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Estrous cycle disruptor effect of an ethanolic extract from *Buddleja globosa* leaves and its main component (verbascoside)

[Efecto disruptor del ciclo estral de un extracto etanólico de hojas de *Buddleja globosa* y de su componente principal (verbascósido)]

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Abstract*:* The estrous cycle disruptor effect of an ethanolic extract (EMATst) from *Buddleja globosa* leaves and standardized in its main component (verbascoside) was determined in rats after the subcutaneous administration of EMATst. Binding of EMATst and verbascoside to the estrogen receptor (ER) of EMATst and verbacoside was also measuredestablished. EMATst produced a significant alteration inof the estrous cycle only at the highest dose (10-5 M), which could be attributed to an antiestrogenic effect. The Bbinding of EMATst and verbascoside to the ER was competitive and occurred in concentrations 1000 times greater than that of 17β-estradiol.

Keywords: *Buddleja globosa*; estrous-cycle; verbascoside; estrogenic-receptor

Resumen: El efecto disruptor del ciclo estral de un extracto etanólico (EMATst) obtenido a partir de las hojas de *Buddleja globosa* y estandarizado en su componente mayoritario (verbascósido) fue determinado en ratas después de la administración subcutánea de EMATst. Se estableció además la unión al receptor estrogénico (RE) tanto de EMATst como de verbascósido. EMATst sólo a la dosis más alta (10-5M) produjo una alteración significativa del ciclo estral, lo que podría atribuirse a un efecto antiestrogénico. La unión al RE de EMATst y verbascósido se produjo a concentraciones 1000 veces mayor que el 17β-estradiol y de forma competitiva.

Palabras Clave: *Buddleja globosa*, ciclo estral, verbascósido, receptor estrogénico

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INTRODUCTION

Endocrine disruptors (ED) are a group of natural or synthetic chemical substances exogenous to the human organism; these compounds interfere with the mechanism of the hormones responsible for the maintenance of homeostasis and the regulation of development and have various origins, structures and uses (Olea *et al.,* 2002). Compounds with estrogenic or antiestrogenic activity present in plants are called phytoestrogens, from which isoflavones, lignans, coumestans, flavonoids and stilbenes have been identified (Papoutsi *et al.,* 2006). Studies on isoflavones have reported that isoflavones exhibit estrogenic and antiestrogenic properties, compete for the estrogen receptor and exhibit biphasic activity: estrogenic action at low concentrations ($< 10^{-6}$ M) and anti-estrogenic activity at high concentrations ($>10^{-6}$ M) (Papoutsi *et al.,* 2006).

Phytoestrogens may or may not be beneficial, depending on the age at exposure and/or whether the compounds act at the intrauterine or postnatal stages of development, especially for those substances that cross the placental barrier or are passed through breast milk (Olea *et al.,* 2002).

Previous studies have reported that farm and wild animals that have consumed large quantities of plants rich in phytoestrogens exhibit problems with reproduction and fertility (Leopold *et al.,* 1976, Berger *et al.,* 1977). These same problems were observed in Australian sheep due to the consumption of phytoestrogens in a species of clover (*Trifolium subterraneum*); equol and coumestrol were identified as being responsible for this effect (Bennetts and Underwood, 1951). These phytoestrogens have also been suggested to be the reason why cheetahs in captivity tend to be infertile, due to the consumption of food rich in soy (Setchell *et al.,* 1987). With respect to the early stages of development, rats and mice exposed before or at birth to coumestrol and genistein have been shown to develop anomalies in the reproductive apparatus, such as changes in ovarian development, sub-fertility, infertility and alterations in the estrous cycle (Delclos *et al.,* 2001; Jefferson *et al.,* 2002; Jefferson *et al.,* 2005; Jefferson *et al.,* 2006).

Buddleja globosa Hope (Buddlejaceae) is a native species (common name: matico) cultivated in Chile, Peru and Argentina that is used in herbal medicine.

