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ESTROGENIC ACTIVITY OF ETHANOLIC EXTRACTS FROM LEAVES OF *ILEX GUAYUSA* LOES. AND *MEDICAGO SATIVA* IN *RATTUS NORVEGICUS*

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Abstract

Ilex guayusa Loes. (guayusa) and *Medicago sativa* (alfalfa) are plants used in Ecuadorian traditional medicine to treat female infertility. It has been verified the estrogenic activity of ethanolic extracts from leaves of guayusa and alfalfa in rats (*Rattus norvegicus*), in the Phytochemistry Laboratory of the Sciences Faculty of the Escuela Superior Politécnica de Chimborazo (ESPOCH). The experimental methodology was the oral administration of treatments, statistically evaluated by ANOVA test ($p < 0.05$) for multiple comparisons in a confidence interval of 99% using Tukey HSD. 25 female albino rats were divided them into five groups called: control, standard and experimental subjects D1, D2 and D3. During treatment received: saline solution (negative control), standardized commercial soy isoflavone extract (positive control) 3,64mg/kg weight, and experimental subjects three doses of 9 mg / kg, 18 mg / kg and 36 mg / kg of guayusa and alfalfa extracts respectively. After treatment serum oestradiol levels were significantly increased for the tested doses, like the ovaries and uteri weights. It is concluded that the three treatments of guayusa and alfalfa extracts produce estrogenic effect in rats both oestradiol levels and weights of reproductive organs.

Keywords: *Ilex guayusa*, *Medicago sativa*, estrogenic, infertility, serum oestradiol, isoflavone.

Introduction

Infertility is the inability to achieve a pregnancy after one year of more of regular unprotected sexual activity [1]. By 1990, in developing countries, one in every four women has been affected by infertility. This has remained similar to 2010 [2-3].

There are many contributing factors to infertility, like infectious, genetic, environmental, dietary, social, etc. These can affect women, men or couples [3]. Anovulatory infertility is treated with ovulation-stimulating agents (OSA), as antiestrogens (clomiphene, tamoxifen) and gonadotrophins (follicle stimulant hormone FSH and luteinizing hormone LH), and aromatase inhibitors [4-5].

According clinical trials, using OSA significantly increases serum oestradiol levels [5]. Estrogens augmented due clomiphene is associated with risk of endometrial and breast cancer for a genotoxic and cellular proliferative effect [6-7]. Oestradiol has demonstrated have a cellular proliferative effect in human breast cancer cell line ZR-75-1 in monolayer and suspension culture [8]. Epidemiological researches in breast cancer reach the same conclusion [9]. The hormonal replacement therapy, a therapy applied in postmenopausal women, increases the risk of development endometrial and breast cancer, due to exposure to tissues to bioavailable estrogens, according to epidemiological studies [10-11]. Postmenopausal women who were using estrogen, increases the risk of breast cancer in comparison with women who had never use hormones [12-13].

International epidemiological research demonstrate that using of estrogens and other hormones is a risk factor for endometrial cancer, according to the unopposed estrogen hypothesis: exposure to estrogens unopposed by progesterone or progestins increases mitotic activity and probabilities of DNA replication errors [14]. On the other hand, controlled and preliminary comparative studies show aromatase inhibitors promote growth of ovarian follicles, stimulating FSH production without increase estrogens levels [15]. Several herbal supplements are used to female infertility, many of which has phytoestrogens [16]. Several compounds have been considered phytoestrogens, like flavonoids, isoflavonoids, coumestans, lignans and anthraquinones [17]. Phytoestrogens has similitude with 17 β-estradiol, therefore, they can binds to estrogen receptors and modulate their effects [18]. Studies conducted to evaluate the effects of the administration of soy extract rich in phytoestrogens in Wistar rats, showed estrogenic activity represented by an increase in the estrous cycle, an increase in uterine weight, and alterations in the ovarian follicle development [19]. In fact, in rat and mouse models with oral and parenteral administration of genistein, a phytoestrogen, it demonstrated uterotrophic activity [20-21].

There are several examples of plants with demonstrated estrogenic activity. *Cimicifuga racemosa*, an American herb, stimulates an increase luteinizing hormone, progesterone and oestradiol, improving the uterine thickness and follicular maturation [22]. The methanol extract of *Polygonum cuspidatum*, a plant used in traditional Chinese medicine, promotes the proliferation of MCF-7 cells sensitive to estrogen, from 30 to 100 ug / mL. Emodin and emodin 8-O-b-d-glucopyranoside are active metabolites that compete with the 17- beta estradiol in the ERa and ERb receptors [23]. *Ilex guayusa* Loes. (Aquifoliaceae) is a tree that grows from southern Colombia to northern Peru. It has reported the use of guayusa leaves in Ecuador for numerous medicinal purpose by Achuar tribe, especially as a stimulant beverage for its high caffeine content [24]. I. guayusa also is traditionally used as treatment to gastritis, headache, body pain, and women infertility in southern and eastern Ecuadorian provinces [25-26].

