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Effects of the Aqueous Extract of *Justicia Insularis* on Reproductive Parameters in Testosterone Propionate-Induced Hyperandrogenic Female Albino Wistar Rats

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ABSTRACT

Background and objective: *Justicia insularis* is a medicinal plant traditionally used in Western Cameroon. It is often combined with *Aloe buettneri, Dicliptera verticillata* and *Hibiscus macranthus* to treat dysmenorrhea and some infertility cases. The present study aimed to evaluate the effects of the aqueous extract of *Justicia insularis* (AEJi) on reproductive parameters in testosterone propionate-induced hyperandrogenic female albino Wistar rats. **Materials and Methods:** Hyperandrogenism was induced in experimental animals by subcutaneous administration of testosterone propionate (10 mg/kg). The rats were then administered 50 or 100 mg/kg of AEJi for 20 consecutive days. Ovarian and uterine weights, serum and ovarian proteins, ovarian cholesterol and serum levels of estradiol, progesterone, FSH and LH were measured. Data were analyzed using Analysis of Variance (ANOVA) and significance was determined through various *post hoc* tests at $p<0.05$. **Results:** The negative control group showed significant decreases in ovarian and uterine relative weights, ovarian and serum proteins, FSH, estradiol and progesterone levels, while LH levels and ovarian cholesterol increased. Animals treated with 50 mg/kg AEJi exhibited notable changes, including body weight growth like the neutral control, significant increases in ovarian and uterine relative weights as well as ovarian and serum proteins, FSH, estradiol and progesterone levels, while LH levels and ovarian cholesterol decreased compared to the neutral control ($p<0.05$). **Conclusion:** The AEJi appears effective in regulating reproductive function in hyperandrogenic females.

KEYWORDS

Justicia insularis, hyperandrogeny, testosterone propionate, sexual hormones, ovary, medicinal plant

INTRODUCTION

Hyperandrogenism is a prevalent medical condition characterized by elevated levels of androgens in the body[1]. This condition is more common in women than in men, affecting approximately 5% of women of reproductive age[2,3]. The symptoms of hyperandrogenism can be quite varied and may include acne,

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seborrhea, hair loss on the scalp, increased body or facial hair and irregular or absent menstruation, which can sometimes lead to infertility[2]. Additionally, hyperandrogenism is a significant factor in follicular arrest in women with polycystic ovary syndrome (PCOS).

In recent years, there has been a global increase in focus on reproductive system dysfunctions caused by hyperandrogenism. This heightened attention aims not only to understand the underlying mechanisms of the condition but also to develop reliable methods to manage this endocrine dysfunction and mitigate its most severe consequence, infertility. Among these methods, the use of medicinal plants has gained prominence due to their potential therapeutic benefits[4].

One such medicinal plant is *Justicia insularis*, whose aqueous extract has shown promising results in various studies. These studies have demonstrated its follicle-stimulating effects in immature female rats[5], FSH-like and/or estrogenic effects[6], improvement in *in vitro* follicular survival, activation of ovine primordial follicles[7] and regulation of the estrous cycle in hyperandrogenic female albino Wistar rats[8]. These properties suggest that *Justicia insularis* could be a valuable candidate for managing hyperandrogenism and its associated reproductive issues.

Despite these promising properties, to the best of our knowledge, no study has investigated the effects of *Justicia insularis* on reproductive parameter regulation in hyperandrogenic rats. This study aims to explore the ameliorative effects of the aqueous extract of *Justicia insularis* (AEJi) on reproductive parameter disorders in testosterone propionate-induced hyperandrogenic female albino Wistar rats. The goal is to elucidate another property of this plant and contribute to its valorization in managing female hyperandrogenism and its related consequences. By doing so, a deeper understanding of the potential benefits of *insularis* would be achieved and new avenues for treatment and management of this condition could be offered.

MATERIALS AND METHODS

Study area: The research carried out at the Faculty of Science of the University of Dschang-Cameroon, from June to November, 2019 included animal handling and laboratory analysis. Prior to implementation, the experimental protocol underwent rigorous evaluation and validation by the faculty's scientific committee.

Experimental protocols used in this study strictly conformed to the internationally accepted standard ethical guidelines for laboratory animals' use and care as described in the European Community guidelines, EEC directive 86/609/EEC of the 24th November 1986[9].