In our laboratory, recent investigations with sequential extracts of leaves have isolated a mixture of β-sitosterol, stigmasterol, stigmastenol, stigmastanol and campesterol from dichloromethane extract, and the standardized ethanol extract in verbascoside (EMATst) is composed mainly of phenylpropanoids, such as verbascoside, and flavonoids, such as the 7-O-glucoside of luteolin (Backhouse *et al.,* 2008a, Backhouse *et al.,* 2008b). Our "in vivo" pharmacological studies of these extracts have demonstrated their anti-inflammatory activity orally and topically, as well as their scarring, anti-oxidant and analgesic effects (Backhouse *et al.,* 2008a, Backhouse *et al.,* 2008b).

The estrogenic effect of verbascoside isolated from *Verbascun macrucurum* was measured by Papoutsi *et al.* (2006), who evaluated its estrogenic effect on osteoblasts, its anti-estrogenic effect on mammalian MCF-7 cancer cells and its anti-proliferative effect on the endometrium using the Ishikawa cell line. The authors also demonstrated its alpha estrogen receptor antagonist properties in HeLa cells. Activity-structure tests "in vitro" to elucidate the relative binding affinity of the estrogen receptor demonstrated that the flavones bind to the estrogen receptor (Heckmann *et al.,* 2008). The 7-O-glucoside of luteolin apparently does not contribute to the estrogenic effect of EMATst due to the substitution of the hydroxyl group in C-7 by glucose (Fang *et al.,* 2001).

The present investigation was undertaken to establish the estrous cycle disruptor effect of EMATst and its main component (verbascoside) to obtain new insights into reproductive performance and to confirm its binding to the estrogen receptor.

MATERIALS AND METHODS

General experimental procedures

^{1}H NMR and ^{13}C NMR measurements were performed in DMSO and CD_3OD at 400 MHz for ^{1}H and 100 MHz for ^{13}C with TMS as internal standard. For HPLC-DAD analysis, the retention time, UV spectra and verbascoside content in EMAT were measured in a HPLC Waters 486 equipment with a coupled UV-VIS diode array detector using a Hibar Purospher Star RP-18 (250×4 mm, 5 μm) column (Merck, Germany) at room temperature. Verbascoside and caffeic acid standards were purchased from Sigma (MO, USA). Thin layer chromatography (TLC) was performed on silica gel GF254 (Merck 5554). Spots were detected under UV light and after spraying with NP/PEG reagent.

Plant Material

The leaves of *Budlleja globosa* were collected in January, 2008 in the Antumapu Campus of the Universidad de Chile, Santiago, Chile; a voucher specimen is kept at the herbarium of the Faculty of Chemical Sciences and Pharmacy (SQF: 22349), Universidad de Chile. The plant was identified by Dr. Carla Delporte.

Extraction and verbascoside isolation

Dry ground leaves of *B. globosa* were sequentially extracted at room temperature with solvents of increasing polarity, according to methodology described by Backhouse *et al.* (2008b).

Air-dried and powdered leaves (1.94 kg) were sequentially extracted at room temperature with n-hexane (H), CH_2Cl_2 (DCM) and MeOH, yielding, after removing the solvents under reduced pressure, 42.2 g of HE (2.1%), 26.3 g of DCME (1.4%) and 163 g of (8.4%) EMAT extracts, respectively.

EMAT (12.4 g) was subjected to CC on silica gel, eluted with solvent mixtures of increasing polarity from 100% CH_2Cl_2 to 100% EtOAc. Fractions eluted with DCM:EtOAc (20:80) and EtOAc 100% yielded verbascoside (72.4 mg), which in the TLC analysis was a light blue spot (NP-PEG reagent); Rf 0.7 (EtOAc:CH_3OH 8:2) was coincident with a standard sample of verbascoside. The presence of verbascoside was confirmed by a) 1H NMR and 13C NMR measurements and b) comparison between the retention time and UV spectrum (obtained between 200 and 400 nm) by HPLC-DAD with a verbascoside standard.

Determination of verbascoside content in EMAT by HPLC-DAD

The solvent system used was formic acid (0.1%):acetonitrile (70:30) at a flow rate of 0.5 mL/min and with UV detection at 365 nm. A simple filtration was performed on the sample using a 0.22-µm PTFE filter before HPLC-DAD analysis. The injection volume was 20 µL with a concentration of EMAT of 1.58 mg/mL dissolved in methanol. This quantitative analysis was performed in triplicate.

