Department of Pharmacognosy[1], School of Pharmacy, Second Military Medical University, Shanghai, China; School of Biomolecular Science[2], Faculty of Sciences, Liverpool John Moores University, Liverpool, UK

Estrogen-like activities in *Vitex* species from China determined by a cell based proliferation assay

Y. Hu[1], Q.-Y. Zhang[1], T.-T. Hou[1], H.-L. Xin[1], H.-C. Zheng[1], K. Rahman[2], L.-P. Qin[1]

Received February 14, 2007, accepted March 29, 2007

Prof. Lu-Ping Qin, Departement of Pharmacognosy, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China
qinsmmu@126.com, lpqin@smmu.edu.cn

Pharmazie 62: 872–875 (2007) *doi: 10.1691/ph.2007.11.7542*

Ethanolic extracts of four Chinese medicinally used *Vitex* species were selected and tested for their estrogen-like activities, using an ER_α-positive MCF-7 cell based proliferation assay (E-screen assay) and cell cycle analysis (flow cytometry). *Vitex negundo* displayed the highest estrogenic-like activity, and could be useful in hormone replacement therapy (HRT).

1. Introduction

Phytoestrogens are plant-derived compounds with estrogenic or antiestrogenic properties (Umland et al. 2000). They seem to be interesting by their possible use as alternative medicines for treating hormonal disorders such as in hormone replacement therapy (HRT). Thus, phytoestrogens could be beneficial in treatment of the symptoms of menopause and hence help to prevent bone resorption.

As part of our continuing search for anti-PMS (premenstrual syndrome) and estrogenic activity from medicinal plants, we focused our studies on *Vitex agnus-castus* – an ancient medicinal plant used for treating a variety of gynecologic conditions. Its fruit extract (VAC) has been traditionally used in Europe for the treatment of conditions affecting females including menstrual disorders, corpus luteum insufficiency, menopause and hormonal imbalance (Daniele et al. 2005). In China, *Vitex rotundifolia L.*, *Vitex trifolia L.*, *Vitex negundo L.*, and *Vitex negundo* var. *cannabifolia* Hands-Mazz., which belong to the same genus as *V. agnus-castus* in Vitex (Verbenaceae), have been used in traditional medicine and have many similarities in their morphological and histological characteristics to *V. agnus-castus* (Wu et al. 1994). The main constituents of the fruits of these four medicinal plants have been determined to be flavonoids, terpenoids, glucosides, which are also similar to the components of *V. agnus-castus* (Singh et al. 2003; Ono et al. 2001; Masateru et al. 2001; Leitao et al. 1999). The fruits, leaves and stems of these *Vitex* species are frequently used in Traditional Chinese Medicine (TCM) to prevent common cold, cough, asthma, chronic bronchitis, and gastrointestinal infections (Li et al. 2005). The aim of this study was to investigate if the four medicinal plants closely related to *V. agnus-castus* possess estrogenic activity similar to *V. agnus-castus.* E-screen assay (Soto et al. 1995) and cell cycle redistributed in MCF-7 human breast adenocarcinoma cells were used.

2. Investigations, result and discussion

In the E-Screen assay using MCF-7 cells, the proliferative effect of the extracts relative to that of estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE) (Fig. 1). The results clearly indicate that the extracts from *V. rotundifolia* and *V. negundo* were able to significantly stimulate MCF-7 cell proliferation at concentrations of 50 μg/mL to100 μg/mL ($P < 0.01$). The highest proliferative effects were achieved by the extracts from *V. rotundifolia* and *V. negundo* at 100 μg/mL ($RPE\% = 106.2 \pm 12.3\%$) and 50 μg/mL ($RPE\% = 96.25 \pm 15.1\%$), respectively, which was almost equivalent to the effect displayed by 1 nM estradiol. While combining the concentration and the proliferative effect, *V. negundo* displayed the most potent estrogenic-like activity and the effect of 50 mg/L of the extract from *V. negundo* could be reversed by co-administration of the pure anti-estrogen ICI 182,780 (Fig. 2). The RPE of the extract from *V. negundo* after treatment with ICI 182,780 decreased to $27.7 \pm 7.0\%$.

