

EFFECT OF THE AQUEOUS EXTRACT OF *JUSTICIA INSULARIS* T. ANDERS (ACANTHACEAE) ON OVARIAN FOLLICULOGENESIS AND FERTILITY OF FEMALE RATSPhelix Bruno Telefo^{*1}; Simon Richard Tagne¹; Olga Elodie Sandrine Koono¹; Didiane M. Yemele¹; Félicité M. Tchouanguép¹¹University of Dschang, Faculty of Science, Laboratory of Biochemistry of Medicinal Plants, Food and Nutritional Science (LABPMAN) P.O. Box: 67 Dschang, Cameroon.

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Abstract

Justicia insularis T. Anders (Acanthaceae) is a medicinal plant whose leaves and those of three other plants are mixed for the preparation of a concoction used to improve fertility and to reduce labour pains in women of the Western Region of Cameroon. Previous studies have demonstrated the inducing potential on ovarian folliculogenesis and steroidogenesis of the aqueous extract of the leaf mixture (ADHJ) of four medicinal plants (*Aloe buettneri*, *Dicliptera verticillata*, *Hibiscus macranthus* and *Justicia insularis*) among which the later represented the highest proportion. This study was aimed at evaluating the ovarian inducing potential of *J. insularis* in immature female rats. Various doses of the aqueous extract of *J. insularis* were daily and orally given, for 20 days, to immature female rats distributed into four experimental groups of twenty animals each. At the end of the experimental period some biochemical and physiological parameters of ovarian function were assayed. The administration of the aqueous extract of *Justicia insularis* significantly induced an early vaginal opening in all treated groups ($P < 0.001$) as well as an increase (at doses of 50 or 100 mg/kg) in the number of hemorrhagic points, *Corpus luteum*, implantation sites, ovarian weight, uterine and ovarian proteins. Ovarian cholesterol level ($P < 0.05$) significantly decreased in animals treated with the lowest dose (12.5 mg/kg). The evaluation of the toxicological effects of the extract on pregnancy showed that it significantly increased pre- and post-implantation losses, resorption index and decreased the rate of nidation as well as litter's weight. These results suggest that the aqueous extract of *Justicia insularis* induces ovarian folliculogenesis thus justifying its high proportion in the leaf mixture of ADHJ.

Keywords: *Justicia insularis*, vaginal opening, ovary, fertility, gestation, resorption index.**List of abbreviations:** ADHJ= *Aloe Buettneri*, *Dicliptera verticillata*, *Hibiscus macranthus*, *Justicia insularis* leaf mixture; EDTA = Ethylene Diamine Tetra acetic Acid; LSD = Least Significant Difference; FSH= Follicle Stimulating Hormone; LH = Luteinizing Hormone; PMSG= Pregnant Mare Serum Gonadotrophin; GnRH= Gonadotrophin Releasing Hormone**Introduction**

The crucial problem of reproduction is the perpetuation of the species. In fact, every species is bound to disappear if it does not reproduce. It is for this reason that humans have not stopped fighting against infertility (Dioulde, 1992). Infertility is characterized by the absence of fertilization or pregnancy in a couple which exercises followed-up sexual relations without the use of contraceptives for a period of two years (Nkounkou et al., 2005). It is a disease of the reproductive system which affects both men and women with almost equal frequency. However, in many African countries and particularly in Cameroon, women are mostly the ones incriminated (Larsen, 2000). That is why the search for remedy against infertility within a couple is more in women. According to their social status, they resort either to modern or traditional medicine (Nkounkou et al., 2005).

Hopefully, many ethnopharmacological studies have proven the implication of medicinal plants or chemical compounds derived from it which biological effects regulate some female reproductive function in mammals. *Sterculia tomentosa* has long been given to immature heifers in order to make them prolific breeders (Dalziel, 1937). Clinical studies on *Aloe buettneri* have proven its usefulness in curing menstrual perturbations and functional sterility in women (Bhaduri et al., 1968; Garg et al., 1970; Gupta, 1972). Many plants of the *Justicia* genus (*J. simplex*, *J. adhatoda*, *J. insularis*) are equally used to cure reproductive ailments in women (Claeson et al., 2000; Badami et al., 2003).

