

Original Article

Casticin, a flavonoid isolated from *Vitex rotundifolia*, inhibits prolactin release *in vivo* and *in vitro*

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Aim: To investigate the anti-hyperprolactinemia activity of casticin, a flavonoid isolated from *Vitex rotundifolia*, and elucidate its molecular mechanism.

Methods: Hyperprolactinemia (MIHP) was induced by administration of metoclopramide dihydrochloride (50 mg/kg, tid, ip, for 10 d) in SD rats and the primary pituitary cells were prepared from the pituitary glands of the SD rats. Prolactin concentrations were measured using a radioimmunoassay. Cell viability was measured using an MTT assay. The mRNA expression of estrogen receptor alpha and beta in rat pituitary cells was measured using semi-quantitative RT-PCR analysis.

Results: The level of serum prolactin in the MIHP model group was 2.1 fold higher than that in the untreated control group ($P < 0.01$). Casticin (10, 20, and 40 mg/kg, ip, for 7 d) reduced serum prolactin levels by 33.9%, 54.3%, and 64.7%, respectively ($P < 0.01$). The positive control drug bromocriptine 1 mg/kg decreased the serum prolactin concentration in MIHP rats by 44.9%. 17β -Estradiol (E_2) significantly increased the proliferation of pituitary cells and casticin (1 and 10 $\mu\text{mol/L}$) markedly inhibited E_2 -induced pituitary cell proliferation by 27.7% and 42.1%, respectively. Stimulation of pituitary cells with E_2 increased prolactin secretion into the cell culture supernatants, and casticin (0.1, 1, and 10 $\mu\text{mol/L}$) significantly inhibited the prolactin release stimulated by E_2 in a concentration-dependent manner. Casticin (1 and 10 $\mu\text{mol/L}$) significantly inhibited $ER\alpha$ mRNA expression in pituitary cells stimulated with E_2 ($P < 0.01$) but increased $ER\beta$ mRNA expression at a concentration of 10 $\mu\text{mol/L}$ ($P < 0.01$). However, casticin had no effects on proliferation and prolactin release of the unstimulated primary pituitary cells *in vitro*.

Conclusion: Casticin inhibited the release of prolactin from pituitary cells of SD rats stimulated with E_2 *in vivo* and *in vitro*. These effects might be related with inhibiting the $ER\alpha$ mRNA expression and increasing the $ER\beta$ mRNA expression.

Keywords: casticin; premenstrual syndrome; prolactin; estrogen; estrogen receptors

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Introduction

Premenstrual syndrome (PMS) involves physical, psychological and behavioral symptoms and occurs during the luteal phase of the menstrual cycle and disappears at the onset of menstruation^[1, 2]. At least one premenstrual symptom occurs in 95% of women of reproductive age^[3]. The main treatment strategies involve ovulation suppression or targeting the central nervous system components that are thought to be involved in the etiology of PMS^[4]. As demonstrated by several clinical studies, PMS patients hypersecrete prolactin in response to the pressures of daily life, which leads to an unphysiological stimulation of the mammary gland, thereby causing mastodynia^[5]. The release of prolactin is associated with a variety of factors such as estrogens, thyroid-releasing factor, vasoactive intestinal peptides, opioids, surgery, illness,

and mental or physical stress^[6]. In the pituitary, estrogen promotes the proliferation of several pituitary cell types and the production of prolactin^[7]. It has been shown that prolactin synthesis is increased by 17β -estradiol (E_2) through direct binding to the promoter region of the prolactin gene^[8]. Studies suggest that changes in follicular or periovulatory estradiol secretion may be critical to the onset of symptoms of PMS^[9, 10]. It has been widely accepted that the action of E_2 is mediated by the nuclear estrogen receptors (ER), $ER\alpha$ and $ER\beta$ ^[11, 12], which function as ligand-dependent transcription factors and regulate the transcription of target genes. $ER\alpha$ plays the major role in estrogen responsiveness in the female rat pituitary^[13] due to it having a higher affinity to estrogen than $ER\beta$ does. However, $ER\beta$ decreases the sensitivity of $ER\alpha$ to estrogen, thereby acting as a physiological regulator of the effects of $ER\alpha$ ^[14].