Experimental animals: In this study, (25) female immature Wistar rats were employed. These animals were bred within the animal facilities of the Department of Biochemistry at the University of Dschang, Cameroon. At the outset of the experiment, the rats were 30 days old and weighed between 55 and 60 g. They received meticulous care, adhering to consistent husbandry practices, including a 12 hrs light/dark cycle and a controlled room temperature of 22±2°C. The animals were provided with a standard laboratory diet and had unrestricted access to tap water.

Plant material and AEJi preparation: Prior to its use, the plant was identified at the National Herbarium of Cameroon under voucher specimen code 34997/HNC. Three kilograms of fresh *Justicia insularis* leaves were collected in June 2019, from Batoufam Village in the Western Region of Cameroon. After washing, the leaves were dried in the shade at room temperature for two weeks. Subsequently, the dried leaves were finely ground using an electric grinder (HR-1500, Zhejiang Harui Industry LTD, China).

To prepare the aqueous extract of *J. insularis*, 100 g of the powdered plant material were infused in 1 liter of hot water (95°C) and subsequently boiled for 30 min, following the protocol reported by Telefo *et al.*[5]. After cooling, the extract underwent filtration and evaporation in a ventilated oven at 45°C for 48 hrs. The final extract was dissolved in distilled water to achieve the desired concentrations, corresponding to doses of 50 and 100 mg/kg for administration to experimental animals.

The determination of *Justicia insularis* concentrations was informed by previous studies conducted by Telefo *et al.*[5] and Goka *et al.*[6]

Testosterone propionate and cyproterone acetate preparation: To prepare a working solution, 1 mL of testosterone propionate (100 mg/mL) from Medi Tech Laboratory was diluted in sesame oil, resulting in a concentration of 10 mg/mL. Additionally, a 50 mg tablet of cyproterone acetate (marketed as "Androcur 50 mg" by Delpharm), an antiandrogen, was dissolved in 5 mL of distilled water to achieve the same working concentration.

Experimental design: The experiment followed the method described by Beloosesky *et al.*[10] and involved the subcutaneous daily injection of testosterone propionate in sesame oil at a concentration of 10 mg/kg to immature female rats for 21 consecutive days. A total of 25 immature female rats (weighing 55-60 g and 30 days old) were divided into five groups of five animals each.

The neutral control group received 1 mL/kg of sesame oil by subcutaneous injection from day 1 to day 21 of treatment and 10 mL/kg of distilled water orally from day 16 to day 35. The negative control group, treated with testosterone propionate, received 10 mg/kg of testosterone propionate from day 1 to day 21 by subcutaneous injection, followed by 10 mL/kg of distilled water.

The reference control group received 10 mL/kg of cyproterone acetate (10 mg/mL) from day 16 to day 35. The experimental groups received an aqueous extract of *Justicia insularis* (50 and 100 mg/kg) orally from day 16 to day 35.

Animals were weighed at two-day intervals throughout the experimental period. On the 36th day, they were euthanized using chloroform anesthesia and their blood was collected via cardiac puncture and centrifuged (2500 g, 15 min). The resulting serum was stored at -20°C for further analysis of proteins, estradiol, progesterone, FSH and LH. Uteri and ovaries were also removed, blotted, weighed and stored at -20°C until use.

Ovaries were homogenized in Tris-sucrose buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) at 1%. The homogenates were then centrifuged at 4000 g at 4°C for 15 min and the collected supernatants were used for protein[11] and cholesterol[12,13] assays. Serum proteins were also assayed using the Bradford method[11].

Sexual hormone assays were performed using direct (FSH and LH) and indirect (estradiol and progesterone) binding techniques[14]. The reagents used for these assays were obtained from GBC (General Biological Corporation, Hsinchu 30077, Taiwan, ROC) and hormonal levels were measured using a microtiter well reader (Lab systems Multiskan RC, 351, FIN-00881, Helsinki, Finland) at 450 nm.

Statistical analysis: The parametric data from the biological assays were recorded as Mean±Standard Error. Statistical differences between the values were analyzed using the ANOVA (analysis of variance) test. When the ANOVA indicated significant differences, the Student-Newman-Keuls test was employed for comparisons between means. Percentage data were analyzed using the Chi-square test. For non-parametric data, the Kruskal-Wallis test was used and the Mann-Whitney test was applied when significant differences were detected. The differences through the tests were considered significant at $p<0.05$.[15].