Medicago sativa, or *alfalfa*, (Fabiaceae) is a plant that has been cultivated as forage crop. This is a good source of phytoestrogens, principally coumestrol and genistein. Has been widely used in South America as diuretic, kidney and vesicular swelling, lung ailments, and reconstituent [27]. In mare, feeding with high amounts of alfalfa causes symptoms of hyperestrogenity [18]. In immature female rats administrate with ethanolic extract of M. sativa, serum progesterone concentrations and ovarian weight has been increased. Therefore, it exhibited estrogen like activity [28]. The peak of luteinizing hormone concentration in ewes feeding with alfalfa has found elevated in the estrus periods [29]. Through this research, we prove the estrogenic effect of both plant species used in Andean traditional medicine by administering their extracts to Wistar rats and the subsequent determination of parameters related to estrogenic activity, as estradiol serum concentration, and ovarian and uterine weight.

Methods

Plant material and extraction procedure:

Ilex guayusa was collected in the canton Puyo, province of Pastaza (924 meters above sea level) and *Medicago sativa* in the canton Colta, province of Chimborazo (2900 meters above sea level). Both

samples were air-dried and individually subjected to maceration in 96% ethanol for 7 days. The extracts were concentrated to dryness under controlled pressure and reconstituted in 0.9% sodium chloride solution (NaCl 0.9%) according to the dose to be administered according to the amount of total solids calculated.

Standard substance

Commercial tablets of standardized soy isoflavones extracts were used, equivalent to 50 mg of active isoflavones. The tablets were solved in saline solution.

Experimental subjects

Wistar female rats were used as experimental subjects, between 2.5 to 3.5 months old, and body weight 170±5 g, obtained from the animal facility center of the Sciences Faculty, ESPOCH. 25 rats were randomly divided into 5 experimental groups of 5 rats each.

Treatment

Based on the protocol of Telefo, P. B. et al [30], after determination of basal oestradiol in blood samples from facial vein by immunoassay Microplate, the subjects were treated daily for 15 days orally with 0.9% sodium chloride solution (negative control), standardized soy isoflavone extract 3,64 mg/kg/day (positive control) or various concentrations of plant extracts each corresponding to 9, 18 and 36 mg/kg/day. At the end of the experiment, it was obtained a sample of blood by cardiac puncture and determined the final concentration of oestradiol. After euthanasia, ovaries and uteri were removed and weighed.

Results

As shown in Table 1 and Figure 1, the administration of the medium and high doses of I. guayusa and M. sativa extracts, caused a significant increase in serum estradiol concentration compared to the negative control. The administration of soy isoflavone extracts did not cause this effect.

A significant increase in the weights of uteri and ovaries in all experimental groups were also observed following a direct relationship with doses of each treatment. In this case, the positive control of soy isoflavones caused an increase in cellular proliferation similar to the administration of the medium dose of both plant extracts.

Discussion

These results indicates that I. guayusa and M. sativa have a significant activity on the production of endogenous estrogen, activity that is different from soy isoflavones. Isoflavones do not promote increased estrogen, but by their structural similarity, they are agonists to estrogen specific receptors. Guayusa and alfalfa phytoestrogens are compounds that are structurally and functionally similar to the 17-β estradiol and bind to its receptor [23].

The main function of estrogen is to cause cell proliferation and growth of tissues of the sexual organs and other tissues related to reproduction. Once ingested phytoestrogens are metabolized in the body, some of them are bound to serum proteins and another portion is available to occupy the estrogen receptor (ER). This free portion exerts its effect via two types of receptors ER alpha and ER beta have different tissue distribution, so that beta is more ubiquitous than alpha. Activation of ER beta receptor is expressed in non-reproductive tissues such as bone, brain, pituitary, tract urinary, vascular system and prostate; and reproductive tissue such as ovary and uterus [23].

At this point, we cannot even say with certainty where the compounds from guayusa and alfalfa involved. However it is believed that could have effect on luteinizing hormone and follicle stimulating hormone, as shown in previous studies in other plant species [29], or even on aromatase and converting precursor's estrogen into active estrogen process. Therefore, guayusa and alfalfa extracts at low dose (9 mg/kg), medium dose (18 mg/kg) and high dose (36 mg/kg) per day, have estrogenic effect manifested in the increased weight of ovaries and uterus, and serum oestradiol concentration in immature Wistar rats. Using these species in traditional Andean medicine for infertility is justified. Both species could be used as a treatment for anovulatory infertility or hormone replacement after menopause, although it is necessary to evaluate the risk of developing breast and endometrial cancer.