Vitex rotundifolia, which belongs to the same genus as *V. agnus-castus*, belongs to the family Verbenaceae. *V. agnus-castus* has been proved to be effective in treating patients with PMS^[15]. In our previous study, we found that the flavone-

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enriched fraction extracted from *V rotundifolia* decreases serum prolactin levels in metoclopramide dihydrochloride-induced hyperprolactinemia (MIHP) mice, and casticin is the active component of this extract^[2]. In order to understand the inhibitory effects of casticin on prolactin release in rats, we used both *in vivo* and *in vitro* models to investigate the effects of casticin on prolactin release and the mechanisms of regulation of estrogen and estrogen receptors.

Materials and methods

Materials

Casticin (3',5-dihydroxy-3,4',6,7-tetramethoxyflavone, Figure 1) was isolated from the methanol extract of fruits of *Vitex rotundifolia* L, and its chemical structure was identified by spectrum analysis. Phenol red-free Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (USA). Bovine serum albumin (BSA) was purchased from Sigma (USA). Bromocriptine was purchased from Novartis Pharma SPA (Italy). Metoclopramide dihydrochloride was obtained from Harvest Pharmaceutical CO. The other chemicals were domestic AR grade.

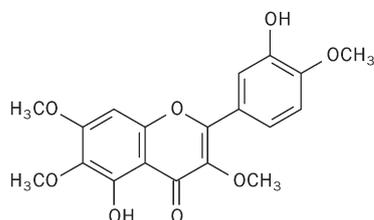


Figure 1. Chemical structure of casticin.

Animal model and drug treatment

Sixty adult female Sprague Dawley (SD) rats (180–220 g) were purchased from the Experimental Animal Center of the Second Military Medical University. These animals had free access to food and water and were housed in a room with a 12-h light/dark cycle. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Ethics Committee of the Second Military Medical University.

Animals were grouped randomly into six groups ($n=10$). Ten rats were not treated and served as the normal control. Hyperprolactinemia was induced in 50 rats using metoclopramide dihydrochloride (50 mg/kg, tid \times 10 d, ip)^[16]. The hyperprolactinemia SD rats were then treated daily with saline (vehicle control), bromocriptine (1 mg/kg) or different doses of casticin (10, 20, and 40 mg/kg, ip) for 1 week. Blood samples were collected from the orbit vein without anesthesia. The sera were obtained by centrifugation at 2500 \times g for 15 min and were stored at -20 °C until the prolactin assay was performed^[16,17].

Pituitary cell proliferation *in vitro*

Primary pituitary cells were prepared from the pituitary glands of the SD rats as described^[18]. In brief, the pituitar-

ies from five rats were cut into small pieces and incubated at 37 °C with 2.5% trypsin for 15 min, then treated with 2 mg/L DNase and trypsin inhibitor. Cells were dispersed, seeded into 96-well culture plates at a density of 2 \times 10⁵ cells/well and cultured in phenol red-free DMEM supplemented with 10% fetal bovine serum, 0.1 mmol/L nonessential amino acids, 25 μ mol/L dexamethasone, 5 g/L insulin, 1 μ g/L fibroblast growth factor, 100000 U/L penicillin, 100 mg/L streptomycin and 60 pmol/L triiodothyronine for 24 h at 37 °C in a humidified atmosphere of 5% CO₂.

To determine the effect of casticin on cell proliferation, the culture medium was replaced with the above phenol red-free conditioned DMEM supplemented as above except for the use of 0.1% BSA instead of 10% fetal bovine serum. Casticin was then added, and the cells were incubated for an additional 72 h. The numbers of metabolically active mitochondria and viable cells were determined colorimetrically at 550 nm using an ELx800 universal microplate reader (Bio-Tek) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described. To analyze the effect of casticin on E₂-driven proliferation, cells were pretreated with E₂ (0.01 μ mol/L) for 4 h and then thoroughly washed with phosphate buffered saline. Casticin was then added, and the cells were incubated for an additional 72 h.