Justicia insularis T. Anders (family Acanthaceae) is an herbaceous and perennial plant of 30 - 75 cm high with opposite ascending branches. Its leaves are simple, opposite, and the flower white, pink or purple (Berhaut, 1971). Studies undertaken by Telefo et al. (2004) revealed the presence of alkaloids, flavonoids and glycosides in their leaves. In Senegal, the leaf decoction of *J. insularis* is given to women during the last month of pregnancy to reduce labour pains. In the Western region of Cameroon, it is used in association with the leaves of three other medicinal plants (*Aloe buettneri*, *Hibiscus macranthus* and *Dicliptera verticillata*), to treat dysmenorrhoea and some cases of women infertility. This aqueous extract mixture has also been proven, in a series of studies to induce ovarian steroidogenesis and folliculogenesis in female rats (Telefo et al., 1998, 2002, 2004). These effects may result from the sum of the biological effects of various compounds present in the mixture, from that of secondary metabolites formed during the preparation of the mixture and eventually from that of the plant presenting the highest proportion in the mixture.

Our main hypothesis is based on the assertion that *Justicia insularis*, which constitutes 42 % of the mixture (Telefo et al., 1998), could induce an ovarian effect similar to that of ADHJ mixture. During the present studies, the effect of the aqueous extract of *J. insularis* on some biological parameters of reproduction was evaluated. Specifically, the inductive potential of the extract on pubertal onset, ovarian follicle growth and some gestational parameters (number of *Corpus luteum*, number of implantation sites,...) was assessed.

Methodology

Extract preparation

The fresh leaves of *Justicia insularis*, which had in a previous study been identified in the National Herbarium of Cameroon under voucher specimen code 34997 (Telefo et al., 1998), were collected in February 2008 from the botanical garden of the University of Dschang (Cameroon). They were washed and air-dried at room temperature and ground into fine powder. 100 g of powder was submerged in 1.5 l of boiling distilled water for 30 mins. After cooling, the extract was filtered and dried in a ventilated oven at 45 °C. The extraction yield (24.33 %) of the powdered extract was calculated. This powdered extract was stored in the refrigerator at -20 °C till use. Before being administered to animals, the powdered extract was prepared at concentrations of 1.25 mg/ml (extract 1), 5 mg/ml (extract 2) and 10 mg/ml (extract 3), in distilled water. Together with distilled water (control group), these preparations (extract 1, 2, 3) were orally administered to animals in a volume of 10 ml/kg body weight, thus corresponding to doses of 12.5, 50 and 100 mg/kg body weight respectively. The dose of 12.5 mg/kg was adopted by reconstitution from the traditional healer's main recipe as described in our previous studies (Telefo et al., 1998).

Animals

The animals used in this study were immature female albino Wistar rats, 21-22 days old, weighing 30-45 g. They were bred in the animal house of the Biochemistry Department (University of Dschang, Cameroon), housed under natural conditions of light (12 h cycles) and temperature (22 ± 2 °C). Animals were fed *ad libitum* with a standard laboratory diet and tap water was given. The care and handling of the animals as well as the experiments were conducted in accordance with the internationally accepted standard guidelines for laboratory animal use and care as described in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Experimental protocols

