The Journal of Phytopharmacology 2022; 11(1):1-6
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2022; 11(1): 1-6
January- February
Received: 18-10-2021
Accepted: 06-12-2021

doi: 10.31254/phyto.2022.11101

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Estrogenic effects of the seeds and stem bark extracts of *Ricinodendron heudelotii* in adult ovariectomized Wistar albino rats

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ABSTRACT

Background: Infertility is a major public health problem in Africa. Most herbal remedies used for the treatment of women infertility are known to be estrogenic. **Objective:** The aim of this study was to investigate the estrogenic effects of the aqueous extracts of *Ricinodendron heudelotii* seeds and stem bark in ovariectomized rats. **Methods:** Adult ovariectomized rats were divided in groups of five animals and treated orally for seven consecutive days with both the seeds extract (SERH) and the bark extract (BERH) at 100 and 300 mg/kg and with Ethinylestradiol (0.02 mg/kg). The control group received distilled water. After treatments, vaginal smears were performed from the rats. Then the uterus, cervix and adrenal glands were weighted and the uterus stored in formalin 10% for histological analysis. **Results:** Ethinylestradiol as well as SERH at 100 mg/kg induced vaginal cornification and highly significant increase of the relative weight of the uterus and cervix in comparison to controls. SERH at 300 mg/kg and BERH at 100 and 300 mg/kg also induced significantly an increase of the relative weight of these organs. In addition, the adrenal glands relative weight was significantly augmented by SERH and BERH treatment. The histological examination of uterus showed the proliferation of the endometrium cells and development of numerous uterine glands in rats treated with Ethinylestradiol and the extracts at 100 mg/kg. **Conclusion:** *R. heudelotii* was found to show estrogenic effects on vagina, uterus, cervix and adrenal glands and the seeds extract exhibited greater estrogenic activities than the bark extract.

Keywords: *Ricinodendron heudelotii*, Estrogenic, Uterus, Endometrium, Vaginal cornification.

INTRODUCTION

Infertility is the inability of a couple to procreate or carry a pregnancy to term within a year of regular unprotected sex [1]. It is increasingly becoming a public health problem [2]. It is estimate that 48.5 million couples are affected by this problem worldwide [3]. This disease affects about 20 to 30% of couple in sub-Saharan Africa [4, 5]. It has negative repercussion on socio-economic development and upsets family stability. Thus, having a child in some African countries becomes a pledge for the survival of a couple [6]. It is therefore in the interest of investing in scientific research in order to provide solutions to this problem. Although infertility affects both men and women, it is very often the latter who are held responsible for the infertility of the couple when they are in a relationship with a man, regardless of whether they are infertile or not [7]. Therefore, women often bear the societal burden of infertility, particularly in societies where a woman's identity and social worth is closely tied to her ability to have children [8]. In the female reproductive system, infertility can be caused by a variety of abnormalities in the ovary, uterus, fallopian tube and endocrine system [7].

Despite the development of modern medicine in the field of reproduction, the means of care in some developing countries may appear ineffective due to the low socio-economic level of the populations, the failure technical platform in hospitals and the high cost of this care. According to a recent World Bank report, 41% of the population in the sub-Saharan Africa live below the poverty line [9]. Thus, many couples from these countries are turning to traditional medicine to find a care for this disease. This medicine is mainly based on the use of many herbal recipes. Indeed, traditional medicine has remained for centuries the most accessible and cheapest health system and medicinal plants contribute significantly to the life of rural populations and to social balance in Africa [10]. A strategic reorientation of research and development focused on medicinal plants for the design of remedies accessible to all and effective is therefore necessary and encouraged by the Ivorian government [11].

Ricinodendron heudelotii (Euphorbiaceous) known locally as ''Akpi'' is a medicinal plant that is part of the Ivorian flora, of which many traditional uses have already been reported. The seeds rich in nutrients

are currently consumed by local populations [12]. The leaves and stem bark are used to treat gynaeco-obstetrical troubles and to facilitate childbirth [13, 14, 15, 16]. Furthermore, the leaves, seeds and stem bark of this plant are used by the Akyé population of Adzope, a region in the southern of Ivory Coast and then by the population of Benin to cure women infertility [17]. Since most of medicinal plants used to treat women infertility was found to have estrogenic properties, *R. heudelotii* could act as a potential estrogenic plant.

The aim of this study was to investigate the estrogenic effects of the aqueous extract of *R. heudelotii* seeds and stem bark in adult ovariectomized Wistar albino rats.

