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Original Article

Mechanism of Action of the Aqueous Leaves Extract of *Sarcocephalus latifolius* (Smith) on the Reproductive System of Female Rat

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Abstract

Prized for its many therapeutic virtues, *Sarcocephalus latifolius* (Smith) is a rubiaceae whose roots alone or in combination with the leaves are used in the treatment of infertility and genital diseases. In the search for mechanisms of action of natural substances, uterotrophic bioassay is essential and frequently used. Thus, ovariectomized impubere female rats received during 7 days, 250; 500 and 1000 mg/kg body weight of aqueous leaves extract of *Sarcocephalus latifolius* and 0, 02 mg/kg body weight (BW) ethinyl estradiol. Variation in animal body weight, vaginal opening, uterine horns, cervix and adrenal glands weights, as well as serum levels of proteins, cholesterol and glucose were assessed. Treatment of the animals at the different doses of extract produced no significant ($p > 0.05$) change in the parameters studied. Only the dose of 1000 mg / kg body weight extract induced a significant ($p < 0.001$) vaginal opening. Administration of ethinyl estradiol to ovariectomized female rats resulted in a significant ($p < 0.001$) reduction in body weight variation, serum protein and cholesterol levels, as well as a significant increase ($p < 0.001$) in the vaginal opening, the uterine horns and cervix weights, and serum glucose. Similarly, the combination of ethinyl estradiol with the different doses of *Sarcocephalus latifolius* extract produced the same significant effects ($p < 0.001$) as previously mentioned in the treated female rats compared to the controls. However, only the variation in body weight, vaginal opening and serum cholesterol levels in these animals decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) with regard to those treated with ethinyl estradiol. These results would assume that the aqueous extract of leaves of *Sarcocephalus latifolius* would possess estrogen-like properties or would act on the hypothalamic-pituitary-gonadal axis.

Keywords: *Sarcocephalus latifolius*, ethinyl estradiol, uterotropic bioassay, estrogen-like and hypothalamic-pituitary-gonadal axis.

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Introduction

Fertility is a crucial problem in the couple. Indeed, it has been shown that infertility may be female cause in 30% of cases, male in 20%, mixed (the couple) in 40% and idiopathic origin (inexplicable) in 10% of cases (Barillier, 2007). To remedy this, given the high costs of modern treatments, or even dissatisfaction, many have recourse to medicinal plants. In fact, according to WHO (2002), 80% of the population of developing countries in the African region use traditional medicine. This medicine has the advantage of being accessible and inexpensive for local populations.

Côte d'Ivoire, like most african countries, has a rich pharmacopoeia. However, these medicinal plants are used empirically in the treatment of ailments without a real scientific basis. The search for the real effects and the mechanisms of action of the extracts of these plants prove indispensable, in order to ensure their valorization. Moreover, the plants of the ivorian pharmacopoeia are for the most part multipurpose. This is the case of *Sarcocephalus latifolius* (Smith), a rubiaceous tropical region of sub-Saharan Africa known to traditional healers for the treatment of various diseases (malaria, pain, cough, epilepsy, fever, stomach ache, diabetes, infertility, intestinal worms…; Adjanohoun *et al.*, 1986; Kerharo and Adam, 1986). All parts of this plant (roots, barks, stems, leaves, fruits) are used for therapeutic purposes.

Scientifically, it has been the subject of numerous studies for its androgenic properties (Rukundo, 2007; Ishimwe, 2008; Majibe, 2008; Djiguibet, 2009; Ngom, 2010), anti-diabetic (Iwueke *et al.*, 2010), antiplasmodial (Benoit-vical, 1998; Badiaga, 2011). This study aims to determine the mechanisms of action of the aqueous extract of leaves of *Sarcocephalus latifolius* (Smith), on the reproductive system of young female rats.

Materials and Methods

Vegetable Material and Preparation of the Extract

The fresh leaves of *Sarcocephalus latifolius* were bought at the market of Cocody (Abidjan, Côte d'Ivoire) among herbalists and authenticated at the national center of floristics (CNF) of the Université Félix Houphouët-Boigny (Abidjan, Côte d'Ivoire).