A calibration curve was constructed with caffeic acid dissolved in methanol (115, 321, 462, 925 and 1850 ppm) as standard substance under the same conditions mentioned as EMAT ($y = 15966\ x - 416796$, $R^2 = 0.9985$ and F calculated 2.07 < F table 3.71). The verbascoside content was 0.61 mg ± 0.01; its equivalent in caffeic acid was 1.58 mg EMAT (38.6% w/w).

Animals and experimental design

Sprague-Dawley rats were used from the Faculty of Chemistry and Pharmaceutical Sciences animal breeding facilities in the Universidad de Chile. The experimental protocol was approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences. To evaluate the pharmacological effect of EMATst on the estrous cycle of rats, we used fourteen 3-months-old virgin females, with an average weight of 280 g, which were maintained under controlled light and temperature. The animals were divided into three groups: control and experimental groups I and II. To experimental group I, we administered a dose of EMATst that was the mole-equivalent to dose of estradiol valerate (EV) (1x) that produces important changes in estrous cycle (Sotomayor-Zarate et al., 2008). To group II, a dosage 10 times greater (10x) was administered. For the *in vitro* pharmacological assay, rat uterus cytosol was obtained from five female rats that were ovariectomized ten days before being sacrificed according to established procedures (National Toxicology Program, 2002). The uterus of each female was extracted and treated with a standard methodology (National Toxicology Program, 2002). The cytosol fractions obtained (11 mL) were stored in 1-mL fractions at -70º C.

Experimental procedure

In vivo pharmacological analysis

EMATst was administered subcutaneously with a hypodermic syringe on days 0, 2 and 7 of the experimental procedure. All rats were killed after 14 days from the last dose. Group I received a dosage of 0.5 mg/300 g/50 µL ethanol (1x) of the extract; group II received a dosage of 5.1 mg/300 g/50 µL ethanol (10x). The estrous cycle was monitored daily beginning 5 days before the EMATst administration (day 0) using vaginal smear samples. Diestrus was characterized by the presence of leukocytes under microscopic observation and a thin non-vascularized uterus, whereas proestrus (P) (day before ovulation) was characterized by a high density of epithelial cells under microscopic observation; this was associated with a ballooned and highly vascularized uterus. During estrus (E) (day after ovulation), microscopic observation revealed a dense accumulation of squamous cells and a uterus that had rapidly decreased in size but maintained its vascularization. Estrual cycling activity was observed over 14 days.

Animals were killed by decapitation, collecting trunk blood for serum, and the ovaries, adrenal gland and celiac ganglion were removed and immediately frozen at -80 ºC for later analysis. Each uterus was extracted, observed and weighed.

In vitro pharmacological assay

We used a methodology to determine the competitive binding to the ER *in vitro* using the cytosol fraction of rat uterus and radiolabeled 17 β- estradiol (^{3}H-E_2) (National Toxicology Program, 2002). We determined the 50% inhibition of ^{3}H-E_2 binding to the ER (IC_{50}) and the binding percentage of each competitor.

Stock solutions of 17 β- estradiol, EMATst and verbascoside were prepared by diluting the substances in ethanol. A final volume of 500 µL was prepared of each serial dilution using a maximum of 0.25 µL ethanol. The ER assay was performed in round-bottomed 1.7-mL Eppendorf tubes prepared in an ice bath with 50 µL uterine cytosol (50 - 100 µg protein per assay) and 10 µL ^{3}H-E_2 to achieve a concentration of 0.5 – 1.0 nM, plus non-radioactive 17β-estradiol or EMATst or verbascoside to compete for the binding in a final volume of 500 µL. Tubes were incubated at 4 ºC for 18-20 hours with rotation. To separate bound from total, ^{3}H-E_2 tubes were mixed with 250 µL of hydroxyapatite solution prepared according to the National Toxicology Program (2002). After washing ^{3}H-E_2 three times, the substances was eluted with 100% ethanol and measured for radioactivity.

Data analysis

The results were expressed as the average ± SEM, and their significance was determined by analysis of variance (ANOVA), followed by a multiple comparison Turkey test using GraphPad Prism Version 5.0. Statistically significant differences were established using $p \leq 0.05$. The same software was also used to prepare the graphs. The IC_{50} was estimated using the data of the E_2 standard and of each sample analyzed by graphing the percentage of bound ^{3}H-E_2 vs the log molar concentration of the competitor.

