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The Aqueous and Methanol Extracts of *Ficus asperifolia* (Moraceae) Improve Reproductive Hormones Profile in High Fat Diet-Induced Obese Rats

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Abstract

Ficus asperifolia (Moraceae), is a plant used as fertility booster. We investigated the effects of *F. asperifolia* extracts on sex hormones profile in high-fat diet (HFD)-induced estrus cycle disturbances in rats. Female Wistar rats were fed either with HFD (n=161) or standard diet (n=8) for 10 weeks. After this period, 70 obese rats with abnormal estrus cycle were distributed into 14 groups of 5 animals each and treated for one and four weeks with distilled water (10 mg kg-1), Tween-80 (5%-10 mg kg-1), lutenyl (0.88 µg kg-1) and aqueous or methanol extract (100 or 500 mg kg-1) of *F. asperifolia*. At the end of each period, plasmatic estradiol and progesterone levels were determined. Acute toxicity parameters were also evaluated. HFD impaired reproductive hormones profile and estrous cycle after 10 weeks of treatment.

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F. asperifolia improved reproductive hormone profile by significantly increasing the plasmatic estradiol and progesterone levels. For instance, the plasmatic estradiol and progesterone levels were significantly increased ($p<0.05$-0.01) in rats treated with the aqueous (100 mg kg-1, four-weeks; 500 mg kg-1, one-week) or methanol (100 and 500 mg kg-1, four-weeks) extract of *F. asperifolia*. No toxic effects of *F. asperifolia* extracts were recorded. Our data support the traditional use of *F. asperifolia* as fertility enhancer.

Keywords: *Ficus asperifolia*; estrus cycle; estradiol; progesterone; acute toxicity; high fat diet Type your.

1. Introduction

Obesity is a global health problem, characterized by an excessive storage of adipose tissue in the organism [1]. Inadequate nutrition impairs reproductive function in many mammalian species [2]. Clinical observation has long suggested that a powerful relationship exists between adiposity and female fertility [3]. Schneider and his colleagues [4] first postulated that a minimal amount of body fat was necessary for both the commencement and the maintenance of ovulatory cycles in young women while obesity may lead to infertility. In addition, obese rats have several endocrine and metabolic abnormalities, including low metabolic rate [5], hyperinsulinemia, [2] progesterone over production [6], subsequent abnormal estrous cyclicity [7], undeveloped uteri [4,7,8], and infertility [9]. Taken together, these findings demonstrate a strong association between obesity and the relative risk for female infertility. Hormone replacement therapy with estrogen may cause severe side effects with regards to risk of cancer [10]. In this light, medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [11]. *Ficus asperifolia* (Moraceae), known as "Ntchach lum" in the west Cameroon, is used for the treatment of some cases of infertility in women. From our previous findings, the aqueous extract of *F. asperifolia* (100 mg kg^{-1}) has been found to have 100% fertility effects in female rat through a significant increase ($p<0.05$) in the implantation sites and litter size of animals as well as uterotrophic-like activities [12]. Also, *F. asperifolia* induced uterotonic effect in the oestrogenised isolated rat uterus [13]. In addition, it has been shown by our research team that the aqueous and methanol extracts of *F. asperifolia* does not disturb normal estrus cycle [14], but have an alleviating effects on female infertility induced by 15% high fat diet. These effects were characterized by a time-dependent correction of irregular estrus cycle, a significant decrease ($p<0.001$) in the total plasma cholesterol and low density lipoprotein (LDL) cholesterol level and a significant increase ($p<0.001$) in high density lipoprotein (HDL) cholesterol in obese female rats [15]. No scientific information is available about the activity of *F. asperifolia* on reproductive hormone concentrations in obese rats. Hence, the aim of the present study was to investigate the effect of the aqueous and methanol extracts of *F. asperifolia* on plasmatic estradiol and progesterone concentrations of obese rat having irregular estrus cycle. The extracts were also evaluated for possible effects on some acute toxicity parameters after two weeks.