Puberty onset and Fertility assays

A total of eighty (80) immature female rats were randomized, based on their body weight, into 4 groups of twenty animals each. They received by oral route, either distilled water (control) or different doses of the aqueous extract of the plant for 20 consecutive days. They were weighed at 2 days interval throughout the experimental period. After two weeks (14 days) of treatment, the vaginal opening of each rat was checked every day until the day it occurred. At the end of the experimental period, 6 animals in each group were randomly sacrificed by anesthetizing with chloroform. Their ovaries and uteri were removed, blotted, weighed and stored at -20 °C until use. The remaining rats (14 per group) were mated the following day and continuously over a period of two weeks, with males of proven fertility. Starting from the mating day, vaginal smears were collected on daily basis in order to assess the presence of sperm. A laparoscopy was undertaken under diazepam (5 mg/ml, 5 mg/kg) and Ketamin (50 mg/ml, 80 mg/kg), ten days after the day of mating to count the number of implantation sites in uterine cords and the number of corpora lutea in ovaries. After delivery, the fetuses were weighed and their number recorded. From these data, the number of resorption sites (number of implantation site – number of viable fetuses), implantation index ($[\text{total number of implantation sites}/\text{number corpora lutea}] \times 100$), resorption index ($[\text{total number of resorption sites}/\text{total number of implantation sites}] \times 100$), preimplantation loss ($[\text{number of corpora lutea} - \text{number of implantations}/\text{number of corpora lutea}] \times 100$), postimplantation loss ($[\text{number of implantations} \times \text{number of viable fetuses}/\text{number of implantations}] \times 100$), antifertility activity ($[\text{number of females without viable fetuses}/\text{total number of females}] \times 100$), antiimplantation activity ($[\text{number of females without implantation sites}/\text{total number of females}] \times 100$), and gestation rate ($[\text{number of females with viable fetuses at birth}/\text{total number of gestational females}] \times 100$) were calculated (Costa-Silva et al., 2007).

Preparation of the uterine and ovarian supernatants and biochemical analysis

Ovaries and uteri were homogenized in Tris–sucrose buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.4) at 1 % and 2 % respectively. The homogenate was then centrifuged at 6000 x g at 4 °C (Beckman model J2–21) for 15 min, and the supernatants collected were used for protein (Bradford, 1976) and cholesterol (Trinder, 1969; Richmond, 1973; Roeschlau, 1974) assays.

Statistical analysis

The data from biological assays were registered as Mean ± SEM (standard error of the mean). The statistical differences between the values were analysed by ANOVA (Analysis of Variance) test. The Fisher LSD test was used for the comparison of means. The analysis of percentages was done by Chi-square (X^2) test. The Kruskal-Wallis test was used for non parametrical data, and the Mann Whitney test used when their differences were significant (Schwartz, 1991).

Results

Effect of *J. insularis* aqueous extract on the age at vaginal opening

Figure 1 illustrates the mean age of animals at vaginal opening and the percentage of those presenting vaginal aperture at a given age. Female rats receiving the aqueous extract of *Justicia insularis* presented vaginal opening one day (12.5 and 50 mg/kg) to two days (100 mg/kg) earlier ($P < 0.01$) as compared to control animals. About 30 % of rats aged 37 days and treated at 100 mg/kg (against 5 % of respective control) presented vaginal aperture ($P < 0.05$). This percentage was significantly increased in all *J. insularis*-treated animals when they were aged 40 or 41 days (80 or 100 % with any plant extract dosage compared to 45 or 65 % with 0 mg/kg). Moreover, complete vaginal opening was observed in all *J. insularis*-treated animals when they were aged 41 days, contrary to those of the control group for which it was observed at the age of 43.

