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Evaluation of the estrogenic activity of the constituents in the fruits of *Vitex rotundifolia* L. for the potential treatment of premenstrual syndrome

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Abstract

The ethanol extract of the fruits of *Vitex rotundifolia* (VRE) and its four major compounds (casticin, luteolin, rotundifuran and agnuside) were tested for their estrogen-like activity by using the modified cell proliferation assay (E-SCREEN assessment system), as well as the estrogen receptor (ER_α), estrogen receptor-regulated progesterone receptor and pS2 mRNA expression in MCF-7 cells. The results showed that only agnuside and rotundifuran could stimulate the proliferation of MCF-7 cells. These actions were dose dependent (range from 100 nM to 10 μM) and could be significantly inhibited by the specific estrogen receptor antagonist ICI 182,780. The estrogen receptor ER_α and the estrogen receptor-regulated progesterone receptor and pS2 mRNA levels were increased by treatment with rotundifuran and agnuside within 24 h, and the effects could be reversed by ICI 182,780. The standardization of the extract and constituents were carried out by means of a high-performance liquid chromatography-fingerprint. It was concluded that VRE and its compounds showed estrogen-like activity and that the estrogenic effects of rotundifuran and agnuside were mediated by the estrogen inducible gene, which may be useful in regulating the hormone levels to treat related diseases. However, further studies are required to assess the physiological significance of VRE in animals and man.

Introduction

Estrogen plays an important role in the growth, differentiation and function of many targets including the female and male reproductive system. Many diseases are estrogen-related, such as premenstrual syndrome (PMS), menopausal syndrome, arteriosclerosis, heart disease, and cancer (Ciocca & Roig 1995; Ososki & Kennelly 2003). The fruit of *Vitex agnus-castus* (chaste-berry) has been traditionally used in Europe for the relief of PMS and menopausal symptoms (Hobbs 1996). As part of our continuing search for anti-PMS and estrogenic activity from medicinal plants we have investigated *Vitex rotundifolia* L., which belongs to the same genus as *V. agnus-castus*, and is also a member of the Verbenaceae, a taxonomically broad family which includes approximately 250 species of *Vitex* in the world (Wu et al 1994). The main difference between *V. rotundifolia* and *V. agnus-castus* is that *V. rotundifolia* is found growing in coastal habitats, hence its implied English name—beach vitex. The fruits of *V. rotundifolia* Viticis Fructus (Manjingzi in the *Pharmacopoeia of the People's Republic of China* (Zheng & Li 2000)) has been used as a folk medicine for headaches, colds, migraine, eye pain, asthma, chronic bronchitis, and gastrointestinal infections such as bacterial dysentery and diarrhoea due to gastroenteritis. The main constituents of the fruits of *V. rotundifolia* are flavonoids, terpenoids and glucosides, which are similar to the components of *V. agnus castus* (Asaka et al 1973; Kondo et al 1986; Zhang et al 1995; Ono et al 2002). We found (unpublished data) that the extract of the fruits of *V. rotundifolia* could reduce peripheral analgesia and the level of prolactin in the serum by use of the acetic acid-induced constriction test and metoclopramide dihydrochloride-induced hyperprolactinaemia in mice.

The fruits of *V. agnus castu*s (VAC) contain a number of diterpenes, which can be bound to the recombinant DA_2-receptor protein and suppress prolactin release from cultivated

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lactotrophs, improving premenstrual mastodynia and possibly other symptoms of premenstrual syndrome. In addition to this dopaminergic principle, there is recent evidence that VAC appears to contain estrogen-like compounds i.e. phytoestrogens (Jarry et al 2000, 2006; Wuttke et al 2003; Liu et al 2004). PMS is a common endocrine (hormonal) disorder characterized by the appearance of physical and/or psychological symptoms specifically before the onset of menstruation. The aetiology of PMS is still unclear, but sex steroids produced by the corpus luteum of the ovary are thought to provoke symptoms, and the therapeutic modality depends on the kind of hormonal disturbances and involves drugs inhibiting the secretion of prolactin, re-uptake of serotonin, or inhibition of ovulation (Mortola 1998; Milewicz & Jedrzejuk 2006). In traditional Chinese medicine, Fructus Viticis is often used to relieve aches, bloating and other symptoms relating to menstruation (Chen 1998; Wu 2000; Wang 2001). However, until now, its estrogen-like activity and its relation to PMS has not been reported.