RESULTS

The evaluation of the pharmacological effect of EMATst on the estrous cycle of rats

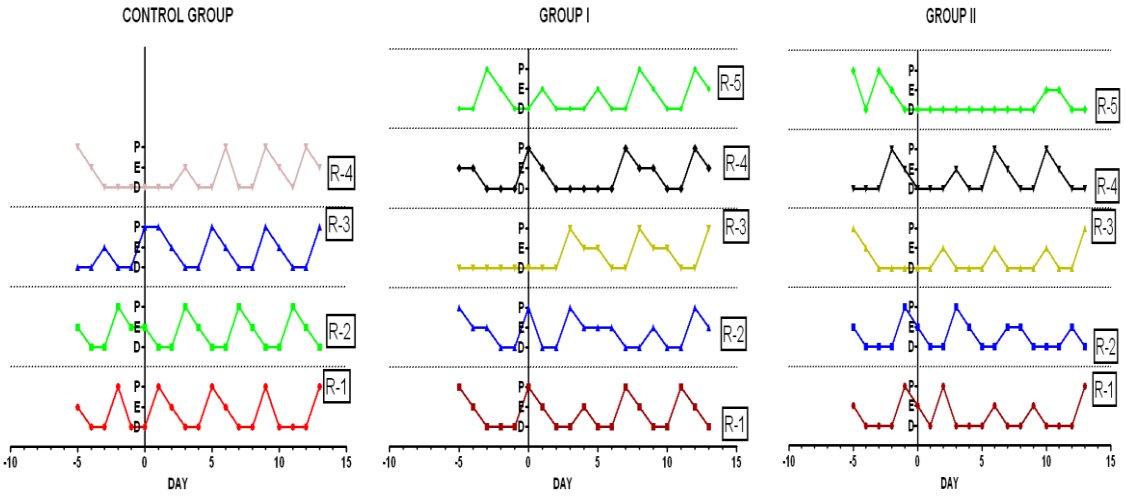
The weight of the animals (300 g) did not change significantly in any of the three groups during the course of the experiment. In the control group, the observed characteristics of the uterus generally coincided with the vaginal smears. In the two experimental groups, however, there were differences in the predicted stage between the gross morphology of the uterus and the vaginal smear. In the group of animals treated with the highest dose of EMATst, a diestrus phase of the cycle was commonly observed in the vaginal lavage observation, but this was contrasted with a clear morphological aspect of proestrus in the uterus of the animals.

Regarding estrous cycling activity (Figure 1), the control group exhibited a normal 4-day estrous cycle, in which P and E lasted one day each and in which D lasted two days. Group I exhibited acyclic estrous cycling activity characterized by alterations in the sequence of the cycle. This difference was clearly demonstrated when a 10x dose of EMATst was used (Figure 1). For example, after administration of EMATst 10x, many rats stopped cycling for the duration of the experiment.

The number of days that each animal was in the different stages of the estrous cycle is shown in Figure 2. Cycles were altered in group I compared with the control group because the percentage of E was greater in group I, whereas P was less than in the control group. A greater alteration was observed in group II because the percentage of D increased compared with the control group, whereas the percentage of P decreased considerably ($p < 0.001$).