RESULTS

Effect of AEJi on weight growth, uterine and ovarian relative weights of testosterone propionate-induced hyperandrogenic female rats: Figure 1 illustrates the body weight variation of the animals throughout the experimental period. From the beginning of cyproterone acetate and extract administration (16th day of treatment), the body weight variation in the neutral control group, as well as in those treated with 50 and 100 mg/kg of AEJi, was less pronounced compared to the negative and reference control groups.

Table 1 presents the ovarian and uterine weight variations after 20 consecutive days of oral administration of cyproterone acetate and AEJi to testosterone propionate-induced hyperandrogenic female rats. The ovarian weight of animals treated with 50 mg/kg AEJi did not significantly differ from that of the neutral control group, although it increased compared to the negative control. A significant increase in uterine weight was observed in all animals treated with either cyproterone acetate or AEJi compared to the negative control group, although these values were not significantly different from those of the neutral control group.

Effect of AEJi on serum and ovarian proteins and ovarian cholesterol levels in testosterone propionate-induced hyperandrogenic female rats: Table 2 illustrates the variations in serum and ovarian protein levels, as well as ovarian cholesterol levels, in testosterone propionate-induced hyperandrogenic female rats treated with either cyproterone acetate or AEJi. In the treated animals, a significant increase in serum protein levels was observed compared to both the neutral control group and the negative control group.

Regarding ovarian proteins, no significant difference was noted between the neutral control group and the animals treated with 50 mg/kg of AEJi. However, a significant increase was observed in animals treated with testosterone propionate alone (negative control), cyproterone acetate and 100 mg/kg of AEJi. A significant decrease in ovarian cholesterol levels was observed in animals treated with 50 mg/kg ($p<0.01$) and 100 mg/kg ($p<0.05$) of AEJi compared to the neutral control group. There was no significant difference between the negative and reference control groups.

Effects of AEJi on sexual hormones in testosterone propionate-induced hyperandrogenic female rats: Table 3 shows that the serum FSH level significantly increased in animals treated with 50 mg/kg of AEJi compared to the control groups, while it decreased in all other treated groups. A similar trend was observed with serum estradiol levels. Except for the animals treated with 50 mg/kg of AEJi, the serum progesterone levels in the other three treated groups significantly decreased ($p<0.05$) compared to the neutral control group. No significant difference in serum LH levels was observed in animals treated with cyproterone acetate and 100 mg/kg of AEJi compared to the neutral control values. However, a significant increase was noted in the negative control animals ($p<0.01$) and in animals treated with 50 mg/kg of AEJi ($p<0.05$).

Table 1: Effect of AEJi on uterine and ovarian relative weights of testosterone propionate-induced hyperandrogenic female rats

Experimental groups	Control	T+distilled water	T+cyproterone acetate	T+AEJi 50 mg/kg	T+AEJi 100 mg/kg
Uterine relative weight (mg/100 g body weight)	0.209 ± 0.040^{b}	0.143 ± 0.009^{a}	0.179 ± 0.011^{b}	0.179 ± 0.010^{b}	0.198 ± 0.010^{b}
Ovarian relative weight (mg/100 g body weight)	0.075 ± 0.004^{c}	0.052 ± 0.003^{b}	0.044 ± 0.004^{a}	0.072 ± 0.004^{c}	0.049 ± 0.003^{c}

Each value represents the Mean±SE for 5 rats. In the same line, values carrying the same letter are not significantly different (Student-Newman-keuls $p<0.05$). TP: Testosterone propionate and AEJi: Aqueous extract of *Justicia insularis*