Estrogens are known to induce S phase transition in breast cancer cells and a number of cell cycle regulatory proteins have been shown to be affected by estradiol treatment during the early cell cycle events (Foster et al. 2001). It has also been reported that phytoestrogens such as icaritin and desmethylicaritin exert their effect on MCF-7 cell proliferation at S and G_2-phase (Zhi-qiang Wang et al. 2004). In this study, we further evaluated the extracts from *V. rotundifolia* and *V. negundo* by flow cytometric analyses of the cell cycle. Although growth-arrested MCF-7 cells are predominantly in the G_0/G_1-phase of the cell cycle, cell proliferation is shown by the marked increase in the percentage of cells in the S and G_2-phase after 24 h of exposure and this effect could be inhibited by 100 nM ICI 182,780. As shown in Fig. 3 and Table 1, the S-phase cell number increased by 29.02% compared to solvent control with *V. rotundifolia* and 22.69% with *V. negundo* and by approximately 30.78% with 1 nM E_2. The G_2-phase cell number increased 97.21% compared to solvent control with *V. rotundifolia* and

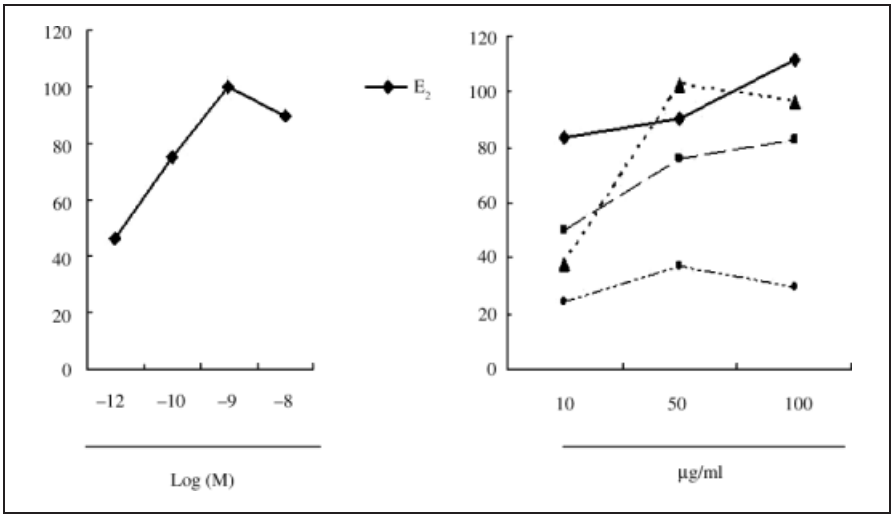

Fig. 1:
Effects of the extract from four medicinal plants on the proliferation in MCF-7 cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum with the extract for 7 days after which the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE)
—◆— V. rotundifolia; – –■– – V. trifolia;
····▲···· V. negundo;
---●--- V. negundo var. cannabifolia

Table 1: Plants collected from different locations in China and their weight/weight yields in terms of crude medicinal materials

Tested materials	Locality	Location (latitude, longitude)	Yield EtOH(%)
Vitex rotundifolia (Fruits)	Xinjian, Jiangxi Province	28°25.41′N, 115°48.61′E	12.1
Vitex trifolia (Fruits)	Shenzhen, Guangdong Province	23°44.60′N, 117°21.25′E	11.6
Vitex negundo (Fruits)	Luzhou, Sichuan Province	31°61.60′N, 105°21.15′E	9.5
Vitex negundo var. *cannabifolia* (Fruits)	Jiande, Zhejiang Province	29°17.65′N, 119°10.12′E	10.9