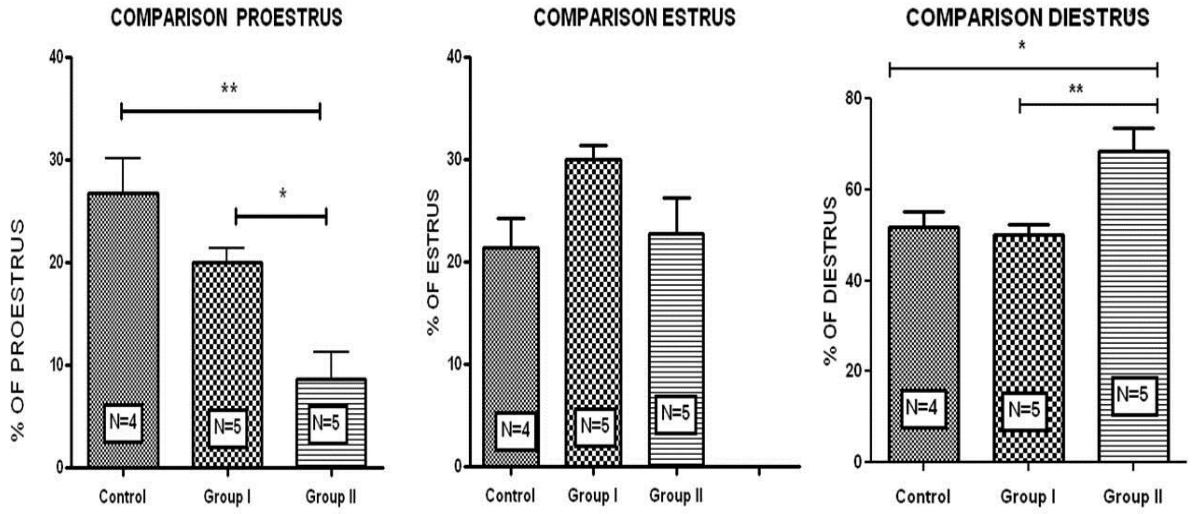
Figure 3 compares the frequency of ovulation, measured as the number of times one P day was followed by one E day, which on physiological grounds is considered to be the number of ovulations. Group 1 exhibited a slight decrease in ovulatory capacity compared with the control group, whereas the ovulatory capacity in group II decreased considerably ($p < 0.001$)**, which suggests an anti-estrogenic effect.

Figure 1



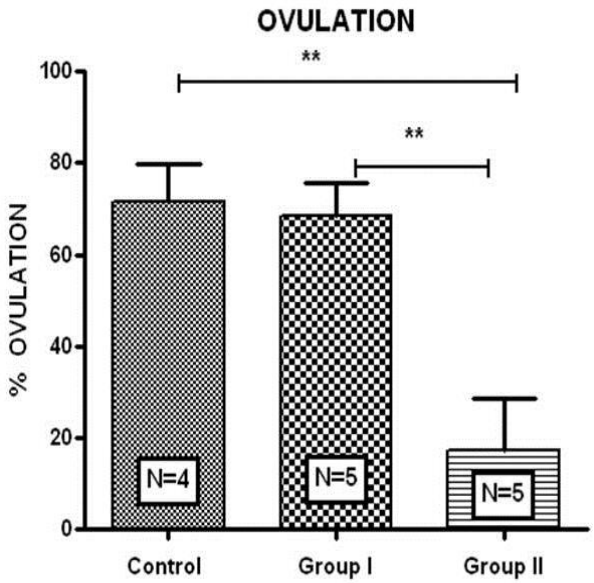
The Eeffect of EMATst on the estrous cycle of Sprague Dawley rats treated with EMATst: groups I (0.5 mg/300 g/50 µL) and II (5.1 mg/300 g/50 µL) compared withto the control group with a normal sequence of proestrus (P), estrus (E) and diestrus (D). A representative examplebehavior of an individual rat's behavior is shown, and the experimental groups are compared withto the control group. The Xx- axis indicates the day of the assay; the y- axis indicates the estrous cycle of each rat. R indicates the number of rats. The behavior is acyclic in group I, whereasile group II exhibitsshows greater activity. Both experimental groups are compared withto the control group.

Figure 2



The Ccomparison of the percentage ofs between days spent in the different stages of the estrous cycle inof rats treated with EMATst. Group I (0.5 mg/300 g/50 µL) and group II (5.1 mg/300 g/50 µL) are compared withto the control group. The bars represent the average percentage for each cycle stage. The Rresults correspond to 5 animals in each group and are expressed as the mean ± SEM. *= p < 0.0 5, vs. control. ** = p < 0.001 vs. control.