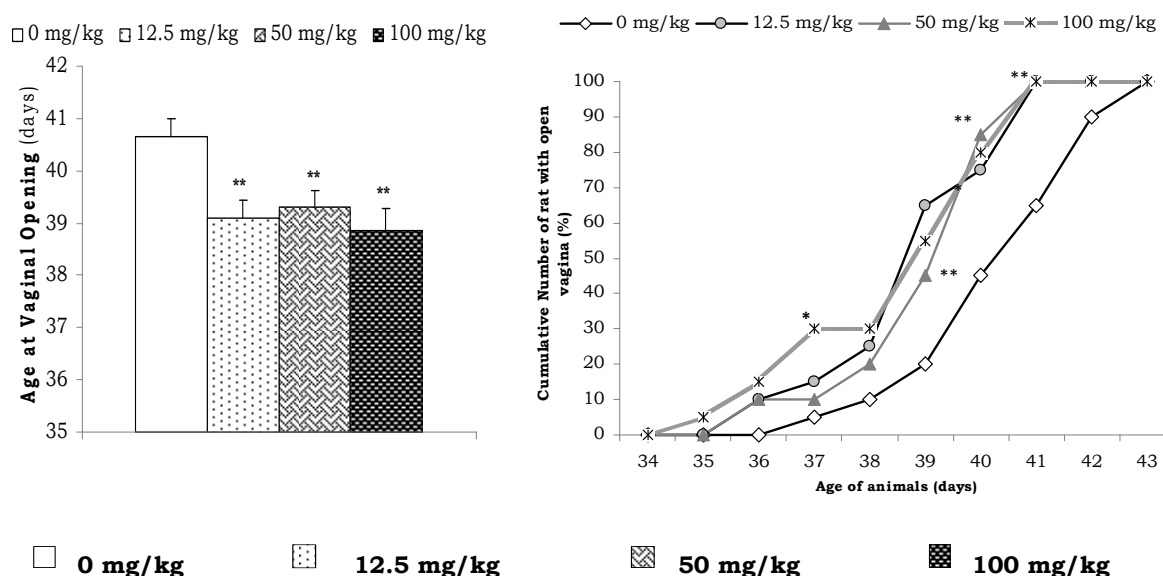


Figure 1: Prepubertal effect of oral administration of the aqueous extract of *Justicia insularis* on the timing of Puberty onset. Shown on the *left* are mean day of vaginal opening in control and plant extract-treated animals. Shown on the *right* are percentages of animal showing vaginal opening as a function of age in the control and plant extract-treated animals. *,**Values significantly different at $p < 0.05$ and $p < 0.01$ from those of the control group (Kruskal-Wallis, Mann-Whitney tests for histograms and Khi Square, Fisher Exact tests for lines); each data represents the mean \pm s.e.m of 20 animals.

Effect of *Justicia insularis* aqueous extract on ovarian weight, protein or cholesterol level and number of hemorrhagic points.

The physiological and biochemical changes obtained in ovaries after oral administration for 20 days of various doses of aqueous extract of *Justicia insularis* to immature female rats are presented in Figure 2. The numbers of hemorrhagic points of animals treated at doses of 50 or 100 mg/kg almost tripled or doubled as compared to that of control animals (Figure 2.D). Significant increases of 35.80 % ($P < 0.05$) at the dose of 50 mg / kg and of 23.56 % at the dose of 100 mg / kg were also recorded in their ovarian relative weight (Figure 2A) and proteins level (Figure 2C) respectively. These increases were slightly correlated with decreases in ovarian cholesterol which level significantly reduced by 70 % in animal receiving the dose of 12.5 mg.kg of *J. insularis* ($P < 0.05$) (Figure 2B).

Effect of *Justicia insularis* aqueous extract on uterine weight and proteins

Oral administration of the aqueous extract of *J. insularis* for 20 days reduced by 38 % the relative uterine weight of animals treated with the lowest dose (12.5 mg/kg). No significant effect of this parameter was obtained at higher doses. However, uterine proteins significantly increased by 58.09 % and 45.64 % for rats treated at the doses of 50 mg / kg and 100 mg / kg respectively ($P < 0.05$; Figure 3).

Effect of the aqueous extract of *Justicia insularis* on some fertility and gestational parameters

As shown in Table 1, the mean weight of foetuses, the fertility and gestation rates, the anti-implantation and anti-fertility activities of the animals remained unaffected after 20 days of oral administration of the aqueous extract of *J. insularis*. Significant increases in the number of *corpus luteum* and implantation sites of *J. Insularis*-treated animals were recorded. There were indeed 3 units (11.60 ± 0.74 vs 8.7 ± 0.33) or 2 units (10.70 ± 0.93 vs 8.70 ± 0.40) increase in the

number of *corpus luteum* or implantation sites of animals treated at the dose of 50 mg/kg as compared to controls. Besides these increases, significant reductions ($P < 0.01$) of the implantation index or the pre and post implantation losses of all *J. insularis* treated-animals were recorded.