In this study, we have evaluated the estrogen-like activity of the ethanol extract of the fruits of *Vitex rotundifolia* (VRE) and its major components, casticin and luteolin (flavonoids), rotundifuran (diterpene) and agnuside (glucoside). To achieve this we measured the relative proliferative effects (RPE) and estrogen receptor (ER_α), progesterone receptor (PR), and pS2 mRNA expression of estrogen receptor (ER)-positive MCF-7 cells in-vitro to elucidate their ability to regulate the level of estrogen, which may be useful in anti-PMS treatments.

Material and Methods

Plant material and compounds

Vitex roundifolia L. was collected at a lakefront in Xinjian, Jiangxi Province, in China (N: 28°25.406, E: 115°48.605) and authenticated by Professor H.-C. Zheng, Second Military Medical University. The voucher specimens of these plants were deposited at the Herbarium of the Department of Pharmacognosy, Second Military Medical University, Shanghai, PR China.

The extraction of the plant materials, the isolation, and the purification of test substances were carried out as described by Xin et al (2006). The powdered fruits of *V. rotundifolia* L. (2900 g) were extracted with 60% EtOH in a percolator and evaporated under vacuum to obtain the VRE. The weight/weight yield in terms of crude medicinal materials was 12%. The extract was then suspended in water and partitioned with dichloromethane, ethyl acetate and aqua-saturated n-butanol. Each fraction was evaporated under vacuum to yield the residues of dichloromethane fraction 61.2 g (2.1%), ethyl acetate fraction 59.5 g (2.1%), and n-butanol fraction 400 g (13.8%). The dichloromethane fraction (60 g) was separated on silica gel column chromatography by sequential elution with petroleum ether/ethyl acetate (25:1–1:5) to obtain subfractions. The subfractions were purified by repeated silica gel column and Sephadex LH-20 chromatography to obtain casticin, luteolin and rotundifuran. The ethyl acetate fraction (50 g) was separated on silica gel column chromatography by sequential elution with petroleum ether/ethyl acetate (30:1–1:5), and purified by repeated silica gel column and Sephadex LH-20 chromatography to obtain agnuside. The structures were identified by a combination of spectral methods (UV, IR, MS and NMR), see Figure 1. All four substances obtained had a purity of over 87%.

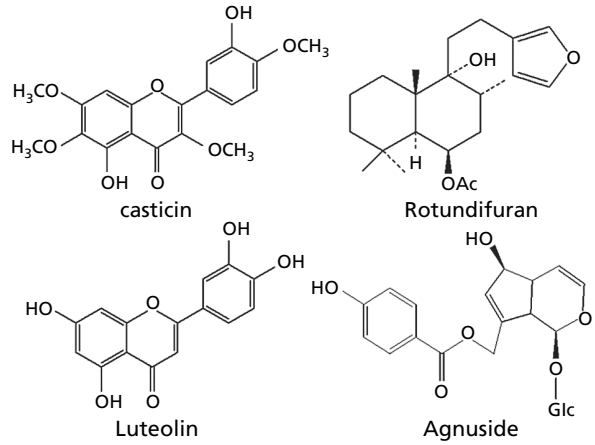

Figure 1 Chemical structures of the related compounds.