The leaves were washed and dried in ambient air in a room, sheltered from the sun, then ground. 150 g of the leaves powder were macerated in 800 ml of distilled water, with micro-vortex stirring at 350 rpm for 24 h. The macerate is then filtered on poplin cloth, then on Wattman paper No. 1, before being dried in an oven at 55 ° C. The extract obtained is stored in the refrigerator, for the subsequent preparation of the various doses of extracts, using distilled water.

Animal Material and Treatment

The animals used for the work are young impubere female rats of Wistar race, from the vivarium of the 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. They were fed to cereal-based granules manufactured by the FACI (Abidjan, Ivory Coast) and to running water, to which they had free access. The experiment was carried out on ovariectomized female rats. Bilateral ovariectomy was performed at 6 weeks of age, according to the OECD Guideline 440, on uterotrophic bioassays on rodents for the short-term screening of estrogenic properties. On the fifteenth day of ovariectomy, animals weighing on average between 100 and 140 g were divided into eight (8) groups of five (5). Treatment was as follows:

Lot 1: control (distilled water).

Lot 2: 0.02 mg / kg bw of ethinyl estradiol.

Lot 3: 250 mg / kg bw of *Sarcocephalus latifolius* extract.

Lot 4: 500 mg / kg bw of *Sarcocephalus latifolius* extract.

Lot 5: 1000 mg / kg bw of *Sarcocephalus latifolius* extract.

Lot 6: 250 mg / kg bw of *Sarcocephalus latifolius* + 0.02 mg / kg bw of ethinyl estradiol.

Lot 7: 500 mg / kg bw of *Sarcocephalus latifolius* + 0.02 mg / kg bw of ethinyl estradiol.

Lot 8: 1000 mg / kg bw of *Sarcocephalus latifolius* + 0.02 mg / kg bw of ethinyl estradiol.

The preparation of the different doses of extract of *Sarcocephalus latifolius* was done with distilled water. Ethinyl estradiol (EFFIK- France) was diluted in olive oil. All these solutions were administered daily, by oesophageal gavage, for 7 days. About 24 hours after the last gavage, the animals are sacrificed by decapitation and then the blood collected in EDTA tubes for possible dosages. The cervix, the uterine horns, are removed and weighted.

Assessment of Body Weight

A daily body weight survey is performed from the first day of treatment to the 7th day. The weight measurements are made on a Satorius scale.

Measuring Vaginal Opening

The state of opening of the vaginal meatus is recorded every morning during the 7 days of treatment. The percentage of females with a vaginal opening is estimated according to the treatment and dose of products and / or extract administered.

Evaluation of the Fresh and Dry Weight of the Organs

Immediately after the euthanasia, cervix, uterine horn and adrenal gland are removed, devoid of any adipose tissue and freshly weighted. They are then placed in an oven at 100 °C for 24 h to determine their dry weight. A precision scale (0.001) of Satorius type was used to evaluate the fresh and dry weight of these organs.

Determination of Serum Protein, Glucose and Cholesterol

The blood samples were centrifuged with a ROTOFIX 32 A (Hettich) centrifuge at 3000 rpm for 4 min. The sera collected are stored at -20° C for the determination of total proteins, glucose and cholesterol by the colorimetric method or endpoint.

Statistical Analysis

The software used are: EXCEL and Graph Pad. All data are expressed on average ± SEM. Analysis of variances (ANOVA) was applied to the different results. The Newman-Keul test was used to compare the different columns. The value $p < 0.05$ is considered significant.

Results

Effects of the Treatment on Body Weight Variation

Administration of the various doses of aqueous extract of *Sarcocephalus latifolius* (250, 500 and 1000 mg / kg bw) and the dose of 20 µg / kg bw of ethinyl estradiol during 7 days of treatment had no significant change ($p > 0.05$) on the body weight of ovariectomized female rats, compared to controls.