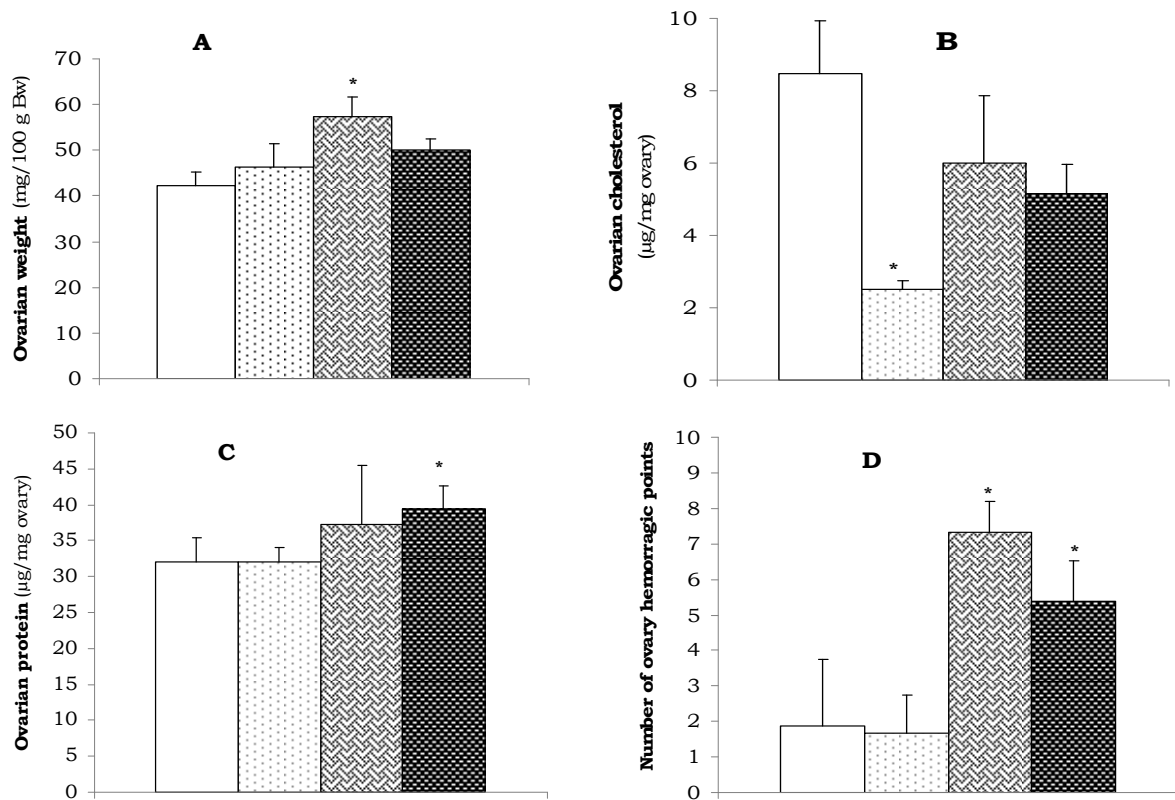


Figure 2: Effect of different doses of *Justicia insularis* aqueous extract on the relative weight of ovaries (A); ovarian protein concentration (C); ovarian cholesterol levels (B) and the number of hemorrhagic points (D). *Values significantly different at ($p < 0.05$) from those of the control group (ANOVA and Fisher LSD). Each histogram represents the mean \pm s.e.m. of 6 animals.