2. Materials and Methods

2.1 Collection of plant material and preparation of extracts

Fresh fruits of *Ficus asperifolia* (L) Hook. ExMiq (Moraceae) were collected in the month of February 2011 from trees in in Dschang, Cameroon. Botanical identification was performed in the Cameroon National

Herbarium (HNC) in comparison with the existing specimen number 338/15240/HNC. The fruits were shade-dried for 5 days and ground into powder. The aqueous and methanol extracts of *F. asperifolia* were used in the present study. In other to obtain an aqueous extract similar to the traditional recommendation, 1 kg of *F. asperifolia* was soaked in distilled water (5 L) and the mixture boiled for 15 minutes. The heated decoction was taken and allowed to cool at room temperature, filtered using Whatman paper No. 3 and oven-dried to give 46.67 g of dried aqueous extract (yield of extraction, 4.66% w/w based on the dried starting weight). To obtain the methanol extract, 1 kg of *F. asperifolia* powder was soaked in 5 L of methanol (95%) for 24 h. The extract was filtered using Whatman paper No. 3 and filtrate was evaporated (87°C) to dryness using a rotary evaporator. 50 g of dried methanol extract was obtained giving an extraction yield of 5% (w/w based on the dried starting weight). For bioactivity investigations, the aqueous and methanol extracts were dissolved in distilled water and 5% Tween 80, respectively.

2.2 Phytochemical screening

The freshly prepared aqueous and methanol extracts were tested to determine the presence of flavonoids (test of Shinoda), sterols (Liebermann Burchard test), phenol (ferric chloride test), alkaloids (Dragendorff test) and saponins (Saponification test) as previously described [16].

2.3 Animals

Healthy non-pregnant adult female Wistar rats (10-11 weeks, 150-160 g) were used in this study. They were housed in groups (five per group in polypropylene cages) and maintained under uniform husbandry conditions having natural photoperiod, humidity, temperature (26±2°C) and free access to food and water. The experiments were conducted in the Laboratory of Physiology and Phytopharmacology of the University of Dschang in accordance to the internationally accepted standards of ethical guidelines for laboratory animal use and care as described in the European Community guidelines [17]. All efforts were made to minimize the number of rats used and their suffering.

2.4 Formulation of high fat diet and standard diet

Control rats were fed on standard diet (SD) consisted of fats (7-10%), carbohydrate (68-70%), protein (18-20%), vitamins (1-2%) and minerals (1-2%). 15% of palm oil was added to the SD to prepare high fat diet (HFD) [15,18,19]. The locally available palm oil used in this study contained saturated fat (lauric acid: <0.5%; myristic acid: 0.5-2%; palmitic acid: 39.5-47.5%; stearic acid: 3.5-6%) monounsaturated fat (oleic acid: 38-45%) and polyunsaturated fat (linoleic acid: 9-12%; α-linoleic acid: <0.5%) [18]. In powder form, chow was mixed with water until it became homogenous in a dough-like consistency. The dough was shaped and used for feeding.

2.5 Experimental design

2.5.1 Induction of obesity

169 female Wistar rats were fed either with HFD (n=161) or standard diet (n=8) for 10 weeks. The hyperlipidic diet was essentially characterized by enrichment in palm oil compared to the standard diet as indicated in the

above composition. The animals were weighed daily. At the end of the 10 weeks diet, increase in body weight (more than 15% of initial body weight prior to hyperlipidic diet) and the Lee index (above 300) were taken in order to validate the obesity status of each animal. The Lee index (LI) was calculated using the following formula: LI= [cube root of the body weight (g)/naso-anal length (cm)] X 100) [20]. Animals declared obese after 10 weeks of HFD were selected and maintained under this special diet during the experiments.