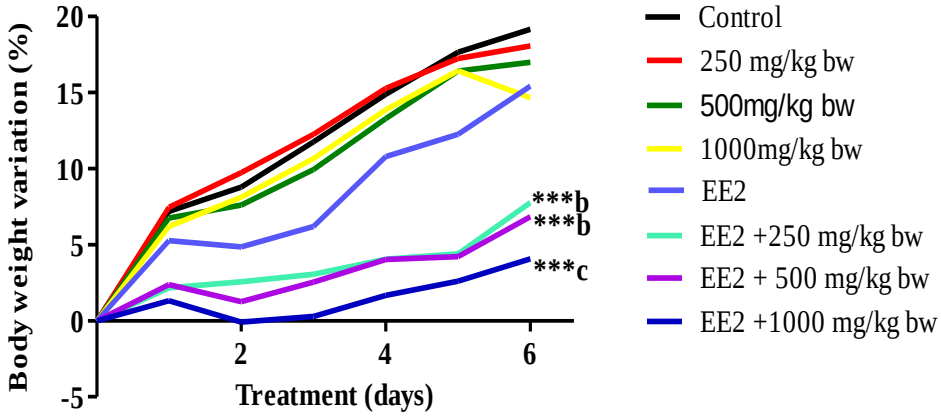

Fig. 1: Body weight variation curve.
EE_2: Ethinyl estradiol; bw: Body weight.
***: $p < 0,001$ significant difference in relation to control (distilled water).
b: $p < 0,01$, **c**: $p < 0,001$ significant difference in relation to ethinyl estradiol.

However, the animals having received the extract-ethinyl estradiol combination showed a very significant decrease ($p < 0.01$ and $p < 0.001$) in the body weight variation compared with the distilled water control and the ethinyl estradiol control; a rate of change of 7.77%; 6.83% and 4.06% for those treated with the respective doses of 250, 500 and 1000 mg / kg bw of aqueous extract of *Sarcocephalus latifolius*, compared to 19.16% for distilled water controls and 15.6% for ethinyl-estradiol controls (Figure 1).

Effects of Treatments on Vaginal Opening

After the 7 days of treatment, 100% of the ovariectomized female rats, treated with ethinyl estradiol, had a vaginal opening, that is to say a highly significant rate ($p < 0.001$) compared to the unchanged controls (0%). Administration of *Sarcocephalus latifolius* doses at, 250, 500 and 1000 mg / kg bw stimulated weakly the vaginal opening of these animals, relatively to the positive controls of 20%, 20% and 40%, respectively. However, the dose of 1000 mg / kg bw had a very significant effect ($p < 0.001$) compared to the negative controls. Similarly, the combination of doses 250, 500 and 1000 mg / kg bw of extract to ethinyl estradiol increased significantly ($p < 0.001$) vaginal opening rates (74%, 63% and 74%), relatively to the distilled water controls. However, they remain significantly low ($p < 0.05$, $p < 0.001$, $p < 0.01$) compared to positive controls (Table 1).

Table 1: Effects of extract of *Sarcocephalus latifolius* and ethinyl estradiol on the vaginal opening of the variectomized female rats.

Treatment	Animals Number	Vaginal Opening Rate (%)
Control	5	0
E_2 (20.10^{-3} mg/kg bw)	5	100^{***}
250 mg/kg of *S. latifolius*	5	20
500 mg/kg of *S. latifolius*	5	20
1000 mg/kg of *S. latifolius*	5	$40^{***\mathbf{c}}$
250 mg/kg of *S. latifolius* + EE_2 (20.10^{-3} mg/kg bw)	5	$74^{***\mathbf{a}}$
500 mg/kg de *S. latifolius* + EE_2 (20.10^{-3} mg/kg bw)	5	$63^{***\mathbf{c}}$
1000 mg/kg of *S. latifolius* + EE_2 (20.10^{-3} mg/kg bw)	5	74^{***b}

EE_2: Ethinyl estradiol; *S. latifolius Sarcocephalus latifolius*; bw: Body weight
***: $p < 0{,}001$ significant difference with regard to control (distilled water).
a: $p < 0{,}05$, **b**: $p < 0{,}01$, **c**: $p < 0{,}001$ significant difference from ethinyl estradiol.

