virosa; batch 3: rats treated with clomiphene citrate 50 mg. The number of ovarian cells was compared independently between treatments according to the period; values followed by the same letter in superscript do not show a significant difference (P > 0.05).

DISCUSSION

Analysis of vaginal smears showed that estrogen index of all batches of rats was less than 60 % (IO < 60 %) before treatment administration. This index indicates that all females were in the same physiological state, corresponding to proestrus phase. Indeed, the fact that all rats were in same phase of the estrous cycle makes it possible to eliminate individual physiological variations that could influence effects of the different treatments. This homogeneity will therefore make it possible to properly assess the action of *F.virosa* aqueous extract on estrous cycle compared to clomiphene citrate and the control batch. These results differ from those of Coulibaly *et al.* (2023) and Bafounguila-Ngala *et al*. (2023) [17,18]. These authors respectively evaluated the effect of an aqueous extract of *Cissus aralioides* leaves in Côte d'Ivoire and a traditionally improved drug (Namikawo®) in Congo on estrous cycle of rats in different physiological states.

Then, from the 5th day of treatment, rats receiving aqueous extract of *F. virosa* moved from proestrus stage to estrus stage (IO > 60 %). They were followed by females treated with clomiphene citrate on the 10th day, compared to untreated rats. Aqueous extract of *F. virosa* promoted persistence of estrus in rats, with very high estrogenic indices reaching 86.66%. This persistence of estrous phase could be explained by the presence of bioactive secondary metabolites in the leaves of *F. virosa*, including total polyphenols, tannins, flavonoids, saponins and alkaloids[19]. Indeed, these phytochemicals have the capacity to modulate hormonal activity. In particular, flavonoids are thought to possess estrogen-mimetic properties by interacting with estrogen receptors to stimulate and prolong the estrous phase [20]. While saponins could improve the bioavailability of estrogens by facilitating their absorption and circulation. Finally, alkaloids may act on the hypothalamic-pituitary-ovarian axis, thereby influencing secretion of GnRH (gonadotropin-releasing hormone), FSH (follicle-stimulating hormone), and LH (luteinizing hormone). All these combined actions could promote follicular maturation, estrogen secretion and stimulate the proliferation of vaginal epithelial cells, hence the persistence of estrus [21,22]. These results are consistent with those obtained by de Zougrou (2017) and Coulibaly *et al*. (2023)[14,17]. These authors showed that aqueous extracts of *Cnestis ferruginea* and *Cissus aralioides* made it possible to block the estrous cycle in the estrus phase and lengthen the duration of this phase, reflecting marked estrogenic activity.

Hormone assay revealed a significant increase in estradiol and progesterone levels in rats treated with *F. virosa*, compared to untreated rats. This hormonal elevation suggests a joint stimulation of estrogenic and follicle-stimulating activities, probably induced by phytochemical compounds present in this plant, such as saponosides, flavonoids and alkaloids. Indeed, these substances would act as phytoestrogens. On the one hand, they would influence aromatase activity by converting androgens into estrogens; on the other hand, they could sensitize estrogen receptors at the target cell level [23]. Furthermore, phytochemical composition of *F. virosa* could justify the high progesterone level observed in treated rats. Flavonoids and saponins are thought to be involved in expression of steroidogenic enzymes, thus promoting progesterone biosynthesis [24]. In addition, saponins may stimulate hypothalamic- pituitary-gonadal axis to trigger the secretion of LH , which is essential for ovulation and the formation of the corpus luteum, the main site of progesterone production [25,26]. These results are similar to those obtained by Bafounguila-Ngala *et al.* (2023), who showed in Congo that the aqueous extract of a traditionally improved phytomedicine increased ovarian hormone levels in rats [18].

Histological analysis of ovaries revealed the presence of a high number of follicular structures, such as Graafian follicles, corpora lutea and corpora albicans in rats treated with the aqueous extract of *F. virosa*, compared to the control group. This abundance of follicular structures could confirm the estrogen -progestational effects of the phytochemical compounds present in the aqueous extract of *F. virosa*. Indeed, the high presence of Graafian follicles would be related to the follicle-stimulating activity of flavonoids and saponosides. These secondary metabolites would act as phytoestrogens, capable of stimulating the secretion of FSH, necessary for follicular growth and maturation [20,23]. After follicular maturation, saponins are also thought to stimulate the hypothalamic-pituitary -gonadal axis, leading to the secretion of LH, which is essential for triggering ovulation and forming corpora lutea [26,27]. Finally, the presence of corpora albicans, resulting from degeneration of unmaintained corpora lutea, could be explained by the active renewal of the ovarian cycle. The high number of these follicular structures could indicate the repeated initiation of non-prolonged luteal phases in absence of fertilization, thus reflecting sustained ovarian stimulation exerted by the aqueous extract of *F. virosa* [28]. These results are consistent with those reported by Zougrou *et al* . (2018), who carried out the counting of follicular structures in rats after their treatment with the aqueous extract of *Cnestis leaves ferruginea* [16] .

CONCLUSION

This study aimed to evaluate the bioactive effects of aqueous extract of *F. virosa* leaves on the estrous cycle and hormone production in female Wistar rats. It revealed a lengthening of estrous phase, stimulation of folliculogenesis, and a significant increase in progesterone and estradiol levels. Rats treated with *F. virosa* extract showed more marked effects than those treated with clomiphene citrate or untreated rats. These results suggest that *F. virosa* could be a promising basis for the development of improved traditional medicines (ATMs) for the management of female infertility.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Ethical approval: Ethical approval was obtained from the Ethics Committee of Biotechnology and Valorization of Agroresources and Natural Substances Laboratory, Peleforo GON COULIBALY University.

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