2.5.2 Estrus cycle monitoring

Vaginal smear was collected and observed (8–10 a.m.) to determine the estrus cycle of each obese animal. This involved sampling the cells of the vaginal canal with sterile saline using a glass pipette. The recovered solution containing cells was placed on microscope slides, stained with methylene blue (0.03%), dried and examined using light microscope (400X, Zeiss). Cell descriptions were used to classify rats based on the stages of the cycle (proestrus, estrus, metestrus and diestrus). Basically, proestrus (the first stage) is characterized by the predominance of nucleated epithelial cells which could appear in clusters or individually; occasionally some cornified cells could be found in the sample. Estrus (the second stage) is distinctively characterized by cornified squamous epithelial cells which occur in clusters, there is no visible nucleus and the shape is irregular. Metestrus (the third stage) is a mix of cell types with a predominance of leukocytes and a few nucleated and/or cornified squamous epithelial cells. Diestrus (the fourth stage) consists predominantly of leukocytes. However, the determination of the estrus stage is based on the proportion among of the above-mentioned three cell types [21]. Only rats with irregular (rats having the four stages of the estrus cycle but in a disorder way) or blocked (rats having less than three phases and one stage coming more than twice per cycle) estrus cycles were selected.

2.5.3 Animal treatment

A total of seventy obese females with disturbed estrous cycle maintained under HFD were randomized into two sets of thirty five animals each and treated for one week (set I) and four weeks (set II). Each set was then divided into seven groups of five animals each and treated as follows: Group 1: distilled water (10 ml kg^{-1}, control 1) ; Group 2: 5% Tween 80 (10 ml kg^{-1}, control 2); Group 3: lutenyl (0.88 µg kg^{-1}, control 3); Group 4-5: aqueous extract of *F. asperifolia* (100 and 500 mg kg^{-1}); Group 6-7: methanol extract of *F. asperifolia* (100 and 500 mg kg^{-1}). The extracts and vehicles were given orally once a day. At the end of the treatment period, animals were killed by decapitation under diazepam/ketamine (10/50 mg kg^{-1} respectively) anaesthesia and blood samples were collected from the catheterization of abdominal artery. After centrifugation (3000 × g for 10 min), plasma was separated, aliquoted and stored at -20°C till biochemical analysis. Acute toxicity parameters were conducted according to the guidelines of Organization for Economic Co-operation and Development (OECD, 423). Forty adult female Swiss albino mice were randomly allocated to four groups of ten animals each and treated as follows: Group 1: distilled water (10 ml kg^{-1}, control 1); Group 2: 5% Tween 80 (10 ml kg^{-1}, control 2); Group 3: aqueous extract of *F. asperifolia* (5 g kg^{-1}); Group 4: methanol extract of *F. asperifolia* (5 g kg^{-1}). The acute toxicity parameters such as diarrhea, aggressiveness, pain sensitivity and noise sensitivity were recorded during the first three hours and mortality was recorded after two weeks. Animals were later sacrificed, liver, kidney, heart, lung and spleen were excised, rinsed and their relative weights were calculated.

2.6 Biochemical analysis

Plasmatic estradiol and progesterone levels were measured using ELISA kit (Accubind, Monobind Inc. Lake Forest, USA) special to animal models according to the manufacturer's instructions. Samples were analyzed in triplicate and data expressed as µg/mg protein. Estradiol and progesterone concentrations were determine as described previously [22,23].

2.7 Statistical analysis

Data were expressed as mean±SEM. One-way analyses of variance (ANOVA) followed by post-hoc LSD was used to analyze statistical difference among groups using Statistica for Software version 8.0. A probability level of less than 0.05 ($p < 0.05$) was considered as statistically significant.

3. Results

3.1 Phytochemical analysis of the aqueous and methanol extracts of F. asperifolia

The results of the phytochemical evaluation of *F. asperifolia* extracts are presented in table 1. Alkaloids, saponins, sterols and triterpens were present in all extracts.

Table 1: Phytochemical analysis of the aqueous and methanol extracts of *F. asperifolia*

Group of compounds	Aqueous extract of *F. asperifolia*	Methanol extract of *F. asperifolia*
Alkaloids	+	+
Saponins	+	+
Flavonoids	-	-
Sterols	+	+
Triterpens	+	+

+: Present, -: Absent

3.2 Effects of HFD on estrus cycle

15% palm oil diet was efficient in promising obesity after 10 weeks of treatment, as demonstrated by a significant increase ($p < 0.05$) in the growth rate and Lee index compared to the SD group. 44.38 % of HFD rats were declared obese while 55.62 % failed to respond. We also noted that 98 % of obese rats exhibited an irregular estrus cycle (Table 2).