Effects of Treatments on Organ Weights

The results show no change in weight (fresh or dry) of the reproductive organs in ovariectomized females rats, treated only with the aqueous extract of *Sarcocephalus latifolius*, at doses of 250; 500 and 1000 mg / kg bw relatively to the negative control. On the other hand, these organs show a very significant reduction in weight ($p < 0.05$, $p < 0.001$, $p < 0.01$) compared to positive controls. However, ethinyl estradiol administered alone, as in combination with doses of 250; 500 and 1000 mg / kg bw of *Sarcocephalus latifolius* induced a very significant increase ($p < 0.001$) of the fresh weight of the uterine horns compared to the control of distilled water (Figure 2).

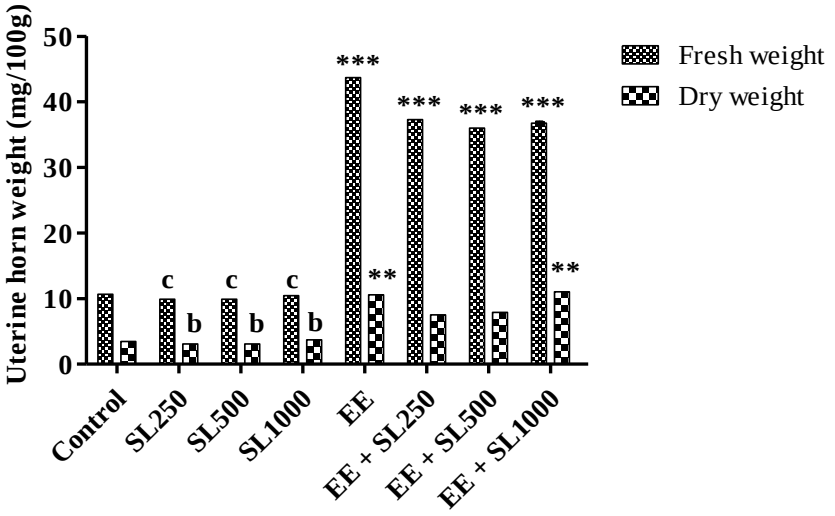

Fig. 2: Effects of different treatments on uterine horns fresh and dry weight of ovariectomized female rats.
**: p< 0,01, ***: p< 0,001 significant difference in relation to control (distilled water).
b: p< 0,01, **c**: p< 0,001 significant difference in relation to ethinyl estradiol.

That is, an increase of 310%, 250%, 238% and 245%, respectively. Treatments with ethinyl estradiol and ethinyl estradiol combined with 1000 mg / kg bw of *Sarcocephalus latifolius* extract produced a significant increase (p <0.01) of the dry weight of the uterine horns of the ovariectomized female rats compared to the negative control, that is a respective increase of 207% and 221%. Similarly, the results of Figure 3 suggest a very significant increase (p <0.001 and p <0.01) in the fresh weight of the cervix of animals treated with ethinyl estradiol alone (147%) or in combination with doses 250; 500 and 1000 mg / kg bw of *Sarcocephalus latifolius* (140%, 114% and 115% respectively) compared to the distilled water control.