Prolactin release from pituitary cells *in vitro*

To test the effect of casticin on E₂-induced prolactin release, primary pituitary cells were seeded into 96-well culture plates at a density of 2 \times 10⁵ cells/well, pretreated with E₂ (0.01 μ mol/L) for 4 h, then treated with casticin at final concentrations of 0.01, 0.1, 1, and 10 μ mol/L for 48 h. The supernatants were collected and stored at -20 °C for measurement of the concentration of prolactin.

Prolactin radioimmunoassay

The prolactin concentration was measured using a double-antibody radioimmunoassay method as previously described^[17]. The assay used human prolactin as a standard, and anti-human prolactin antiserum from rabbits (UCB-Bioproducts, Brussels, Belgium) that interacted with the ¹²⁵I-labeled human prolactin (Dupont New England Nuclear, Boston, MA, USA) served as a tracer. A sheep anti-rabbit gamma-globulin (UCB-Bioproducts) was used as the second antibody. The immunotitration curve for common rat serum prolactin was found to be parallel to the human prolactin standard curve^[17]. The assay sensitivity was 0.5 μ g/L, and the inter- and intra-assay coefficients of variation were 10% and 4%, respectively.

ER α and ER β mRNA expression analysis using RT-PCR

Pituitary cells were seeded into 6-well tissue culture plates at a density of 2 \times 10⁶ cells/well. Cells were primed with 0.01 μ mol/L E₂ for 4 h at 37 °C and then treated with casticin at final concentrations of 0.01, 0.1, 1, and 10 μ mol/L for 48 h. Total RNA was isolated using a TRIzol reagent kit and was reverse transcribed to form cDNA with a Revert Ace- α -R kit (TOYOBO, Osaka, Japan) according to the manufacturer's rec-

ommendations. The primer sequences were as follows: ER α sense primer: 5'-AATTCTGACAATCGACGCCAG-3'; ER α antisense primer: 5'-GTGCTTCAACATTCTCCCTCCTC-3'; ER β sense primer: 5'-AAAGCCAAGAGAAACGGTG-GGCAT-3'; ER β antisense primer: 5'-GCCAATCATGTG-CACCAGTTCCTT-3'; β -actin sense primer: 5'-TTGTAACCAACTGGGACGATATGG-3'; and β -actin antisense primer: 5'-GATCTTGATCTTCATGGTTGCTAGG-3'. The PCR reaction mixture, in a total volume of 25 μ L, contained 2.5 μ L 10 \times buffer, 2 μ L dNTPs (0.2 mmol/L each), 2 μ L forward primer (2.5 mmol/L), 2 μ L reverse primer (2.5 mmol/L), 0.5 μ L rTaq polymerase, 2 μ L cDNA template and 14 μ L dH₂O. The PCR conditions used for ER α and ER β were as follows: initial denaturation at 94 $^{\circ}$ C for 4 min; 32-33 cycles of 94 $^{\circ}$ C for 45 s, 63 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 60 s; and a final extension at 72 $^{\circ}$ C for 7 min. The expression of β -actin was used as an internal standard. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide staining. The densities of the bands were determined using a bio-image analyzer (Bio-Rad), and quantification of the products was performed using Quantity One version 4.2.2 software (Bio-Rad).

Statistical analysis

Data were evaluated for statistical significance using the Student's *t*-test. A value of $P < 0.05$ was considered significant. All data were expressed as the mean \pm SD of triplicate independent experiments, and representative experiments are shown.

Results

Anti-hyperprolactinemia activity *in vivo*

The animals were either left untreated or were given metoclopramide dihydrochloride (50 mg/kg, tid \times 10 d, ip) to induce hyperprolactinemia (MIHP). The level of serum prolactin in the MIHP model group was 2.1 fold higher than in the untreated control group ($P < 0.01$). The elevated serum prolactin levels were reduced by 33.9%, 54.3%, and 64.7% with casticin at doses of 10, 20, and 40 mg/kg, respectively, and by 44.9% with bromocriptine at a dose of 1 mg/kg (Figure 2).