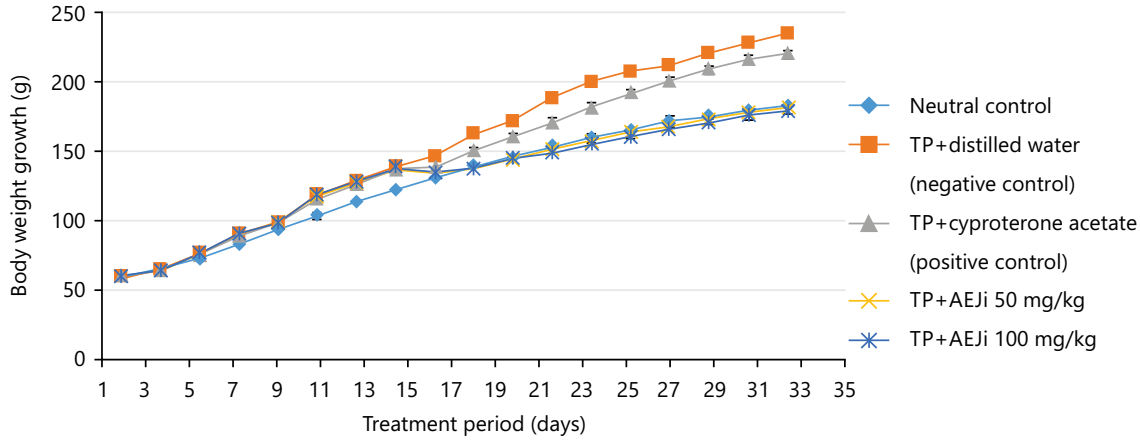

Fig. 1:Effects of AEJi on animals' body weight variation through the treatment

TP: Testosterone propionate and AEJi: Aqueous extract of *Justicia insularis*

Table 2: AEJi effects on serum and ovarian parameters in hyperandrogenic rats

Experimental groups	Control	T+distilled water	T+cyproterone acetate	T+AEJi 50 mg/kg	T+AEJi 100 mg/kg
Serum protein level (mg/kg body weight)	3.144±0.051^{b}	3.066±0.032^{a}	3.258±0.027^{c}	3.229±0.022^{c}	3.631±0.025^{d}
Ovarian protein level (µg/mg ovary)	31±2.13^{a,b}	42.07±2.16^{c}	43.78±2.96^{c}	29.60±1.29^{a}	34.55±2.49^{b}
Ovarian cholesterol level (µg/mg ovary)	1.027±0.107^{c}	0.978±0.093^{c}	0.940±0.070^{c}	0.436±0.034^{a}	0.615±0.086^{b}

Each value represents the Mean±SE for 5 rats. In the same line, values carrying the same letter are not significantly different (Student-Newman- keuls p 0.05). TP: Testosterone propionate and AEJi: Aqueous extract of *Justicia insularis*

Table 3: Effects of AEJi on gonadotropins and sexual hormones in testosterone propionate-induced hyperandrogenic female rats

Experimental groups	Control	T+distilled water	T+cyproterone acetate	T+AEJi 50mg/kg	T+AEJi 100mg/kg
Serum FSH level (UI/mL)	2.426±0.028^{c}	1.906±0.030^{a}	2.300±0.045^{b}	2.628±0.031^{d}	2.314±0.019^{b}
Serum LH level (UI/mL)	0.716±0.011^{a}	0.884±0.024^{c}	0.702±0.019^{a}	0.756±0.029^{b}	0.692 ±0.024^{a}
Serum estradiol level (pg/mL)	2.204±0.021^{c}	1.510±0.022^{a}	2.098±0.019^{b}	2.662±0.033^{d}	2.090±0.022^{b}
Serum progesterone level (pg/mL)	1.6±0.019^{d}	1.102±0.019^{a}	1.408±0.029^{c}	1.522±0.029^{d}	1.350±0.025^{b}

Each value represents the Mean±SE for 5 rats. In the same line, values carrying the same letter are not significantly different (Student-Newman- keuls p 0.05). TP: Testosterone propionate and AEJi: Aqueous extract of *Justicia insularis*

DISCUSSION

In the current study, the aqueous extract of *Justicia insularis* (AEJi) was investigated for its beneficial effects on abnormal reproductive conditions caused by hyperandrogenism induced by testosterone propionate in albino Wistar female rats. Hyperandrogenism is a common disorder affecting 5 to 10% of women of reproductive age[3]. Its main manifestations include oligo-ovulation or anovulation and polycystic ovary syndrome (PCOS), which are closely associated with obesity, hirsutism, acne, infertility and endometrial cancer[16]. These physiological dysfunctions are strongly related to disruptions in gonadotropins and sexual hormones. Therefore, targeting the improvement of physiological and biochemical reproductive parameters using medicinal plants is relevant for discovering effective agents to manage hyperandrogenic conditions at an affordable cost. Testosterone propionate-induced hyperandrogenism is one of the experimental models used for this purpose[10].