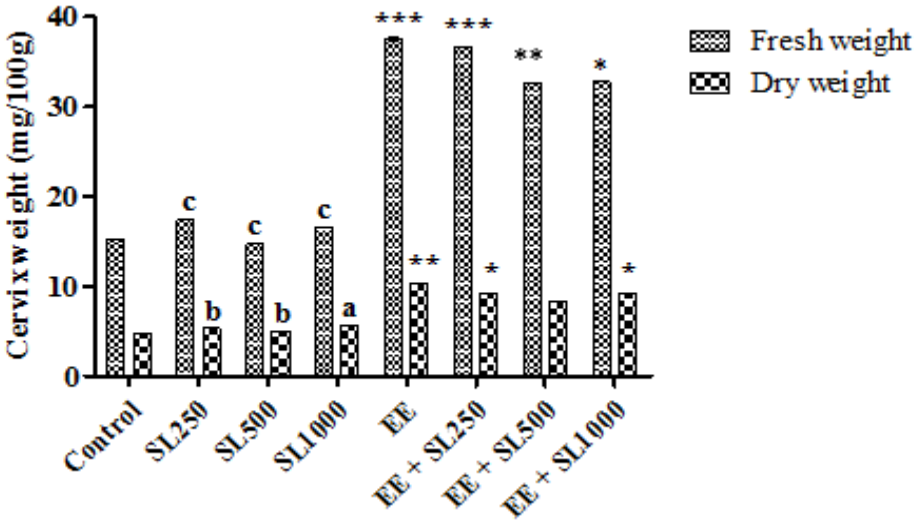

Fig. 3: Effects of different treatments on cervix fresh and dry weight of ovariectomized female rats.
*: p< 0, 05, **: p< 0, 01, ***: p< 0,001 significant difference in relation to control (distilled water).
a: p< 0,05, **b**: p< 0,01, **c**: p< 0,001 significant difference in relation to ethinyl estradiol.

As for the dry weight of the cervix, only rats having received ethinyl estradiol alone or in combination with 250 or 1000 mg / kg bw of aqueous extract produced significant results (p <0.001).That is to say a respective increase of 116%, 94% and 93%. On the other hand, the fresh and dry weight of the adrenal glands did not change in comparison with the controls (Figure 4).

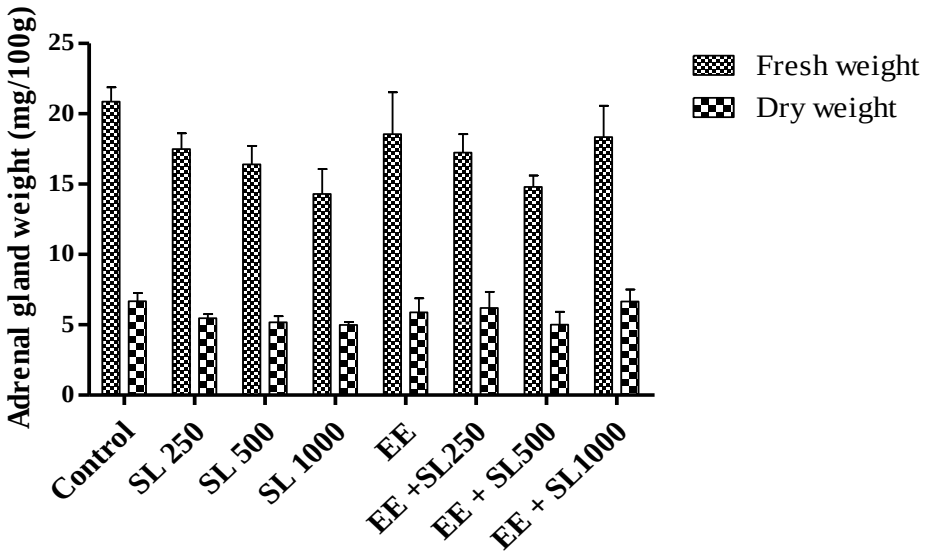

Fig. 4: Effects of different treatments on adrenal glands fresh and dry weight of the ovariectomized female rats.

Effects of Treatments on the Level of Proteins, Glucose and Cholesterol Serum

The level of serum proteins was not affected by the different doses of *Sarcocephalus latifolius* extract. However, treatment with ethinyl estradiol and ethinyl estradiol in combination with doses of 250; 500 and 1000 mg / kg bw of *Sarcocephalus latifolius* significantly decreased (P <0.001, p <0.01 and p <0.05) serum proteins level by 13.3%, 11.7%, 9.1% and 9.4%, respectively, as compared to the negative control (Figure 5).