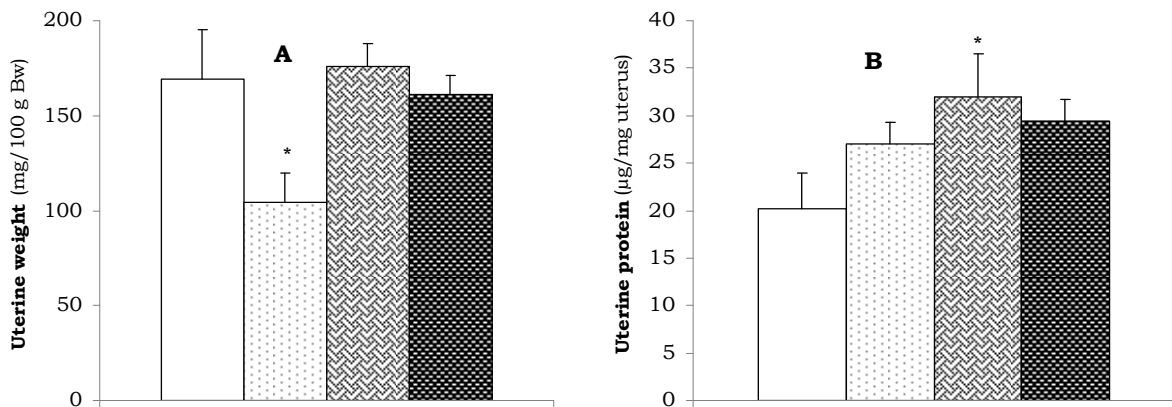


Figure 3: Effect of the different doses of *Justicia insularis* aqueous extract on the relative weight of the uterus (A) and the uterine protein level (B). *Values significantly different at ($p < 0.05$) from those of the control group (ANOVA and Fisher LSD). Each histogram represents the mean \pm s.e.m. of 6 animals.

Discussion and Conclusion

Justicia insularis, which is the plant of interest in this study is mixed with three other plants (*Aloe buettneri*, *Dicliptera verticillata*, *Hibiscus macranthus*) and used by traditional healers of the Western Region of Cameroon to regulate

menstrual dysfunctions and functional sterility in women (Telefo et al., 1998, 2004). Its inductive effect on the ovarian folliculogenesis and a physiological parameter of the onset of puberty (age at vaginal opening) has been evaluated. The choice of these parameters was not only directed by the influence of the gonadotrophic hormones (FSH, LH, PMSG, GnRH) in the induction of the follicular growth and the precocious onset of puberty in immature female rats, but also by the clinical usage of these hormones in the treatment of various forms of infertility (ovulatory defects or hypogonadal infertility) (La Rochebrochard, 2004)

Table 1: Effect of the aqueous extract of *J. insularis* on some fertility and gestational parameters. Each value represents the mean \pm s.e.m of 14 animals. Data with superscript *, **, *** are significantly different at $p < 0.05$; $p < 0.01$ and $p < 0.001$ respectively compared to that of the control group (ANOVA; Fisher LSD and Khi Square tests).

Studied parameters	Dose (mg/kg/)			
	0	12.5	50	100
Nb.°Corpora Lutea	8.70 \pm 0.33	10.81 \pm 0.32**	11.60 \pm 0.74***	11.36 \pm 0.54***
Nb.°Implantation sites	8.70 \pm 0.40	9.54 \pm 0.34	10.70 \pm 0.93**	9.45 \pm 0.45
Nb.°Fetuses Alive	7.90 \pm 0.37	8.36 \pm 0.65	7.90 \pm 0.67	8.54 \pm 0.45
Nb.°Resorption sites	0.35 \pm 0.16	0.85 \pm 0.37	1.35 \pm 0.75	0.71 \pm 0.28
Mean weight of fetuses (g)	5.20 \pm 0.12	5.15 \pm 0.15	4.77 \pm 0.60*	5.07 \pm 0.16
Implantation index	96.34 \pm 1.87	88.32 \pm 2.17	90.18 \pm 2.17	83.31 \pm 1.51***
Preimplantation Loss	3.65 \pm 1.87	11.67 \pm 2.17*	9.81 \pm 2.77**	16.68 \pm 1.51***
Postimplantation Loss	5.61 \pm 2.65	12.87 \pm 5.37	23.31 \pm 6.69**	9.29 \pm 3.53
Antiimplantation Activity (%)	28.57	21.43	28.57	21.43
Antifertility Activity (%)	28.58	21.43	28.53	21.43
Resorption Index (%)	40.00	54.54	90.00*	45.45
Gestation Rate (%)	100.00	100.00	100.00	100.00
Fertility Rate (%)	71.43	78.57	71.43	78.57

Each value represents the mean \pm s.e.m of 14 animals. Data with superscript *, **, *** are significantly different at $p < 0.05$; $p < 0.01$ and $p < 0.001$ respectively compared to that of the control group (ANOVA; Fisher LSD and Khi Square tests).