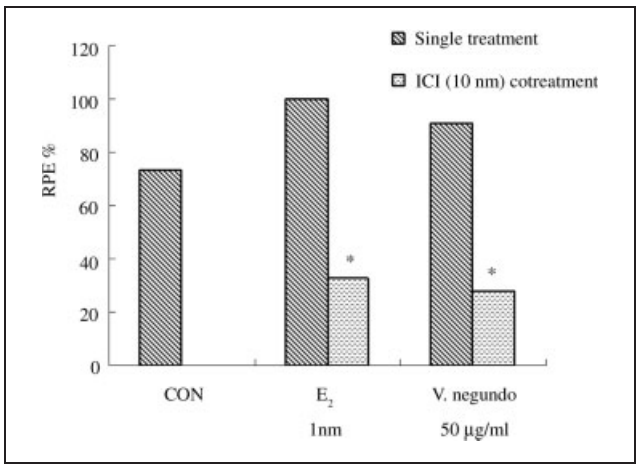

Fig. 2: Effect of cotreatment with pure antiestrogen ICI 182,780 on cell proliferation induced by the extract of *V. negundo* in MCF-7 cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum with or without 100 nM ICI 182,780 for 7 days after which the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE). Significance was set at $^{*}P < 0.01$ vs. the same dose of extract without treatment of ICI 182,780

65.23% with *V. negundo*, but only by 54.99% with 1 nM E_2. These results corroborated the findings from the E-screen assay and demonstrate the reinduction of cell proliferation of growth-arrested MCF-7 cells within a much shorter exposure period, which is similar to estodial.

Phytoestrogens are polyphenolic non-steroidal plant derived compounds with estrogen-like biological activity, and have been associated with a variety of changes in the reproductive system and certain hormone-dependent diseases, such as prostate cancer, colon cancer, breast cancer and PMS (Lippitt et al. 2001; Cos et al. 2003). The results show that among the four medicinal plants tested the ethanolic extracts of *V. negundo* could significantly stimulate the growth of MCF-7 cells and significantly increase the percentage of cells in the S and G_2-phase. The proliferation stimulatory effect and cell cycle redistribution could be reversed by co-administration of a pure anti-estrogen, ICI 182,780. Thus, it can be concluded that the extract of *V. negundo* possesses potential estrogen-like activity in MCF-7 cells and acts like a phytoestrogen. Thus, it may play a role in the treatment of PMS and menopausal disorders by regulating the levels of the estrogen and progesterone. Further investigations of the estrogenic activities of the constituents in *V. negundo* are currently being conducted in our laboratory.

3. Experimental

3.1. Plant material and extraction

Plants listed in Table 1 were collected from different sites in China and authenticated by Prof. Hanchen Zheng, Second Military Medical University. The voucher specimens of these plants were deposited at the Herbarium of Department of Pharmacognosy, Second Military Medical University, Shanghai, PR China. The powder from the fruit (2900 g) was successively infiltrated with 60% EtOH at room temperature for 2 weeks and evaporated under vacuum to obtain the EtOH extract.

3.2. Cell culture

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.

3.3. Charcoal-dextran stripped human serum preparation

In order 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.

3.4. Proliferation assay of MCF-7 cells (E-screen assay)

Confluent MCF-7 cells were washed twice with D-Hanks solution before the addition of 0.25% trypsin-EDTA. The flask was left for at room tem-