Figure 3



The percentage of ovulations of rats treated with EMATst: groups I (5.1 mg/300 g/50 µL) and II (5.1 mg/300 g/50 µL) are, compared withto the control group. To calculate theis percentage of ovulation, the number of times that a proestrus day was followed by an estrus day was considered. The bars represent the average percentage. The R results correspond to 5 animals in each group and are expressed as the mean ± SEM
**** = p < 0.001 vs control.**

In vitro pharmacological assay

Concentration of 17β-estradiol between 10^{-11} and 10^{-7} M competed with ^{3}H-E_2 bound to the receptor fraction in a dose-dependent manner, but the same doses of EMATst did not.. Higher concentrations of verbascoside and EMATst (10^{-3} to 10^{-4} M) in a range 1000 times greater were able to compete with 17β-estradiol. After calculating the percentage of binding to the receptor, expressed in pmol bound estradiol/mg protein (which represents the quantity of E_2 bound to the receptor that has not been displaced by the competitor), we found that both compounds exhibited a displacement of ^{3}H-E_2 (Figure 4). For verbascoside and EMATst, the phenomenon was produced at concentrations 1000 times greater than those used for cold 17β-estradiol. There was also a maximum concentration of verbascoside and EMATst at which the ^{3}H-E_2 displacements was produced. Interestingly, at higher concentrations, the phenomenon was reversed.

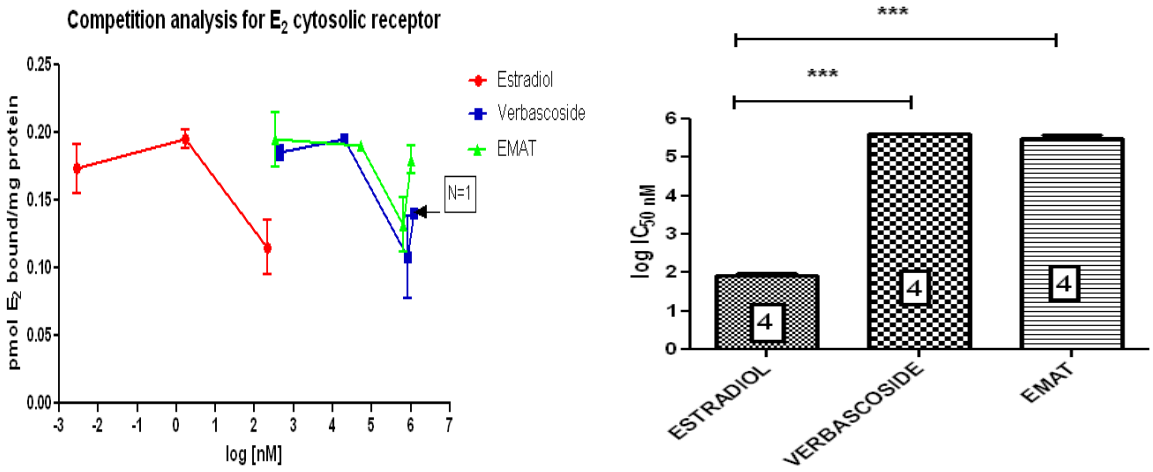
The values of percent binding in Figure 4 were used to estimate the IC_{50} value of displacement for each competitor in each of the experiments performed. The graph of the values of IC_{50} for each competitor with respect to the concentration indicates a significant increase in the IC_{50} value both for verbascoside and EMATst compared with cold estradiol. Therefore, there was displacement of radiolabeled estradiol and thus binding of EMATst and verbascoside to the estrogen receptor, but this activity was in concentrations much greater than that for 17β estradiol.

DISCUSSION

We found evidence that the exposure of adult rats to an EMATst of *B. globosa* leaves disrupts estrous cycling activity, most likely in a dose-dependent manner and through its binding to the estrogen receptor. Although the lower dose of the EMATst used was equimolar to that of 17β-estradiol (with a concentration of verbascoside of 10^{-6} M), it was not able to compete with 17β estradiol in our binding study but was able to slightly modify the estrous cycling activity of the rats after chronic exposure for 14 days. Low-dose extract exposure produced an increase in the number of estrus cycles observed in the rats, suggesting that, at this dose, the extract could acts as an endogenous estrogen to accelerate the transition between proestrus and estrus. This effect was clearly observed at the level of vaginal cytology, as the histological appearance changed from epithelial to cornified cells. The fact that a 10-fold increase in the dose of EMATst (with a concentration in verbascoside of 10^{-5} M) produced a clear change in estrous cycling activity and in the ovulatory performance gives further support to the possibility of a direct effect of the extract as a plant-derived estrogen. Significant evidence suggests that high doses of estradiol produced anovulation in rats and that chronic exposure acts at the ovary level as well as in the reproductive hypothalamus to block GnRH-induced gonadotropin surge (Barría *et al.*, 1993; Weiland *et al.*, 1997; Herbison, 1998; Schuiling *et al.*, 1999; Tsai and Legan, 2002; Petersen *et al.*, 2003; Kasturi *et al.*, 2009). If this is the case, we can understand why chronic treatment with a 10x dose of the EMATst not only affected the estrous cycling activity by increasing