VRE high-performance liquid chromatography (HPLC)-fingerprint

To ensure the quality of the extract of fruits of *V. rotundifolia* L, after thermal treatment, VRE was dissolved with solvent (methanol: H_2O=1:1) and filtered through a membrane filter (0.45 μm, Alltech, Germany). Suitable dilutions were prepared with each sample for HPLC analysis. Instrument: Agilent 1100 HPLC (Palo Alto, CA) including a quaternary pump, vacuum degasser, thermostatic column compartment and a diode array detector. Zorbax Extand–C18 4-Pack (12.5 mm×4.6 mm i.d., 5 μm) guard column (Columbia, MD) and an Agilent Zorbax SB-C18 column (250 mm×4.6 mm i.d., 3.5 μm) were maintained at 32°C for analysis. Mobile phase: A, H_2O (0.2% methanoic acid), B, CH_3CN; gradient: 10–20% B linear in 10 min; isocratic 20% B linear in 10 min; 20–40% B linear in 20 min; 40–90% B linear in 25 min; flow rate: 0.8 mL min^{-1}; detection wavelength: 300 nm; injection volume: 20 μL.

Cell culture

MCF-7 cells were provided by the Institute of Biochemistry and Cell Biology. Estrogen receptor-positive human breast adenocarcinoma MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin), supplemented with 10% fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2 and the medium was renewed 2–3 times per week.

Charcoal–dextran stripped human serum preparation

To minimize the estrogenic activity of serum, steroid hormones were stripped from pooled human serum by treatment

with charcoal and dextran (purchased from Gibco BRL). The charcoal–dextran stripped human serum was filtered and stored at −20°C until used.

Proliferation assay of MCF-7 cells

Confluent MCF-7 cells were washed twice with D-Hanks solution before the addition of 0.25% trypsin-EDTA. The flask was left for 2–3 min at room temperature (close to 20°C), after which the cells were detached, resuspended in full medium, counted and seeded into 96-well plates at a density of 1×10^4 cells/well in normal growth medium. After 48 h, the cells were completely attached to the well bottom. The cells were then washed with D-Hanks and the estrogen-free medium (phenol red-free DMEM with 5% charcoal–dextran stripped human serum) was added and cultured for 24 h; the different concentrations of test compounds were also added to this medium. In the antagonistic test of the cell proliferation assay, the pure estrogen receptor antagonist, 0.1 μM ICI 182,780 (7α-[9(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl)-estra-1,3,5(10)-triene-3,17β-diol)), was added with the other test compounds. Cell proliferation was assessed after seven days, during which the medium was changed every three days. In the assessment method, cells were incubated with 100 μL 5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution for 4 h. The medium was then discarded and replaced with 600 μL dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm in an ELx800 universal microplate reader (Bio-TEK, USA), and cell proliferation was expressed as absorbance values. The results were expressed as proliferation compared with that induced by treatment with 1 nM estradiol.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

MCF-7 cells were grown in 25 cm^2 plastic flasks in phenol red-free DMEM containing 10% charcoal–dextran stripped human serum for 48 h and treated with vehicle or different concentrations of compounds for 24 h, after which the cells were harvested. Extraction of total RNA and its reverse transcription to cDNA were performed using Trizol reagent (Gibco BRL) and ReverTra Ace–α-R kit (TOYOBO Biotech Co. Ltd), respectively, according to the manufacturer's protocol. PCR was carried out with Taq DNA polymerase (Sangon, China) under the following reaction conditions: 20 mmol L^{-1} Tris-HCl, pH 8.4, 50 mmol L^{-1} KCl, 2 mmol L^{-1} $MgCL_2$, 0.2 mmol L^{-1} dNTP mix, 0.025 U mL^{-1} recombinant Taq DNA polymerase in an Eppendorf Mastercycler Gradient (Eppendorf, Germany). The PCR conditions were initial denaturation for 3 min at 94°C, cycling: 45 s at 94°C, 45 s at 55°C, 60 s at 72°C for 30 cycles for estrogen and progesterone receptor, 25 cycles for pS2, and 20 cycles for β-actin using Mastercycler gradient (Eppendorf, Germany), and final elongation at 72°C for 10 min. The primers were designed as follows: progesterone receptor sense primer 5′-AGTTGTGAGAGCACTGGATGC-3′, progesterone receptor antisense primer 5′-GATCTGCCACATGGTAAGGC-3′, pS2 sense primer 5′-TGGAGAACAAGGTGATCTGC-3′, pS2 antisense primer 5′-ATCTGTGTTGTGAGCCGAGG-3′, ER_α sense primer 5′-AGACATGAGAGCTGCCAAC C-3′, ER_α antisense primer 5′-GCCAGGCACATTCTAGAAGG-3′, β-actin sense primer 5′-TGACGGGG TCACCCACACTGTGCCCATCTA-3′, β-actin antisense primer 5′-CTAGAAGCA TTTGCGGT-GGACGATGGAGGG-3′ (Otsuki et al 2000). The PCR products were analysed by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and quantified using a bio-imaging analyser (Bio-Rad). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad).