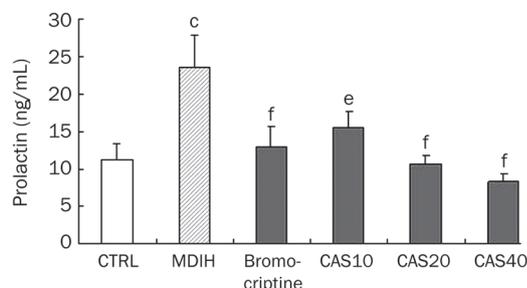


Figure 2. Effects of casticin on MDIH rats. CAS10, CAS20, and CAS40 represent the prolactin levels, expressed as the percentage of production by the controls, of pituitary cells treated with casticin at doses of 10, 20, and 40 mg/kg, respectively. The data are represented as the mean \pm SD ($n=10$). ^c $P < 0.01$ vs CTRL group, ^e $P < 0.05$, ^f $P < 0.01$ vs MDIH group.

Effects on pituitary cell proliferation

To assess whether casticin affects pituitary cell proliferation, we first analyzed the effect of casticin on unstimulated pituitary cells. Casticin did not show any effect on the proliferation of pituitary cells at the tested concentrations. However, E₂ significantly increased the proliferation of pituitary cells and casticin (1 and 10 μ mol/L) markedly inhibited E₂-driven pituitary cell proliferation by 27.7% and 42.1%, respectively. These results indicate that casticin could counteract the stimulatory effects of E₂ on pituitary cell proliferation (Figure 3).

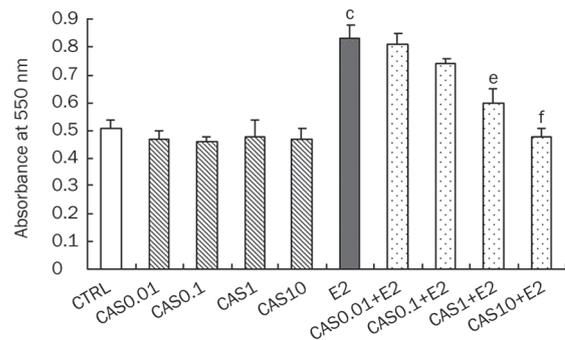


Figure 3. Effects of casticin on pituitary cell proliferation. CAS0.01, CAS0.1, CAS1, and CAS10 represents the prolactin levels, expressed as the percentage of production by the controls, of pituitary cells treated with casticin for 48 h at doses of 0.01, 0.1, 1, and 10 μ mol/L, respectively; E₂ represents the prolactin level, expressed as the percentage of production by the controls, of pituitary cells stimulated with E₂ (0.01 μ mol/L) for 4 h; E₂+CAS0.01, E₂+CAS0.1, E₂+CAS1, and E₂+CAS10 represent prolactin levels, expressed as the percentage of production by the controls, of pituitary cells stimulated with E₂ (0.01 μ mol/L) for 4 h and then treated with casticin for 48 h at doses of 0.01, 0.1, 1, and 10 μ mol/L, respectively. The data are represented as the mean \pm SD ($n=10$). ^c $P < 0.01$ vs CTRL group, ^e $P < 0.05$, ^f $P < 0.01$ vs E₂-treated group.

Effect on prolactin release from pituitary cells *in vitro*

Because casticin decreased the serum levels of prolactin in the MIHP model, we investigated whether this decrease was due to a direct effect of casticin on the pituitary cells. Casticin did not decrease prolactin release from unstimulated pituitary cells at the tested concentrations. We then explored the effect of casticin on prolactin secretion by the pituitary cells induced by E₂. Stimulation of pituitary cells with E₂ resulted in an increase in prolactin secretion into the cell culture supernatants, and casticin (0.1, 1, and 10 μ mol/L) significantly inhibited prolactin release from E₂-stimulated pituitary cells in a concentration-dependent manner (Figure 4). These results indicate that casticin attenuated the stimulatory effects of E₂ and regulated prolactin release from pituitary cells.