Table 2: Obesity and estrus cycle parameters in normal (SD) and high fat diet (HFD) rats after 10 weeks of treatment.

Outcome measure	SD rats	HFD rats
Obesity parameters		
Body weight gain (%)	5.52 ± 2.47	26.89 ± 3.93*
Lee index	297 ± 1.05	367.32 ± 2.57*
Percentage of obese rats (%)	0	44.38
Estrus cycle parameters (%)		
Normal cycle	100	2
Disturbed cycle	0	98
Backward cycle	0	12
Proestrus constant	0	17
Diestrus constant	0	6
Metestrus constant	0	5
Metestrus-Diestrus	0	6
Diestrus-Proestrus	0	11
Proestrus-Metestrus	0	8

* $p < 0.05$: significantly different compared with SD rats.

3.3 Effets of F. asperifolia on plasmatic estradiol concentration in obese rats

Compared to control groups (distilled water and Tween 80), lutenyl or aqueous and methanol extracts of *F. asperifolia* increased plasmatic estradiol level after one and four weeks of treatment. This increase was significant in the animals treated with aqueous (dose 100 mg kg^{-1}, four weeks, $p < 0.05$; dose 500 mg kg^{-1}, one week, $p < 0.01$) or methanol (dose 100 and 500 mg kg^{-1}, four weeks, $p < 0.01$) extracts of *F. asperifolia* and lutenyl (four weeks, $p < 0.001$). However, the aqueous extract of *F. asperifolia* at the dose 500 mg kg^{-1} and lutenyl exhibited the highest effects after one and four weeks of treatment respectively (Figure 1).

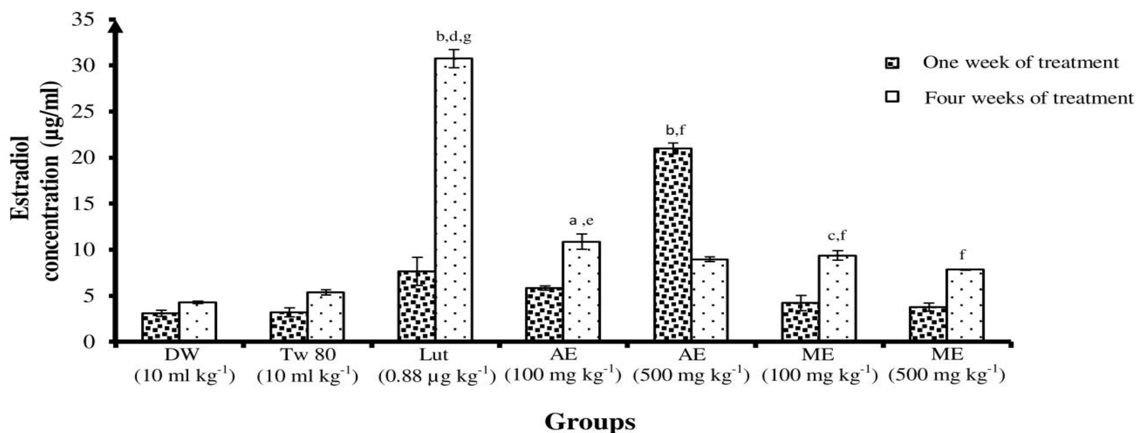

Figure 1: Effects of aqueous and methanol extracts of *F. asperifolia* on plasmatic estradiol level in obese rats after one and four weeks of treatment.

Each bar represents the mean±SEM. Number of rats in each group = 5. DW: distilled water, Tw= Tween, Lut: Lutenyl, AE: aqueous extract, ME: methanol extract. $^{a}p < 0.01$; $^{b}p < 0.001$: *significantly different compared*

with distilled water. ${}^{c}p < 0.05$; ${}^{d}p < 0.01$: significantly different compared with Tween. ${}^{e}p < 0.05$; ${}^{f}p < 0.01$; ${}^{g}p < 0.01$: significantly different compared with Lutenyl.