The daily oral administration of the aqueous extract of *Justicia insularis* over a period of 20 days induced, although not dose dependently, an early vaginal opening and significantly increased the weight of the ovaries and the levels of uterine and ovarian proteins. These increases may be attributed to the presence of estrogenic compounds in the plant extract. Indeed, several studies have shown that the administration of oestradiol or estrogenic compounds to immature female rats induces the synthesis of ovarian and uterine bio-macromolecules such as DNA, RNA and proteins (Jensen and Desombre, 1972; Katzenellenbogen et al., 1979); leading to the increase in the weight of these reproductive organs (ovary, uterus). This statement is strengthened by the 70.23 % decrease at the dose of 12.5 mg/kg in the ovarian cholesterol level. Cholesterol constitutes the main precursor of steroids hormones during their biosynthesis. Its reduction clearly proves its utilisation in the biosynthesis of oestradiol which will then contribute in the stimulation of ovarian cells growth. Besides, it is well established that follicular development ends at ovulation. This important physiological landmark of the ovarian function is followed by a stratum transformation of broken follicles into hemorrhagic points (Tavernier et al., 1983). The increase by 300 % and 193 % in the number of hemorrhagic points of *J. Insularis*-treated animals at the doses of 50 mg/kg and 100 mg/kg respectively may be connected to the induction of folliculogenesis by some estrogenic compounds found in the plant extract. Altogether, the above observations suggest the inductive effects of the aqueous extract of *Justicia insularis* on ovarian folliculogenesis or steroidogenesis. This induction is not related to the amount of extract administered as it may probably result in the synergistic effect of various biochemical compounds in the plant aqueous extract. To better appreciate the inductive effects of the plant extract, fertility test were carried out. Follicular cells growth generally culminates up to the ovulation of well differentiated ones which are then transformed into *corpus luteum*. Thus, the number of corpus luteum in the ovary is an efficient and excellent parameter of ovarian folliculogenesis induction. The significant increase in the number of *corpus luteum* recorded

during the fertility assay of our experiment constitute another clear indication of the inductive effect of *J. insularis* aqueous extract on ovarian follicles growth.

The oestradiol molecule, one of the various compounds implicated in the inductive effects of the plant extract, controls multiple physiological functions not only at ovarian levels but also in the hypothalamus, pituitary, bone and uterus. It plays crucial role in uterine smooth muscle contraction and could thus improve or affect eggs nidation or embryo resorption (Roberts et al., 1989). Significant decreases in the implantation index as well as pre and post implantation losses were recorded in *J. Insularis*-treated animals. This result could be attributed to the fast migration of fertilized eggs through the oviduct. This fast migration is stimulated by the contraction of oviduct and uterine smooth muscle which are under high oestrogenic environment, following the induction of ovarian folliculogenesis or steroidogenesis by the plant extract. In such conditions, the eggs will enter the uterine lumen in an immature state and could not easily adhere to the endometrium. This result is in agreement with the data of Telefo et al. (2005), who demonstrated that the aqueous extract of *Justicia insularis* induces the *in vitro* contraction of rat uterine smooth muscle.

Globally, the results obtained demonstrate the inductive effect of the aqueous extract of *Justicia insularis* on the ovarian folliculogenesis, justifying its high proportion (42 %) in the aqueous extract of the mixture of the leaves of *Justicia insularis*, *Aloe buettneri*, *Dicliptera verticillata* and *Hibiscus macranthus* (Telefo et al., 1998). This extract could thus substitute the mixture to improve folliculogenesis or ovulation. However, further studies on its effects on various stages of gestation would allow us to evaluate the margin of security that accompanies its use.

Acknowledgments

Authors are thankful to the members of Laboratory of Biochemistry of Medicinal Plants and Food and Nutritional Science (LABPMAN), for technical support during the experimental part of the work.

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