MATERIALS AND METHODS

Plant material

The seeds and stem bark of *R. heudelotii* were collected from the region of Adzope in the southern of Ivory Coast, precisely in the village called Ande. The plant was authenticated in the National Center of Floristic where a voucher specimen was deposited under the number UCJ006326.

Animals

Adult female albino Swiss mice and Wistar rats aging from two to three months obtained from the vivarium of the Superior Normal School of Abidjan were used for the experiments. The animals were raised under room temperature (28±2°C) with a photoperiod of 12 hours light dark. The hygrometry was 50-60% and they were free allowed to water and commercial food (15% protein, 5.3% fat) provided from IVOGRAIN industry (Abidjan, Ivory Coast). All the animals were acclimatized for 14-days under the above laboratory conditions before experiments beginning. Experiments were conducted according to the EU Directive 2010 /63/ EU for animal experiments.

Preparation of the aqueous extract

The stem bark and seeds of *R. heudelotii* were dried under ambient temperature without exposure to sun light and pulverized separately using an electric grinder (RETSCH SK100/C, Germany). Fifty grams (50g) of each plant material were macerated in 1 L of distilled water under a magnetic agitator (JANKE & KUNKEL RH, Germany) for 24 h and double filtered using hydrophilic cotton and Whatman filter paper number 1. Each solution obtained was evaporated in an air circulating oven (MEMMERT UF55, Germany) at 50°C until total dryness. The aqueous extract of stem bark and seeds was separately recovered and stored at 4°C in a refrigerator for the different tests.

Phytochemical screening

The qualitative evaluation of the chemical composition of the stem bark and the seeds of *R. heudelotii* was carried out using standard methods [18]. Various compounds such as alkaloids, polyphenols, tannins, quinones, flavonoids, sterols, polyterpenes, and saponosides were tested.

Acute toxicity study

This study was carried out according to the guideline 423 of the organization for the economic cooperation and development [19]. Three months aged female healthy mice weighting between 25 and 30g and divided in 3 groups of 3 animals each were used for this test. They were fasted for 3 h prior to dosing but free allowed to water and were individually marked for recognition. The principle of limit test was used in this experiment. The first group served as control and was given distilled water. The second group was administered with the unique dose of 2000 mg/kg b.w. of the seed extract of *R. heudelotii* (SERH) while the third group was treated with the same dose of the stem bark extract (BERH) of this plant. All the treatments were given by the oral route using an intragastric sound. After the treatments, animals were observed individually at least once during the first 30 minutes, periodically for 24 hours and daily for a total of 14 days to record the mortality or signs of toxicity [19].

Determination of the estrogenic effects

For this study, the uterotrophic bioassay in rodent was used according to OECD guidelines 440 [20]. Eight weeks old female Wistar albino rats were used for the experiment. The rats were bilaterally ovariectomized using the method of Khajuria et *al*. (2012) [21]. After a 14-day rest period, the rats were divided in six groups of five animal each and treated as following:

Group 1: control, distilled water;
Group 2: 0.02 mg/kg b.w. of Ethinylestradiol;
Group 3: 100 mg/kg b.w. of SERH;
Group 4: 300 mg/kg b.w. of SERH;
Group 5: 100 mg/kg b.w. of BERH;
Group 6: 300 mg/kg b.w. of BERH.

All the treatments were administered daily for 7 consecutive days by oral route using an intragastric sound. The rats were weighted every two days and at the day after the last treatment, vaginal smears were performed on the animals and they were sacrificed by cervical dislocation under ether anaesthesia. An incision was made in the abdominal cavity to remove organs such as uterus, cervix and adrenal glands. Immediately after collection, these organs were weighted and the uterus was stored in formalin 10% for histological analysis.

Histological examination

After 48 hours of storage in 10% formalin, the uterus was dehydrated in alcohol (80°, 90°and 100° successively) and embedded in paraffin at 58°C. Sections of 4-5 µm in thickness were cut by a microtome and stained with haematoxylin-eosin. The sections were then observed under a microscope (Olympus CK41SF, Philippine) for the analysis of the structure of endometrium and uterine glands.

Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism version 8.4.3 (686) software (Microsoft, USA). Values were presented as mean ± standard error on the mean. The data were evaluated by the one-way ANOVA analysis method followed by the Tukey multiple comparison test at the 5% level to assess the significance of the observed differences. If $p < 0.05$ the difference between the values is considered significant and if $p < 0.01$ this difference is highly significant.

RESULTS

Phytochemical screening

The phytochemical analysis revealed that the bark extract of *R. heudelotii* contains sterols, polyterpenes, polyphenols, flavonoids, tannins, alkaloids, quinones and saponosides whereas the seeds extract contains sterols, polyterpenes and alkaloids. Hence, BERH was found to be rich in phytoconstituents than SERH in this analysis (Table 1).