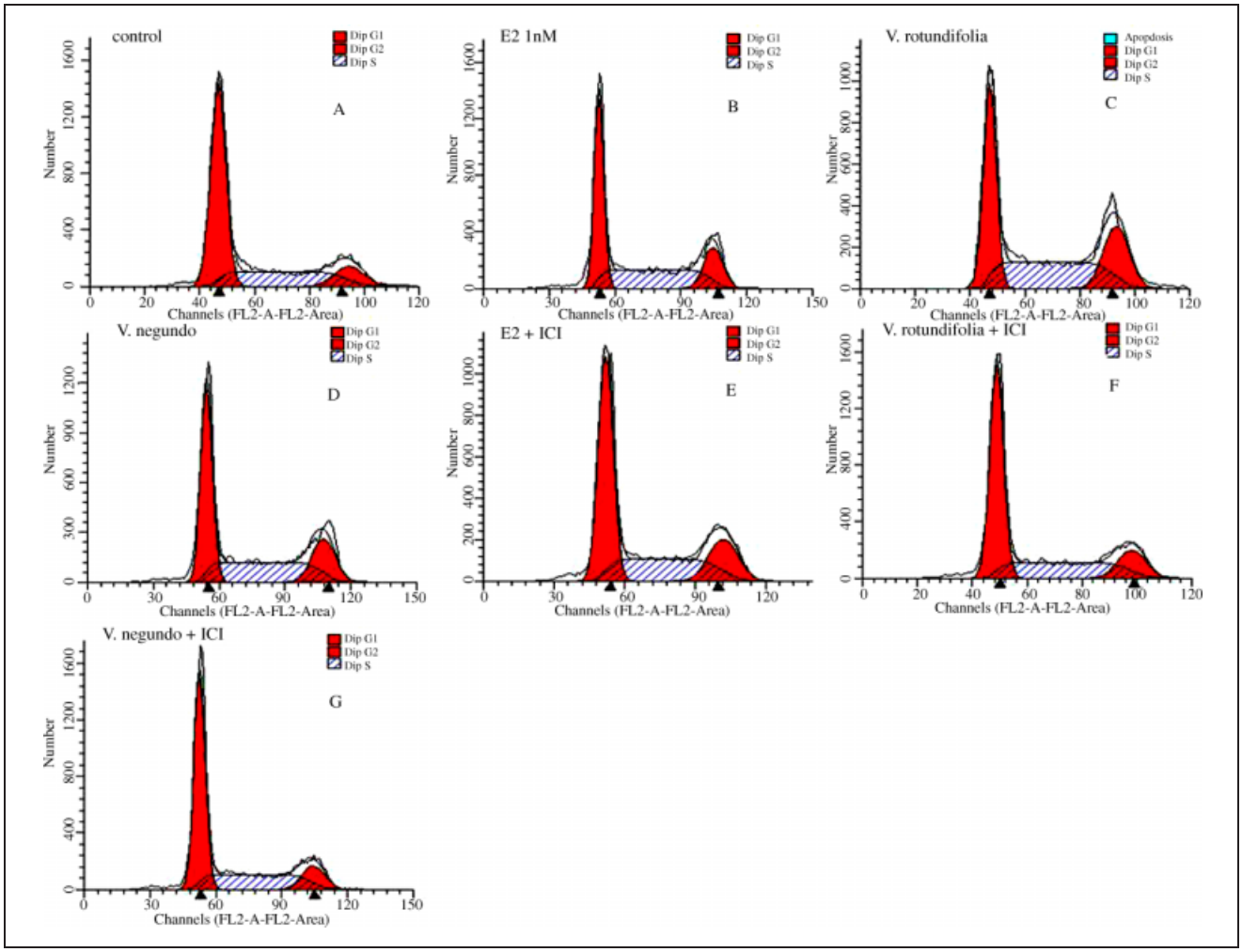

Fig. 3: Effects of *V. rotundifolia* and *V. negundo* on cell cycle distribution of MCF-7 cells. DMSO was used as solvent control and MCF-7 cells were cultured in DMEM with 10% fetal bovine serum for 24 h. The medium was then changed to one without the hormone for 72 h and for another 24 h with test compounds. Cell-cycle analysis was performed by Flow Cytometry. Results are expressed as percentage of cells in G1 phase, S phase and G2 phase. (A) Solvent Control; (B) 1 nM E2; (C) 100 μg/mL *V. rotundifolia*; (D) 50 μg/mL V. *negundo*; (E) 1 nM E2 + 100 nM ICI 182,780; (F) 100 μg/mL V. rotundifolia + 100 nM ICI 182,780; (G) 50 μg/mL V. negundo + 100 nM ICI 182,780. These data are representative of three separate experiments

Table 2: Results of cell cycle analyses of MCF-7 cells exposed to concentrations of fluorotelomer alcohols given as the percentage of cells by phase

Groups/Cell cycle phase	G_1 Phase %	S Phase%	G_2 Phase%
Solvent control	58.65	29.53	11.82
1 nM E_2	43.06	38.62	18.32
100 μg/mL *V. rotundifolia*	38.59	38.10	23.31
50 μg/mL *V. negundo*	44.24	36.23	19.53
1 nM E_2 + 100 nM ICI 182,780	50.25	31.17	18.58
100 μg/mL *V. rotundifolia* + 100 nM ICI 182,780	54.77	30.78	14.45
50 μg/mL *V. negundo* + 100 nM ICI 182,780	56.63	30.73	12.65

perature (close to 20 °C) for 2–3 min, 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 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 materials 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 test compounds. Cell proliferation was assessed after 7 days, during which the medium was changed every 3 days. In the assessment method, cells were incubated with 100 μl of 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 DMSO. The absorbance was measured at 540 nm in an ELx800 universal micro plate reader (Bio-TEK, USA), and cell proliferation was expressed as absorbance values and the results were expressed as proliferation compared with that induced by treatment with 1 nM estradiol.