Data analysis

The data was analysed by two-way analysis of variance and tests of significant differences were determined by Tukey's test at $^*P < 0.05$ and $^{**}P < 0.01$. All data were expressed as means ± s.d. and have been given in the symbols and columns in the figures.

Results

Concentration of the constituents in HPLC fingerprint

HPLC analysis showed that the concentration of the four major compounds casticin, luteolin, rotundifuran and agnuside were 0.13% (50.9 min), 0.038% (37.7 min), 0.033% (61.1 min) and 0.097% (20.3 min), respectively (Figure 2).

Effects on MCF-7 cell proliferation

In the cell proliferation assay, we used estradiol as a positive control, and the RPE value of 100% represented the maximum growth of MCF-7 cells obtained in the presence of estradiol (10^{-9} M). At 200 μg mL^{-1}, VRE had shown maximal cell proliferation and was almost equivalent to the effect of 1 nM estradiol (RPE = 96.6%), while at concentrations of more than 200 μg mL^{-1}, the cell proliferation was decreased (Figure 3). Of the four major constituents, only agnuside and

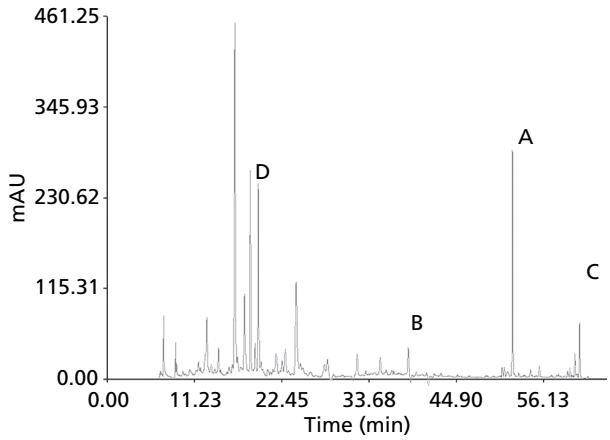

Figure 2 HPLC chromatograms of extracts and online recorded UV-spectra. A, casticin; B, luteolin; C, rotundifuran; D, agnuside.

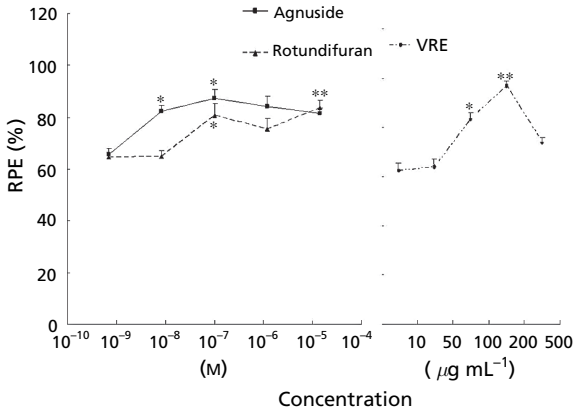

Figure 3 Effects of VRE and its major components on the proliferation of ER-positive human breast cancer cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum with VRE and test compounds for seven days. After incubation for seven days, the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as relative proliferative effect (RPE). Results are expressed as means ± s.d. of three separate experiments for each data point. Significance was set at $^{**}P < 0.01$ ($^{*}P < 0.05$, two-way analysis of variance) vs solvent control.