3.4 Effets of F. asperifolia on plasmatic progesterone concentration in obese rats

The effects of lutenyl and aqueous or methanol extract of *F. asperifolia* on plasmatic progesterone concentration are shown in figure 2. It is observed that both compounds induced a significant increase in plasmatic progesterone level at all doses after one and four weeks of treatment. In all groups, the four weeks treatment was more efficient in increasing progesterone level, with the highest effect observed in rats treated with lutenyl (Figure 2).

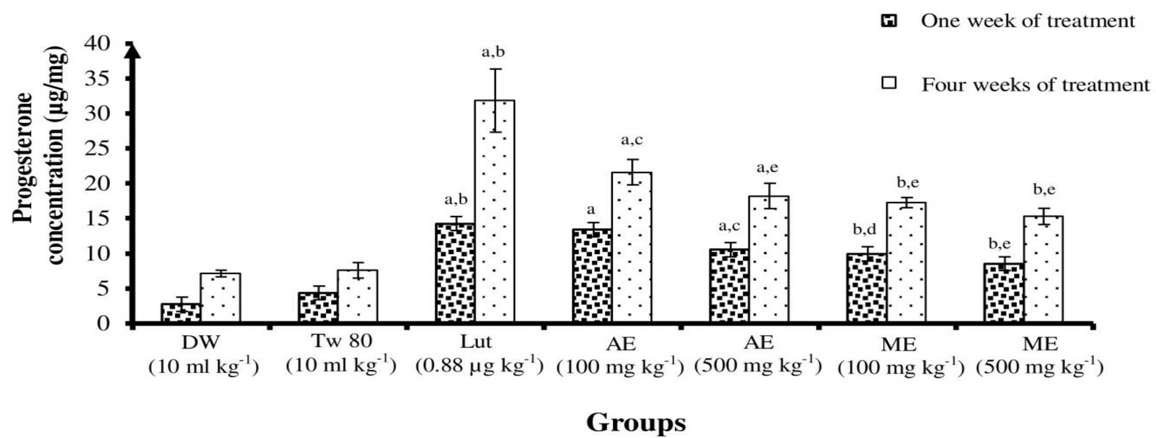

Figure 2: Effects of aqueous and methanol extracts of *F. asperifolia* on plasmatic progesterone elevel in obese rats after one and four weeks of treatment.

Each bar represents the mean±SEM. Number of rats in each group = 5. DW: distilled water, Tw = Tween, Lut: Lutenyl, AE: aqueous extract, ME: methanol extract. ${}^{a}p < 0.001$: significantly different compared with distilled water. ${}^{b}p < 0.001$: significantly different compared with Tween. ${}^{c}p<0.05$; ${}^{d}p<0.01$; ${}^{e}p<0.001$: significantly different compared with lutenyl. Based on the results obtained, the aqueous extract of *F. asperifolia* was more active than methanol extract in improving estradiol and progesterone levels.

3.5 Effects of F. asperifolia on acute toxicity parameters

From the result obtained, no toxicity parameters such as the presence of diarrhea, aggressiveness, pain sensitivity, noise sensitivity and death was observed after treatment with the aqueous or methanol extract of *F. asperifolia* (Table 3). No significant change in the relative organ weights was also noted in all groups (Figure 3).