the length of time in which rats stayed in diestrus, thereby blocking the preovulatory surge of gonadotropins, but also why this effect was sufficient to block the ovulatory performance of the rat. Estradiol could act locally at the ovary and also centrally at the hypothalamus. We could likely observe both effects by chronic exposure to EMATst. The consequences of the disruption of ovulation by chronic exposure to an estrogen-disrupting chemical seems similar to the one we have previously observed after two months of exposure to a single estradiol dose in adults rats. The rats developed an acyclic condition, an increased sympathetic nerve activity and polycystic ovary morphology (Sotomayor-Zarate *et al.*, 2008). Likely, we would have observed a similar effect with EMATst, a possibility currently under study. The results obtained with the two doses suggest a dose-dependent estrogenic effect.

Figure 4



Competition analysis for the E2 cytosolic receptor: The displacement of 3H-E2 by 17β-estradiol, verbascoside and EMATst is presentedshown on the left. The values of IC50 are shown on the right. The Rresults correspond to 6 animals in each group and are expressed as the mean ± SEM. *** = p < 0.05 vs. control.

The possibility of an estrogenic effect from EMATst was strongly supported with the binding experiments. Rat uterus cytosol has been amply used to study binding to estradiol receptors (Baker *et al.*, 1999; Blair *et al.*, 2000; Fang *et al.*, 2001). Rat uterus expresses principally the alpha subtype of the estrogen receptor, and this receptor is up-regulated by estradiol (Wang *et al.*, 1999). The fact that EMATst competed for ER suggests that it acts through the alpha-estradiol receptor. This is a very interesting possibility because it could explain why the lower dose of EMATst increased the number of days in which cornified cells appeared in the vaginal lavages, indicating the local effect of EMATst in the vagina through alpha receptors, the predominant receptor in this tissue (Wang *et al.*, 2000). The increased dose exhibited a more pronounced effect in the cycling activity and at the reproductive hypothalamus, as was clearly observed in the present study. Notably, the pivotal behavior of the highest dose of EMATst and verbascoside in the binding to the cytosolic fraction of rat uterus was important. The fact that higher doses not only failed to displace the estradiol but also increased the binding to the receptor suggests the up-regulation of the alpha estrogen receptors, as has been previously found in ovariectomized rats (which have low levels of estradiol) treated with estradiol (Nephew *et al.*, 2000; Wanda *et al.*, 2007).

As we previously demonstrated, EMATs exhibit anti-inflammatory and analgesic effects (Backhouse *et al.*, 2008a; Backhouse *et al.*, 2008b). These data are well correlated with the results obtained in this work and those obtained by Zamorano *et al.* (1994), who demonstrated that vascular reactivity and vascular prostanoids synthesis are influenced by the hormonal changes occurring during the estrous cycle of normal female rats and could thus be involved in

the effect on estrous cycling activity found in the present work.

One study correlated the availability of verbascoside in plasma after oral administration to laboratory rats (Funes *et al.*, 2009). This study concluded that verbascoside is absorbed rapidly through the gastrointestinal tract, reaching a maximum concentration in the first 20 minutes. Although the availability of verbascoside by oral administration is quite low, it is probable that other metabolites present in the extract, such as flavones and other metabolites derived from the intestinal biodegradation of verbascoside, caffeic acid and other undetermined compounds may contribute to the *in vivo* biological activity. The concentration of bioavailable verbascoside is in the micromolar range, which allows us to suggest that the dosage administered to laboratory rats subcutaneously in the study of the alteration of the estrous cycle is equivalent to the concentrations used in the binding assay.

CONCLUSIONS

The result of the pharmacological assay on the estrous cycle of the laboratory rats may be attributed to an antiestrogenic effect of ematst at the highest dose evaluated.

The results of the binding assay to the estrogenic receptor of ematst and verbascoside strongly suggests that both compounds bind to the estrogenic receptor in concentrations 1000 greater than that of 17β-estradiol.

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