3.5. Cell cycle analysis

MCF-7 cells were seeded in 6 well plates with standard growth medium at a density of 1×10^5 cells/flask. The cells attached overnight, after which the growth medium was replaced by phenol red-free DMEM containing 10% Charcoal-dextran stripped human serum. After incubation in estrogen-free medium for 72 h, cells were exposed to E_2 or test materials at concentrations corresponding to the highest observed effect during the E-screen assay. After 24 h, cells were harvested by trypsinization and washed twice in PBS and the cell nuclei were isolated and stained with propidium iodide

(PI) for 1 h as described by Vindelov et al. (1983). Flow cytometric analysis of cell cycle distribution and apoptosis was performed with a BD FACSCalibur flow cytometer with a 488-nm argon-ion laser (Becton Dickinson, San Jose, CA, USA) and the PI fluorescence was collected at bandpass 575/26 nm (FL_2, red fluorescence channel) in the linear mode. For each measurement, data from 10,000 single cell events were collected, whereas cell aggregates and doublets were gated out in the two-parameter histograms of pulse height to pulse width of PI fluorescence. The cell cycle histograms were analysed using ModFit LT 3.0 software (Variety Software House, Topsham, ME, USA).

Acknowledgements: This research was supported by the Shanghai Modernization of TCM Foundation of China (Grant No. 04DZ19810). We were indebted to Ting Han and Yan Huo for technical assistance.

References

Cos P, De Bruyne T, Apers S, Vanden Berghe D, Pieters L, Vlietinck AJ (2003) Phytoestrogens: recent developments. Planta Med 69: 589–599.

Daniele C, Thompson CJ, Pittler MH, Ernst E (2005) *Vitex agnus castus*: a systematic review of adverse events. Drug Saf 28: 319–332.

Foster JS, Henley DC, Ahamed S, Wimalasena J (2001) Estrogens and cell-cycle regulation in breast cancer. Trends Endocrinol Meta 12: 320–327.

Lippitt LL, Besinque K et al. (2001) Treatment of Premenstrual Syndromes: PMS, PMDD. PMM Journal July:10–14.

Leitao SG, Fonseca EN, Dos STC (1999) Essential oils from two Brazilian *Vitex* species [J]. Acta Hortic 500: 89–92.

Li Chunzheng, Su Yanfang, Jin Xianjun (2005) Advances in studies on chemical constituents from plants of *Vitex* and their bioactivities. Chin Trad Herbal Drug 36: 930–938.

Masateru O, Yasuyuki I, Toshihiro N (2001) Four new halimane type diterpenes.vitetrifolins D–G, from the fruit of *Vitex trifolia* [J]. Chem Pharm Bull 49: 1220–1222.

Ono M, Yamamoto M, Yanaka T (2001) Ten new labdane type diterpenes from the fruit of *Vitex rotundifolia* L [J]. Chem Pharm Bull 49: 82–86.

Singh V, Dayal R, Bartley JP (2003) Chemical constituents of *Vitex negundo* leaves [J]. Jivied Arom Plant Sci 25: 94–98.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO (1995) The E-SCREEN assay as a tool to identifyestrogens: an update on estrogenic environmental pollutants. Environ.Health Perspect 103 (Suppl. 7): 113–122.

Umland EM, Cauffield JS, Kirk JK, Thomason TE (2000) Phytoestrogens as therapeutic alternatives to traditional hormone replacement in postmenopausal women. Pharmacotherapy 20: 981–990.

Vindelov LL, Christensen IJ, Nissen NI (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3: 323–327.

Wu ZY et al. (1994) In: Flora of China (first fascicule of the sixty-fifth volume). Edited by board of Flora of China science academy, Beijing: Science Press, p. 131–150.

Zhi-qiang Wang, Yi-jia Lou (2004) Proliferation-stimulating effects of icaritin and desmethylicaritinin MCF-7 cells. Eur J Pharmacol 504: 147–153.