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Table 1. Effect of the administration of M. sativa and I. guayusa extracts in rats for 15 days, in serum oestradiol, uterine weight and ovarian weight.

Treatments		Serum oestradiol (pg/mL)	Uterine weight (mg)	Ovarian weight (mg)
Negative control (0,9% NaCl)		12.7±0.01	344.5±3.6	140.0±0.6
Positive control soy isoflavones extract (3.64 mg/kg)		14.5±1.15	472.8±2.1	226.2±2.3
***Ilex guayusa* Extract**	**Low dose (9 mg/Kg)**	23.7±0.06	489.3±0.1	198.7±0.1
	Medium dose (18 mg/Kg)	41.7±0.08	521.7±0.1	221.3±0.1
	High dose (36 mg/Kg)	53.5±0.03	630.3±0.03	253.7±0.03
***Medicago sativa* extract**	**Low dose (9 mg/Kg)**	17.8±0.16	446.0±0.2	243.0±0.2
	Medium dose (18 mg/Kg)	24,0±0.21	560,0±0.2	252.0±0.2
	High dose (36 mg/Kg)	50.2±0.44	678.0±0.4	265.0±0.4

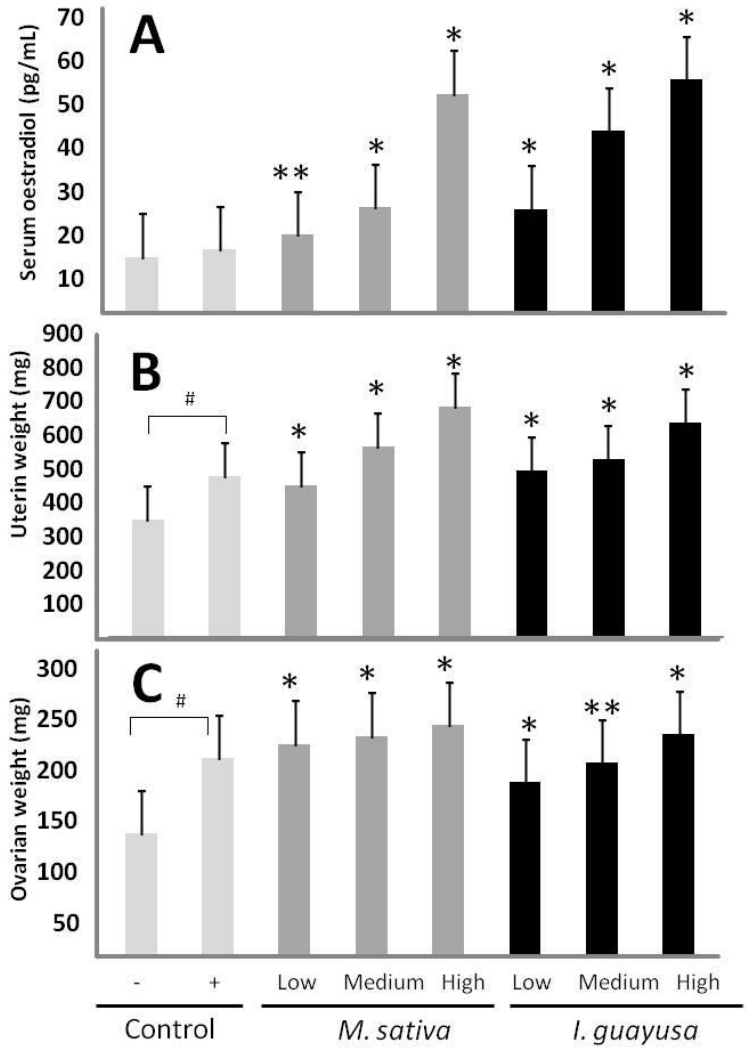

Figure 1. Effect of the administration of M. sativa and I. guayusa extracts in rats for 15 days, low dose (9 mg / kg), medium dose (18 mg / kg) and high dose (36 mg / kg) (A) Level of serum oestradiol (pg / mL). (B) Uterine Weight (mg). (C) Ovarian weight (mg). C + = soy isoflavones extract (3.64 mg / kg). C- = 0.9% NaCl. n = 3. * p <0.01. ** p <0.05. (Tukeys HSD test). # p <0.01 (Students t-test)