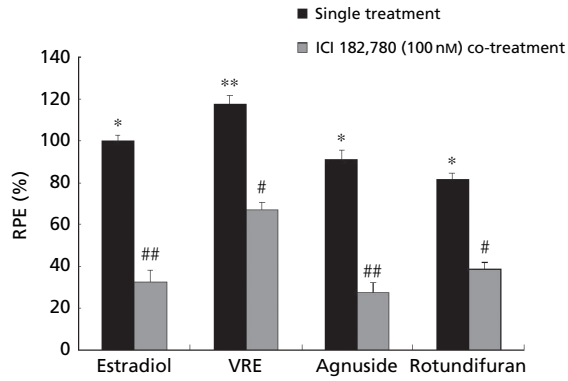

Figure 4 Effect of co-treatment with pure anti-estrogen ICI 182,780 on cell proliferation induced by VRE and its major components in MCF-7 cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum without or with 100 nM ICI 182,780 for seven days. After incubation for seven days, the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as relative proliferative effect (RPE). Results are expressed as means ± s.d. of three separate experiments for each data point. Significance was set at $^{**}P < 0.01$ ($^{*}P < 0.05$, two-way analysis of variance) vs solvent control and $^{\#\#}P < 0.01$ ($^{\#}P < 0.05$, two-way analysis of variance) vs the same dose of compound without ICI 182,780 treatment.

rotundifuran were able to significantly stimulate MCF-7 cell proliferation at concentrations of 10^{-9}–10^{-5}M in a dose-dependent manner ($P < 0.01$) (Figure 3), compared with the vehicle control. The maximal proliferative effect of agnuside and rotundifuran was achieved at 10^{-7}M and 10^{-5}M (The RPE was 89.9 ± 4.3 and 85.3 ± 3.3), respectively. The proliferation effects of 1 nM estradiol, 200 μg mL^{-1} VRE, 10^{-7}M agnuside or 10^{-5}M rotundifuran on MCF-7 cells were blocked by addition of 100 nM ICI 182,780—the pure estrogen receptor antagonist (Figure 4). The RPE of estradiol, VRE, agnuside and rotundifuran after treatment with ICI 182,780 decreased to 32.6 ± 3.2, 67.4 ± 1.3, 27.2 ± 3.6 and 38.6 ± 2.1, respectively.

Gene expression

To further characterize the molecular mechanisms, we studied the effects of VRE on ER_α, progesterone receptor (PR) and pS2 mRNA expression in MCF-7 breast cancer cells in comparison with estradiol and ICI 182,780 (Figure 5). Estradiol (1 nM) and VRE (200 μg mL^{-1}) significantly increased the expression of mRNA for the estrogen-responsive genes (ER_α, PR and pS2) compared with the control. These effects were inhibited by 100 nM ICI 182,780 (Figure 5). Agnuside (10^{-7}M) and rotundifuran (10^{-5}M) significantly increased the expression of mRNA for the PR and pS2 compared with the control. These effects were inhibited by 100 nM ICI 182,780 (Figure 5B), except for rotundifuran in the PR expression. ER_α mRNA expression was not significantly affected by agnuside or rotundifuran, but was significantly stimulated by VRE in our experiments.

Discussion

This is the first report on the estrogenic activity of the fruits of *V. rotundifolia*. In our study, we found that VRE and its compounds agnuside and rotundifuran had estrogenic activity on MCF-7 cells. Although saturation ligand binding analysis of estrogen receptors was not conducted, coadministration of ICI 182,780 virtually blocked the proliferation stimulatory effects induced by VRE and its two compounds. Both proliferation stimulatory effect and up-regulated mRNA levels of PR and pS2 of VRE and its compounds could be reversed by coadministration of a pure anti-estrogen, ICI 182,780. This complete reversal indicated that VRE exerted its effect through estrogen receptors in MCF-7 cells.