ER α and ER β mRNA expression

To further understand the underlying mechanism of casticin's effects on prolactin secretion, we evaluated the effects of casticin on the expression of ER genes in rat pituitary cells. Stimulation of pituitary cells with E₂ resulted in an increase in

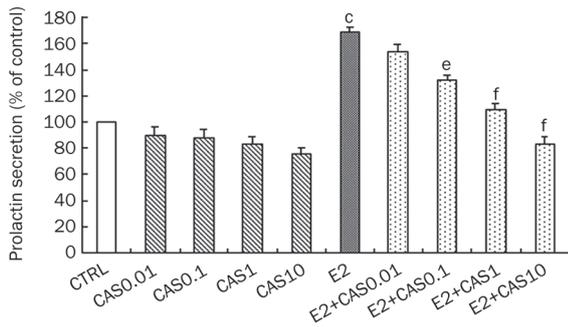


Figure 4. Effects of casticin on prolactin secretion of normal and E₂-treated pituitary cells. CASO.01, CASO.1, CAS1, and CAS10 represent the prolactin levels, expressed as the percentage of production by the controls, of pituitary cells treated with casticin for 48 h at doses of 0.01, 0.1, 1, and 10 μmol/L, respectively; E₂ represents the prolactin level, expressed as the percentage of production by the controls, of pituitary cells stimulated with E₂ (0.01 μmol/L) for 4 h; E₂+CASO.01, E₂+CASO.1, E₂+CAS1, and E₂+CAS10 represents the prolactin levels, expressed as the percentage of production by the controls, of pituitary cells stimulated with E₂ (0.01 μmol/L) for 4 h and then treated with casticin for 48 h at doses of 0.01, 0.1, 1, and 10 μmol/L, respectively. The data are represented as the mean±SD (n=10). ^cP<0.01 vs CTRL group, ^eP<0.05, ^fP<0.01 vs E₂-treated group.

ERα and ERβ mRNA expression. Casticin (1 and 10 μmol/L) significantly inhibited ERα mRNA expression in pituitary cells stimulated with E₂ (P<0.01) but increased ERβ mRNA expression at a concentration of 10 μmol/L (P<0.01, Figure 5).

Discussion

It has been reported that women with very severe PMS have higher mid-cycle estrogen levels^[10] and that the symptom severity is strongly associated with high luteal-phase estradiol levels^[19]. PMS patients experience significant symptom relief after administration of the gonadotropin-releasing hormone agonist leuprolide; in contrast, the addition of either estrogen or progesterone results in the recurrence of symptoms^[9]. Estrogenic stimulation of prolactin secretion is the primary

cause of higher serum prolactin concentrations in women^[20, 21]. In our study, casticin significantly inhibited E₂-stimulated pituitary cell proliferation and prolactin production *in vitro*, but casticin did not affect either the proliferation or the prolactin production of normal pituitary cells. These results suggest that casticin decreased prolactin production by counteracting the stimulatory effects of estrogen.

Other investigations have shown that the actions of estrogen on pituitary cells during the estrous cycle are probably mediated by ERα, and prolactin can stimulate estrogen receptor expression and increase the number of estradiol-binding sites^[22]. In our study, casticin inhibited the mRNA expression of ERα in pituitary cells stimulated with E₂. Hence, the prolactin-reducing effect of casticin might be due to its inhibition of ERα expression in pituitary cells. Further, the stimulatory effect of E₂ decreased in the casticin-treated pituitary cells and led to a reduction in the proliferation and prolactin production of these pituitary cells. In addition, inhibition of pituitary cell proliferation by casticin might also contribute to the observed decrease of ER transcription and the reduced prolactin production.