Oral acute toxicity

All the mice administered with the unique dose of 2000 mg/kg b.w. of BERH or SERH did not show any sign of mortality nor behavioural abnormalities during the 14 days of observation in comparison to the control mice. In addition, no significant change in the body weight gain of mice was recorded at the end of this study (Table 2).

Table 1: Chemical constituents of aqueous extract of stem bark and seeds of *R. heudelotii*

Chemical Composition	Extracts	
	BERH	SERH
Sterols	+	+
Polyterpenes	+	+
Polyphenols	+	-
Flavonoids	+	-
Tannins	+	-
Quinones	+	-
Alkaloids	+	+
Saponosides	+	-

BERH: bark extract; SERH: seeds extract; (+): Presence; (-): Absence

Table 2: Body weight gain of mice after 14 days of observation

Body weight	Control group	BERH group	SERH group
Initial weight (g)	27.75±1.59	28.52±1.06	28.27±1.04
Final weight (g)	30.19±1.63	30.74±1.17	30.52±0.99
Weight gain (%)	8.79±0.92	7.78±0.61	7.95±0.55

Results are presented as means ± SEM (n=3)

Table 3: Effects of treatments on vaginal epithelium cells

Treatments	Doses	Eosinophil cells
Control	-	-
Ethinylestradiol	(0.02 mg/kg)	++
BERH	(100 mg/kg)	+
	(300 mg/kg)	+
SERH	(100 mg/kg)	++
	(300 mg/kg)	+

(++): high presence; (+): low presence; (-): absence.

Table 4: Effects of treatments on the body weight gain of the rats

Treatments	Doses (mg/kg)	Body weight gain (%)			
		Day 2	Day 4	Day 6	Day 8
control	-	4.56±1.26	13.26±2.11	18.16±2.06	23.89±1.89
BERH	100	3.06±0.62	10.62±2.02	12.39±1.88*	14.55±2.66*
	300	6.76±1.09	11.73±1.67	15.18±2.78	20.81±2.91
SERH	100	4.52±0.42	9.42±2.79	14.24±1.11*	16.31±1.59*
	300	6.11±2.03	11.05±1.16	17.98±3.31	19.44±3.98
Ethinylestradiol	0.02	2.61±0.07	2.23±0.47**	1.22±0.13***	0.4±0.02***

Results are presented as means ± SEM (n=5); *p<0.05 vs control; **p<0.01 vs control; ***p<0.001 vs control.

Table 5: Effects of treatments on the relative weight of organs

Treatments	Doses (mg/kg)	Relative weight of organs (g/100g b.w.)		
		Uterus	Cervix	Adrenal gland
Control	-	10,49 ± 0,51	6,99 ± 0,34	19,44±1,21
BERH	100	45,36 ± 1,69*	31,68±1,08*	20,89±0,46
	300	35,25 ± 2,16*	23,5 ± 1,44*	26,12±0,78*
SERH	100	112,80±8,05***	75,19±5,36***	27,12±2,08*
	300	41,83 ± 1,52*	27,89 ± 1,01*	20,56±1,49
Ethinylestradiol	0.02	146,20±14,89****	97,44±9,93****	22,94±0,50

Results are presented as means ± SEM (n=5); *p<0.05 vs control; ***p<0.001 vs control.

Estrogenic activity

Effect on the vaginal epithelium cells

High presence of eosinophil cells was observed in female rats treated with the 100 mg/kg b.w. of SERH as well as those treated with Ethinylestradiol. However, the treatment with 300 mg/kg b.w. of SERH and with BERH at 100 and 300 mg/kg b.w. induced a low presence of eosinophil cells in the vaginal epithelium (Table 3).

Effects on the body weight of rats

During treatments, the body weight gain of control rats and both the bark and seeds extracts treated rats gradually increased over time. However, the body weight gain of rats administered with the dose of 100 mg/kg b.w. of these extracts was significantly lower ($p<0.05$) than that of the controls from day 6 to day 8. Additionally, the body weight of the rats treated with Ethinylestradiol varied very little and the weight gain was high significantly less ($p<0.01$) than that of the controls from day 4 to day 8 (Table 4).

Effects on the relative weight of organs

Treatment of rats with Ethinylestradiol resulted in a highly significant ($p<0.001$) increase in relative weights of uterus and cervix compared to controls. The bark extract also induced a significant increase ($p<0.05$) in the weight of these organs at doses of 100 and 300 mg/kg b.w. while only the dose of 300 mg/kg increased significantly the relative weight of the adrenal glands.