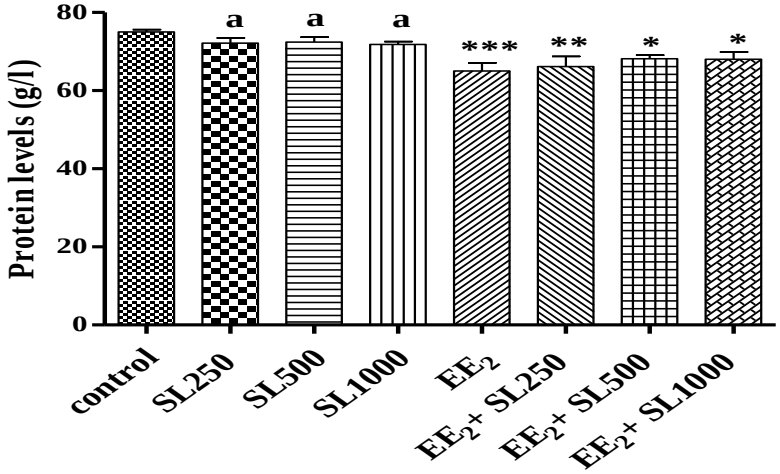

Fig. 5: Effects of different treatments on the level of serum proteins of ovariectomized female rats.
*: p< 0,05, **: p< 0,01, ***: p< 0,001 significant difference with regard to control (distilled water).
a: p< 0,05 significant difference with regard to ethinyl estradiol.

Similarly, serum cholesterol was not influenced at the end of 7 days of treatment at the different doses of *Sarcocephalus latifolius* extract. As observed previously, administration of ethinyl estradiol alone or in combination with the different doses of extract produced a very significant (p <0.001) decrease in serum cholesterol, 30.4%, 41.9%, 54.6% and 50.9% respectively (Figure 6), compared with the control distilled water. Compared with estradiol, doses of 500 and 1000 mg / kg bw have led to a significant decrease (p <0.01 and p <0.05 respectively) in serum cholesterol.

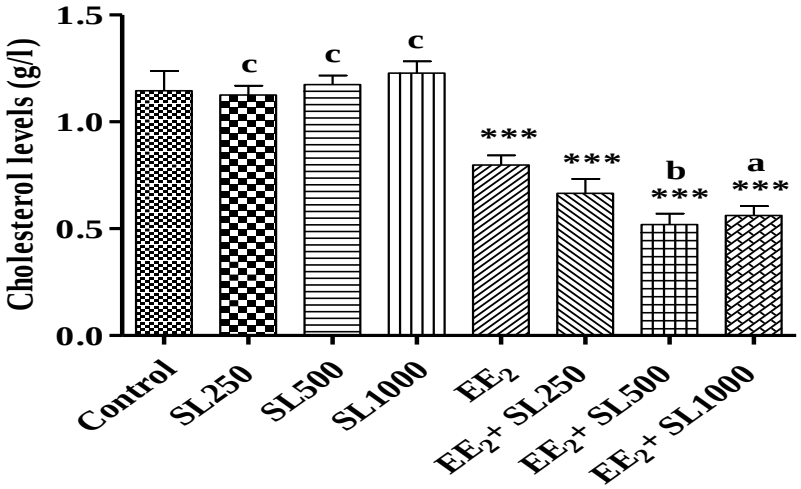

Fig. 6: Effects of different treatments on serum cholesterol in ovariectomized female rats.
***: p< 0,001 significant difference with regard to control (distilled water).
a: p< 0,05, **b**: p< 0,01, **c**: p< 0,001 significant difference with regard to ethinyl estradiol.

On the other hand, the level of serum glucose was not modified by the different doses of extract. However, the results show a highly significant (p <0.001) increase in serum glucose levels of 128%, 124%, 114% and 147%, respectively, following the treatments with ethinyl estradiol alone, as well as its combination at the various doses of extract, relative to the negative control (Figure 7).