Many premenstrual syndromes, particularly premenstrual mastalgia (mastodynia), are associated with latent hyperprolactinemia^[23–25]. In addition, casticin has been demonstrated to reduce abnormally high serum prolactin levels *in vivo* (Figure 2). The level of prolactin *in vivo* is controlled not only by prolactin-releasing factor (PRF) and prolactin-inhibiting factors (PIF) such as dopamine but also by several possible stimulatory pathways that respond to serotonin reuptake, opiates, cocaine, and antihypertensive medications^[26]. In previous studies on extracts of *V. agnus-castus* using the corpus striatum membrane dopamine (DA) receptor binding assay *in vitro*, however, diterpenes (not casticin) were reported to be the active constituents, acting as DA agonists and inhibiting the release of prolactin^[15, 24]. Therefore, the prolactin-reducing effect of casticin described in the hyperprolactinemia model *in vivo* may be due to other compounds counteracting the stimulatory effects of estrogen on pituitary cells. Unfortunately, we did not provide *in vivo* evidence for E₂ involvement in the

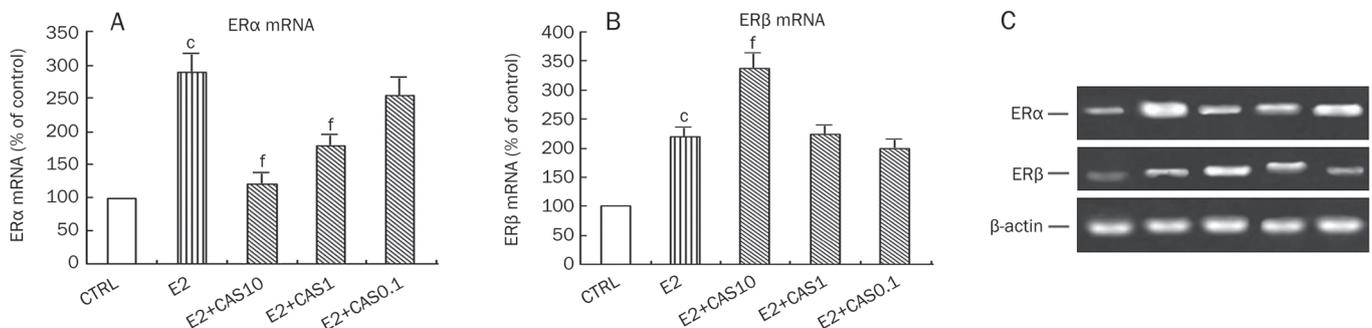


Figure 5. Effects of casticin on the mRNA expression of ERα and ERβ in pituitary cells stimulated with E₂. Pituitary cells were stimulated with E₂ (0.01 μmol/L) for 4 h and then treated with casticin for 48 h. E₂+CASO.1, E₂+CAS1, and E₂+CAS10 represent the expression of ERα or ERβ mRNA, expressed as the percentage of expression by the controls, in pituitary cells treated with casticin at doses of 0.1, 1, and 10 μmol/L, respectively. ^cP<0.01 vs CTRL group, ^fP<0.01 vs E₂-treated group.

mechanism of action of casticin. Further studies should focus on these effects of casticin. Despite this limitation, our *in vitro* data provide deeper insight into the mechanisms underlying casticin-reduced prolactin production. In summary, in the fruits of *Vitex rotundifolia* and *V. agnus-castus*, diterpenes inhibit the release of prolactin by acting as DA agonists, and the current study indicated that casticin counteracted the stimulatory effects of estrogen to reduce the production of prolactin.

In this study, we observed the effects of casticin on the release of prolactin *in vivo* and *in vitro*. Casticin inhibited the release of prolactin from pituitary cells stimulated with E₂. These results further support the hypothesis that casticin might be the active constituent in *Vitex rotundifolia* extracts. This plant could be the source of an efficient alternative phytotherapeutic drug in the treatment of PMS.

Acknowledgements

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Author contribution

Lu-ping QIN designed research; Qi YE performed research; Cheng-jian ZHENG and Yang WANG contributed new analytical tools and reagents; Qiao-yan ZHANG analyzed data; Qi YE wrote the paper.

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