Phytoestrogens, a term coined to describe plant derived chemicals that exert estrogenic activity, include a vast variety of structurally diverse compounds, including isoflavones and flavones, lignans, stilbenes, etc. In research involving *V. agnus-castus*, it has been reported that linoleic acid obtained from the methanol extract of fruits of *V. agnus-castus* could bind to estrogen receptors and induce certain estrogen inducible genes (Liu et al 2004). Of the four major constituents, the flavones casticin and luteolin did not show any effects on proliferation of MCF-7 cells at the dose of 10^{-9}–10^{-4} M (compared with the control, data not shown). Casticin is used as the standard for quality control in Viticis Fructus and its drug preparations in China (Zheng 2000). It inhibited mouse lymphocyte growth and proliferation (You et al 1998), and has been reported to block effects of histamine released from sensitized mast cells (Gemini et al 2002). In our research, casticin did not display proliferative activity in MCF-7 cells, which may be coincident with its anti-proliferative effect on

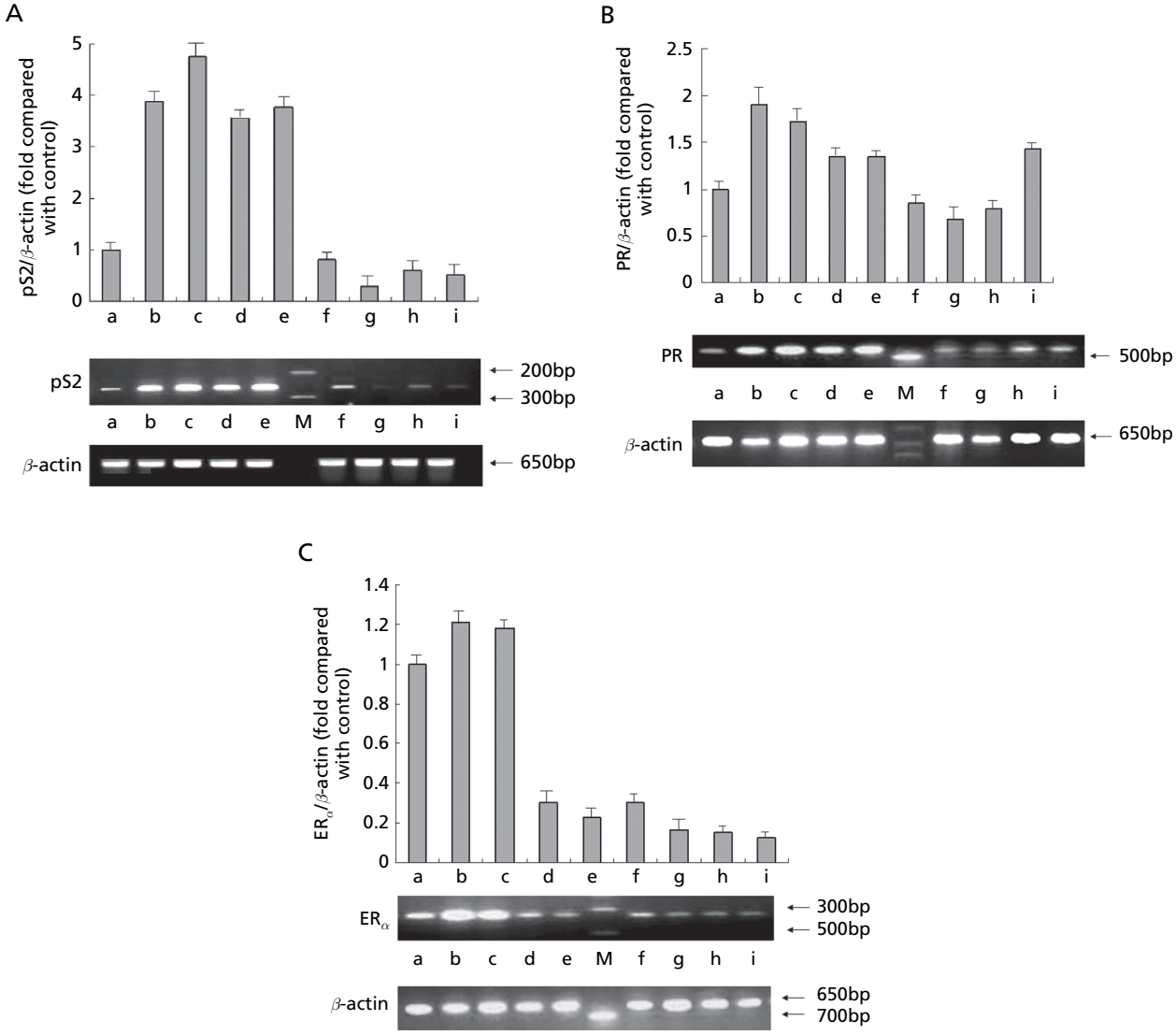

Figure 5 Effect of VRE and its compounds on mRNA expression of estrogen-responsive genes in MCF-7 cells. MCF-7 cells were treated with (a) DMSO; (b) 1 nM 17-β estradiol; (c) 200 μg mL^{-1} VRE; (d) 10^{-7} M agnuside; (e) 10^{-5} M rotundifuran; (f) 1 nM 17-β estradiol + 100 nM ICI 182,780; (g) 200 μg mL^{-1} VRE + 100 nM ICI 182,780; (h) 10^{-7} M agnuside + 100 nM ICI 182,780; (i) 10^{-5} M rotundifuran + 100 nM ICI 182,780. After incubation with test compounds for 24 h, total RNA was extracted using TRIzol reagent (Gibco BRL). The mRNA levels of (A) pS2, (B) progesterone receptor (PR) and (C) ER_α were measured by reverse transcriptase-polymerase chain reaction (RT-PCR) and normalized using β-actin as an internal control. RT-PCR products were run on an ethidium bromide-stained 1.5% agarose gel, which was scanned using a bio-imaging analyser (Bio-Rad) and the density of the products was quantited using Quantity One version 4.2.2 software (Bio-Rad). Results expressed as fold relative to DMSO (solvent control). All data are expressed as means ± s.d.

KB human epidermoid carcinoma cells (Kobayakawa et al 2004). Luteolin also showed its anti-tumour activity on the cell (Lim et al 2006; Samya et al 2006) and when it was present conjugated with glucose as the flavone, luteolin displayed estrogenic activity (Garritano et al 2005).