Concerning animals treated with the seeds extract, a highly significant increase ($p<0.001$) in the relative weight of the uterus and cervix was observed for the dose of 100 mg/kg b.w. as well as a significant increase ($p<0.05$) for the dose of 300 mg/kg. The relative weight of the adrenal glands was significantly elevated ($p<0.05$) only at a dose of 100 mg/kg b.w. (Table 5).

Histological structure of uterus

The histological examination showed that the uterus of control rats appeared relatively atrophied with a low thickness of endometrium and small uterine glands. That of rats treated with Ethinylestradiol was characterized by a very thick hypertrophied endometrium with numerous tortuous glands. Furthermore, both the stem bark and seeds extract of *R. heudelotii* at doses of 100 and 300 mg/kg b.w. induced endometrium proliferation and uterine glands multiplication similar to the effect of Ethinylestradiol. These effects were dose dependant since the effects at 100 mg/kg was greater than those obtained at 300 mg/kg. Then the endometrium hypertrophy and hyperplasia observed on the uterus of rats treated with the seeds extract was more important than that of rats treated with the stem bark extract (Figure 1).

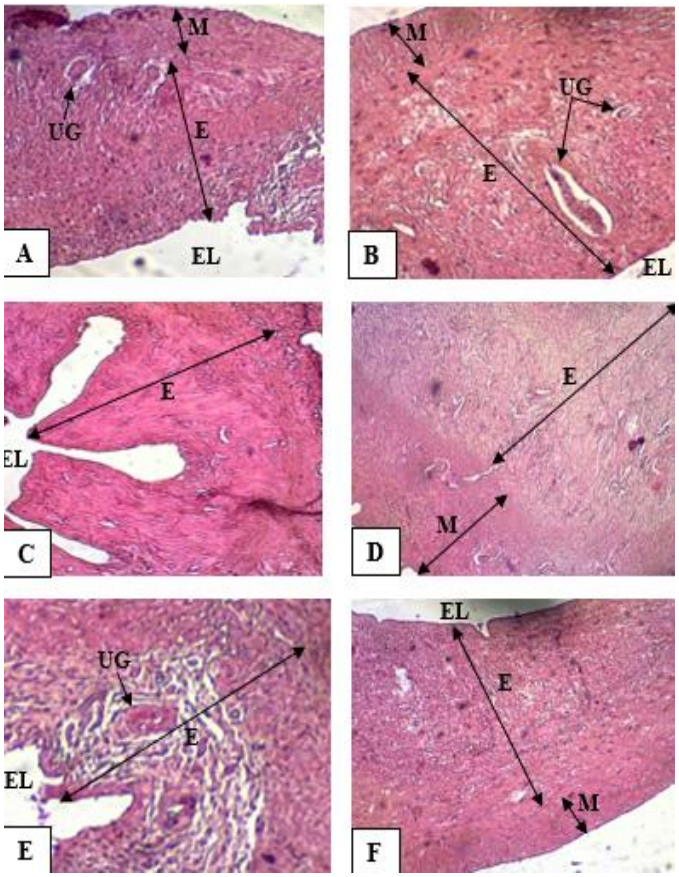

Figure 1: Photomicrograph of the histological structure of uterus of rats Uterus of control rats (A) was atrophied unlike to that of rats treated with EE (B), SERH at 100 mg/kg (C) and 300 mg/kg (D), BERH at 100 mg/kg (E) and 300 mg/kg b.w. (F) which exhibited hypertrophied endometrium and developed glands. E: endometrium, EL: endometrium lumen, M: myometrium, UG: uterine gland. Original magnification x10, stained: haematoxylin-eosin.

DISCUSSION

The phytochemical screening showed that the aqueous extract of *R. heudelotii* stem bark contains sterols, polyterpenes, polyphenols, flavonoids, tannins, quinones, alkaloids and saponosides. This result was similar to that obtained by Oyono et al. (2014) [22]. and by Uzoekwe and Hamilton-Amachree (2016) [23]. respectively with the methanol and the ethanolic bark extract of the same plant. Moreover, the aqueous extract of seeds contained only sterols, polyterpenes and alkaloids. This chemical composition was different from that of seeds analysed by Odinga et al. (2016) [24]. which highlighted in addition to previous constituents the presence of terpenoids, tannins, flavonoids and saponins. The diversity of secondary metabolites known for various potentials [25, 26]. in the anatomical structures could explain the many pharmacological properties and medicinal uses of this plant.

The toxicological study demonstrated that when administered orally with the unique dose of 2000 mg/kg b.w. of the aqueous extract of both the stem bark and seeds to mice, any sign of mortality or abnormality was recorded. The LD_{50} of these extracts were therefore found to be greater than 2000 mg/kg.