At the end of the treatment, a significant increase in FSH and estradiol levels was observed in animals treated with 50 mg/kg of AEJi compared to the negative control group, where these parameters were significantly lower ($p<0.5$) compared to the neutral control. High levels of androgens lead to a reduction in FSH levels and consequently estrogens due to impaired follicular growth and estrogen production.

Conversely, the antiandrogenic activity of AEJi[8] appears to stimulate the production of FSH and estrogens at the administered dosage. The binding of FSH to its receptor regulates the expression of certain genes involved in the proliferation of ovarian cells and steroidogenesis, leading to increased estrogen production[17]. When these estrogens bind to receptors in their target organs (ovaries, uterus, bones, hypothalamus, etc.), the hormone-receptor complex attaches to DNA, promoting protein transcription and translation, resulting in high protein production and accelerated tissue growth[18-20]. This explains the significant increase in relative ovarian and uterine weights in animals treated with 50 mg/kg of AEJi compared to the negative control, aligning these values with those of the neutral control group. This significant increase in relative ovarian weight corroborated the results from different studies[5-21] after oral administration of an aqueous extract mixture of *Aloe buettneri*, *Dicliptera verticillata*, *Justicia insularis* and *Hibiscus macranthus* for 20 days to immature female rats.

Cholesterol, being the precursor of steroid hormones (estrogens and progesterone), showed a significant drop in its level in rats treated with the extract, further explaining the intensification of ovarian steroidogenesis. The relatively high cholesterol level in the negative control group and the significant decrease ($p<0.5$) in uterine relative weight indicate a deceleration of this reaction in that group, which also correlates with the greater body weight growth observed in the affected rats.

Progesterone is exclusively produced by the corpus luteum. The significant decrease in its level in the negative control group corroborates the findings of Beloosesky *et al*.[10], who reported anovulation and the lack of progesterone production in rats after 35 days of testosterone propionate administration. This suggests an alteration in follicular growth in this group, correlating with the drop in FSH levels, which is a key regulator in this biological process[22].

No significant difference was observed in the serum LH levels in the positive control and 100 mg/kg AEJi groups compared to the neutral control. However, a significant increase ($p<0.05$) was noted in the negative control group and those treated with 50 mg/kg AEJi. This result can be explained by the negative feedback mechanism of estrogens on the hypothalamic-pituitary system. In the negative control group, a decrease in estrogen levels would have led to a significant increase in LH levels. Hypersecretion of LH is also a known phenomenon associated with hyperandrogenism[23,24].

Herbs are readily available to humans and have been extensively explored for their medicinal properties. As a result, herbal medicines are increasingly sought as alternatives to synthetic pharmaceutical products, leading to a rise in their demand as natural remedies[25]. In this study, the effects of the aqueous extract of *Justicia insularis* (AEJi) were investigated on hyperandrogenism induced by testosterone propionate in female albino Wistar rats. The AEJi showed promising results, including increased FSH and estradiol levels, reduced ovarian cholesterol and improved relative ovarian and uterine weights. Implications include potential applications in managing conditions like polycystic ovary syndrome (PCOS) and infertility. However, limitations such as the animal model and the need for further mechanistic understanding should be considered.

CONCLUSION

Overall, these findings demonstrate that the aqueous extract of *Justicia insularis* effectively regulates physiological and biochemical parameters in testosterone propionate-induced hyperandrogenic female albino Wistar rats. It proves to be an effective treatment for ameliorating reproductive parameter disorders in hyperandrogenic females by normalizing sexual hormone and gonadotropin levels, as well as ovarian and uterine relative weights. These results also explain its restorative effects on estrous cycles and fertility in hyperandrogenic females, as reported in previous studies. This makes the extract an excellent candidate for managing dysfunctions linked to hyperandrogenism in women.

SIGNIFICANCE STATEMENT

Research exploring African traditional pharmacopoeia has turned attention to *Justicia insularis*, a plant renowned for its therapeutic properties. Recent findings highlight its aqueous extract's promise against Polycystic Ovary Syndrome (PCOS), urging further investigation. The present study honors tradition while embracing scientific innovation for women's health.

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