Since VER induced ER_α, PR and pS2 mRNA transcription, and its components rotundifuran and agnuside induced PR and pS2 mRNA transcription, it may be conferred that these compounds could affect the transcription of estrogen and estrogen-responsive genes, thus displaying estrogen-like activity. In a report from the National Center for Drug Screening of China, PR competition ability (IC50 = 4 μM) compared with the PR ability (IC50 = 18.1 nM) (unpublished data). This implied that rotundifuran could regulate the PR at the protein level. In this study, rotundifuran induced PR mRNA transcription, and this effect was not inhibited by 100 nM ICI 182,780. Therefore, rotundifuran could up-regulate mRNA levels of PR, and this indicated it regulated the PR at protein and RNA levels. In a recent report, the bio-guided fractionation of rotundifuran did not display any significant activity to reduce the level of prolactin through binding with dopamine D_2-receptor, which is relevant for the amelioration of PMS

(Jarry et al 2006). However, in this study it has displayed its ability to up-regulate mRNA levels of PR and pS2, hence it is likely that it would regulate the balance between estrogen and progesterone to treat PMS.

In conclusion, VRE and its major components displayed estrogenic activity via the estrogen response pathway through interaction with the ER, PR and pS2. These could be useful in balancing the changes in the reproductive system and for treating certain hormone-dependent diseases, such as prostate cancer, colon cancer and breast cancer (Cos et al 2003), and to regulate the ratio of estrogen and progesterone to treat PMS. VRE manifested stronger estrogenic activity compared with its constituents, hence there may be other compounds with estrogenic activity present. As the purity of the major constituents was approximately 87%, the estrogen-like activity may have been dependent on themselves or on a synergistic effect with the other constituents (<13%). The other constituents may have also displayed antagonism, which could have decreased the activity of the major constituents. This supported that notion that the herbal medicine exerted its effects through multiple and synergistic mechanisms in the treatment of disease. However, further studies are required to assess the physiological significance of VRE in man.

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