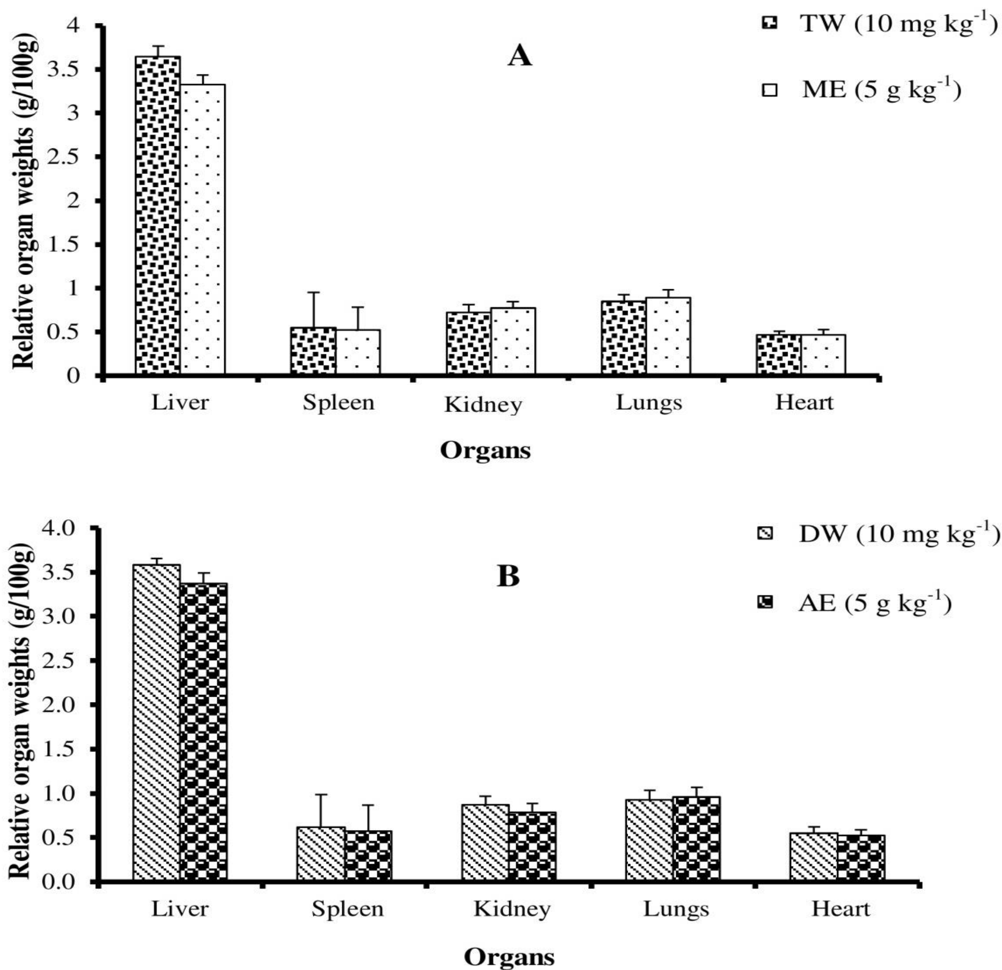

Figure 3: Effects of aqueous (A) and methanol (B) extracts of *F. asperifolia* on relative organ weights in acute toxicity.

Each bar represents the mean±SEM. Number of rats in each group = 10. DW: distilled water, Tw = Tween, AE: aqueous extract, ME: methanol extract.

3.6 Discussion

The aim of this work was to evaluate the effect of *F. asperifolia* extracts on plasma concentration of estradiol and progesterone in obese female rats. Reproduction in general and the estrus cycle in particular, is under strict control of ovarian steroids, namely estrogen and progesterone. Progesterone is produced by the ovaries, adrenal glands and placenta (during pregnancy) regulates the estrus cycle, enhances endometrial cell growth prior to the nidation and is essential during pregnancy as the dominant ovarian hormone [3,24]. Progesterone and estrogens regulate their secretion by exerting a feedback effect on gonado-stimulins release [25]. In obese rats, hyperlipidemia disrupts this mechanism and causes an overproduction of sexual hormones which in turn inhibits through a negative feedback the secretion of LH and FSH, explaining high infertility risk in obese females [26].In the present study, obesity status was induced by 10 weeks continuous feeding with a standard rat diet by 15% palm oil. This approach was efficient as attested by a significant increase in the growth rate and Lee index compared to the SD group. 44.38 % of HFD rats were declared obese while 55.62 % failed to respond.