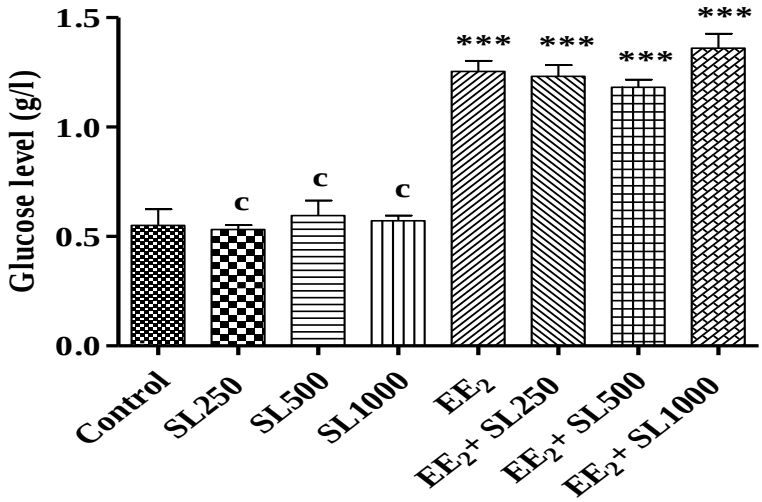

Fig. 7: Effects of different treatments on serum glucose level of ovariectomized female rats.
***: p< 0,001 significant difference with regard to control (distilled water).
c: p< 0,001 significant difference with regard to ethinyl estradiol.

Discussion

Sarcocephalus latifolius is a rubiaceae widely used in the african pharmacopoeia, notably in the treatment of many ailments, including those of the male or female reproductive system, as well as infertility. In order to evaluate the biological activity of the leaves of this rubiaceae and especially to determine the mechanism of action of the extract, uterotrophic tests are necessary. Uterotrophic bioassay is an *in vivo* and short-term screening test, allowing to assess the estrogenic activity of a chemical substance (OECD, 2007). The estrogenic activity is manifested by a morphological, biochemical and histological change of the uterus (DIEL, 2002).

The aqueous extract of *Sarcocephalus latifolius* leaves, as well as ethinyl estradiol, had no significant effect ($p < 0.05$) on the body weight evolution of animals. This result is similar to that of Bayala *et al.*, (2006), on macerated leaves of *Holarrhena floribunda* in ovariectomized female rats. This effect is due to the very low toxicity of the aqueous extract of *Sarcocephalus latifolius* leaves. Indeed, previous work on acute toxicity, orally, conducted according to OECD guideline 423 (2001), have allowed the classification of this extract in category 5, since the 5000 mg / kg body weight dose did not induce any mortality or apparent signs of toxicity. This could mean that the LD50 (lethal dose 50%) would be greater than 5000 mg / kg body weight. However, no significant increase in body weight was observed in animals treated with *Sarcocephalus latifolius* extract. This would mean that this extract would not contain any substance capable of increasing the weight on one side and the other, the effects of the extract would be well targeted and not general.

However, when the extract is in the presence of estradiol, the body weight of the animals increases ($p < 0.001$) very weakly. The association of extract of *Sarcocephalus latifolius* with estradiol would affect the metabolism of animals or food intake, which would influence their weight gain, hence the small variation in body weight. Ovariectomized female rats treated with the dose of 1000 mg / kg of body weight extract, as well as those having received estradiol or estradiol-extract, showed significant ($p < 0.001$) important vaginal opening, after the 7 days of treatment, while the vagina of the control female rats remained closed. This result is similar to that of Bleu (2013) about immature ovariectomized female rats treated with *Passiflora foetida* extract. This would then suggest an estrogenic effect of the aqueous extract of *Sarcocephalus latifolius*. At this dose, the extract would be have as an analogue of estrogen.

However, this vaginal opening produced by the extract is significantly ($p < 0.001$) lower than that induced by estradiol. The extract would therefore use other routes of action than those of estradiol. The combination of estradiol at different doses of extract induced a significant ($p < 0.05$; $p < 0.01$ and $p < 0.001$) decrease in vaginal opening. The extract would interfere with the effects of estradiol. These results are contrary to those of Koneri *et al.*, (2007), about the ethanolic extract of *Momordica cymbalaria* roots. In the works of these authors, the root extract did not induce vaginal opening, whereas the combination of estradiol with the extract of *Momordica cymbalaria* caused a vaginal opening similar to that induced by estradiol.