Basing on this result, the doses of 100 and 300 mg/kg b.w. were used for the study of the pharmacological effects of these extracts on the female genital tract of Wistar ovariectomized rat. In this study, vaginal smears performed from the rats at the end of treatments showed high presence of eosinophil cells in the vaginal epithelium of rats treated with Ethinylestradiol and SERH at 100 mg/kg b.w. This result indicated that SERH induced important vaginal cornification as well as Ethinylestradiol. The same effect was obtained with the ethanol extract of *Lannea acida* (Anacardiaceous) [27]. The vaginal epithelium is one of the numerous target organs of estrogenic where it induces epithelium proliferation and cornification through estrogenic receptors α [28]. Abundance of eosinophil cells means elevated estrogenic levels in rats. The effects of SERH suggested that this extract exerted effective estrogenic activity on vaginal cells at the dose of 100 mg/kg. BERH at 100 and 300 mg/kg b.w. and SERH at 300 mg/kg induced a slight vaginal cornification. In addition, Ethinylestradiol as well as extracts of the bark and seeds of *R. heudelotii* at a dose of 100 mg/kg b.w. caused a significant decrease in the body weight of the rats compared to the controls. This decrease is greater in rats treated with Ethinylestradiol and could be linked to a decrease in appetite induced in animals by these treatments, causing a decrease in their food consumption. Estrogenic is well known to have a depressive effect on appetite in ovariectomized rats [29]. The decrease in body weight caused by the seeds and bark extracts could also be due to the presence of estrogenic substances or phytoestrogens in these extracts.

Moreover, treatments of rats with Ethinylestradiol and SERH at 100 mg/kg resulted in highly significant increase ($p<0.001$) in uterine and

cervix weights. The treatment with SERH at 300 mg/kg and BERH at 100 and 300 mg/kg also caused a significant increase ($p<0.05$) in the weight of these organs. Similar results were obtained by Ybañez-Julca et al (2020) [30]. with the aqueous extract of *Lepidium meyenlii* (Brassicaceae) and by Zingue et al (2017) [31]. with the phytoestrogen genistein and propolis, a naturel mixture produced by *Apis mellifera.* As well as the vaginal epithelium, the uterus is one of the target tissues for estrogenic. At high levels, it induces endometrial proliferation, multiplication of the uterine glands which become tortuous as well as an accumulation of fluid in the uterine cavity thus causing an increase in the weight of the uterus; the proliferation of the endocervix epithelium is also stimulated, showing a thicker epithelium [32, 33, 34]. All these morphological changes may explain the increase of the weight of uterus and cervix induced by the extracts of *R. heudelotii*. The result on the uterus weight was corroborated by the histological architecture of his organ. Indeed, the photomicrograph of this organ highlighted an increase in the thickness of the endometrium, reflecting the hypertrophy and/or hyperplasia of this tissue as well as numerous tortuous glands in rats treated with Ethinylestradiol and the seeds and bark extract (100 mg/kg). The increase in the weight of the uterus by extracts of *R. heudelotii* and its effects on the histological structure of this organ would also be linked to the estrogenic effect of these extracts and confirmed the presence of phytoestrogens in the seeds and bark of this plant. These extracts also increased the adrenal glands weight, showing a stimulating activity on this gland similar to that of chromatographic fraction of *Citrus medica* (Rutaceae) [35]. seeds which illustrated the estrogenic nature of *R. heudelotii*.

CONCLUSION

The qualitative evaluation of the phytoconstituents of *R. heudelotii* showed that it contained various secondary metabolites such as sterols, polyterpenes, polyphenols, flavonoids, tannins, quinones, alkaloids and saponosides. This phytochemical could explain the estrogenic effects of both the seeds and stem bark extracts on vagina, cervix, uterus and adrenal glands demonstrated in this study. However, the seeds extract was found to exhibit the most effective estrogenic activity on these organs. A fractionation of the chemical constituents of this extract deserves to be carried out in order to identify the compounds at the origin of its estrogenic effects and their pathways of action.

Acknowledgement

The first author would like to thank the head of the vivarium of the Normal Superior School of Abidjan for providing the animals for all the experiments.

Conflict of Interest

None declared.

Financial Support

None declared.

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HOW TO CITE THIS ARTICLE

Michel BG, Fidele KZ, Marie-Odile TW, koffi K, Flavien T. Estrogenic effects of the seeds and stem bark extracts of *Ricinodendron heudelotii* in adult ovariectomized Wistar albino rats. J Phytopharmacology 2022; 11(1):1-6. doi: 10.31254/phyto.2022.11101