This difference in the response of the animals issued from the same husbandry and submitted to the same stimulating factors could be related to the intraspecific responses among those animals [27]. This success in POD-induced obesity matches the view of many researchers who previously demonstrated that high fat diet is capable of inducing obesity after 6 weeks [28], 7 weeks [29], 10 weeks [14] or 16 weeks [18,19]. It is well established that obesity seriously impairs female reproductive hormones synthesis [30,32], which may lead to estrus cycle imbalance and infertility in some cases. The aqueous and methanol extracts of *F. asperifolia* improved the plasmatic progesterone level after treatment. This result is in accordance with the studies of Shapiro and his colleagues [3] who showed that the aqueous extract of *Lepidagathis longifolia* and *Phyllagathus rotundifolia* induced a significant increase in the plasmatic progesterone and estradiol levels in non-pregnant and pseudo-pregnant rats. These effects could be attributed to a variety of compounds described in plants extracts. In fact, plant sterols are known for their regulatory effect on progesterone synthesis [33,34]. Thus, the increase in progesterone concentration by the aqueous and methanol extracts of *F. asperifolia* could be due to the presence of this class of compounds as demonstrated by NPC [35]. These compounds would have operated by promoting hypothalamic and pituitary hormones release, improving ovarian steroidogenesis in obese rats. Since hormonal dysregulation may be due to that of the lipid profile, the modulation of the lipid profile by the plant extracts as shown in previous studies may also justify the hormonal regulation observed in the present study. Produced by the ovaries, estradiol stimulates uterine growth, enhances the proliferation of the uterine lining during the pre-ovulatory phase through its mitotic action. Estradiol is the main hormone responsible for the development of reproductive organs. In synergy with FSH, estradiol stimulates proliferation of granulosa cells during follicular development [25]. Plants with estrogenic properties can directly influence pituitary action by peripheral modulation of LH and FSH, increase the secretion of these hormones and stimulate ovulation [36,37]. Thus, the increase in plasmatic estradiol concentration observed in the present study could be attributed to increased aromatase activity or substrate supplementation during estradiol synthesis [34,38]. Our results are in line with those of Orsi and his colleagues [39] who demonstrated that extracts of Shakuyaku (*Paeniae radix*), Keihi (*Cinnamomi cortex*) and Botanpi (*Mutan cortex*) stimulate aromatase activity in human granulosa cells and increase estradiol secretion. De and his colleagues [16] reported that several alkaloid-containing plants activate aromatase activity *in vitro*, thereby increasing the production potential of steroids and increasing reproductive performance. Alkaloids present in *F. asperifolia* extracts could be responsible for the increase of estradiol level, probably by activating the aromatase activity. This result indicates that aqueous and methanol extracts of *F. asperifolia* could contain phytochemical compounds with beneficial endocrine effects. *F. asperifolia* effects on ovarian hormones strongly support pour previous study in which we demonstrated the alleviating effect of these extracts on high fat diet-induced female infertility [15] . These results suggest that active compounds found in this plant could act locally to promote ovarian steroidogenesis or centrally by activating LH and FSH release. But these hypotheses have not been investigated in the present study. Other mechanisms of action may also be involved in these results, including the modulation of gonadotropin hormones regulation and activity, steroidogenesis stimulation and lipid profile regulation. Additional studies to identify the exact mechanism of action and the related bioactive compounds of *F. asperifolia* are therefore needed. Whatever being the therapeutic potential of a product, it is essential to measure its biosafety before any recommendation to persons in need. This process helps to determine the potential side effects which could arise from the acute or chronic use of the product. Despite the frequent use of *F. asperifolia*, no information about its

toxicity is reported. To evaluate the potential harmful effect of this plant, a single dose of 5 g/kg was orally administered as recommended by OECD protocols and acute toxicity parameters were observed. In all groups, no toxicity signs were noted after 2 weeks. According to numerous legislations aiming at regulating the commercial use of toxins, an LD50 values above 5 g kg^{-1} correspond to merely non-toxic substances [40]. Thus the aqueous and methanol extracts of *F. asperifolia* are non-toxic. However, other studies on chronic toxicity must be carried out to access the long-term safety of the plant.

4. Conclusion

These findings show that the altered level of reproductive hormones profile in female rats caused by a high fat diet (15%) was regulated by the extracts of *F. asperifolia. F. asperifolia* extracts are non-toxic. These findings may justify the traditional use of *F. asperifolia* as female fertility enhancer.

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