However, the relative weights of the uterine horns and cervix (fresh and dry), of the animals treated at the different doses of extract did not undergo any significant change. This result is contrary to that of many other authors (Kouakou, 2000; Bayala *et al.*, 2006 ; Sharangouda *et al.*, 2008 and Bleu 2013), but similar to those of Santos *et al.*, (2015) on the ethanolic extract of *Cenostigma macrophyllum* Tul. And those of Njamen *et al.*, (2008) on the methanolic extract of root barks of *Sarcocephalus latifolius* in the ovariectomized female rat. About Njamen *et al.*, (2008), the root bark extract of *Sarcocephalus latifolius* had no effect on uterine weight or thickness of the uterine epithelium, but increased significantly ($p < 0,05$) of 15% in the thickness of the vaginal epithelium compared to the control. Indeed, the administration of so-called estrogenic or estrogen-like substances to immature or ovariectomized rats induces the increase in the weight of the uterine horns, the cornification of the epithelial cells and the vaginal opening (Couse et Korach, 1999; Pinilla *et al.*, 2001; Shivalingappa *et al.*, 2002 and Bayala *et al.*, 2006). However, the comparison of the batches

treated with estradiol and estradiol combined with the extract does not reveal any significant change either. Knowing that vaginal opening is a qualitative measure of the estrogenic potential of a substance (Koneri *et al.*, 2007), it would be appropriate to suggest that the aqueous extract of leaves of *Sarcocephalus latifolius* would possess estrogen-like activity or act on the hypothalamic-pituitary-gonadal axis (HPG), to cause vaginal opening. However, an utero-trophic test on immature female rats would confirm this result.

Indeed, the method on immature females, whose HPG axis is intact, covers a wider field of investigation than that practiced on ovariectomized animals, because it is sensitive to substances that interact with the HPG axis and not only with estrogen receptors (OECD, 2007). The adrenal glands also did not change weight (fresh and dry) after the 7 days of treatment. This result corroborates those of Bayala *et al.*, (2006) in ovariectomized female rats and Kouakou (2000) in normal female rats. However, it is contrary to those of some authors on ovariectomized female rats (Kouakou, 2000; Mariotti *et al.*, 2011).

The level of serum proteins of animals treated with the *Sarcocephalus latifolius* extract also did not vary with respect to the vehicle. However, this rate remains significant and higher than in estradiol treated female rats. This result is contrary to that of Ahmad *et al.*, (2012), whose hydro-methanolic extract of *Linum usitatissimum* L. induced in immature female rats a low concentration of total serum proteins compared to that induced by estradiol.

Serum cholesterol levels in animals treated with *Sarcocephalus latifolius* extract also showed no significant change with regard to the control. However, this rate is very high and highly significant ($p < 0.001$), compared to that induced by estradiol. Indeed, according to Vaissaire (1977), estradiol decreases the cholesterolemia. It is well known that cholesterol is the lipid precursor of the biosynthesis of steroid hormones, especially testosterone and estradiol (Johnson & Everitt, 1988). Its abundance in blood would be synonymous with its unused and the reduction of an assimilation on the level with the gonads. For Ahmad *et al.*, (2012), short-term treatment of rats with estrogen-containing plants could increase total serum cholesterol, while long-term treatment may have the opposite effect.

Blood glucose showed no change in the treatments. This extract of leaves would be neither hyperglycemic nor hypoglycemic and would contribute to the homeostasis which allows the maintenance of the glycemic balance. Contrary to our results, the work of Ouédraogo (1996) on the aqueous extract of root barks of *Terminalia macroptera* showed a decrease in blood glucose in the rabbit, thus highlighting the hypoglycemic properties of this plant.

Conclusion

In view of all these results, it appears that the aqueous extract of leaves of *Sarcocephalus latifolius* Sm. possess estrogen-like activity or act on the hypothalamic-pituitary-gonadal axis.

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