The effect of xanthorrhizol, curcumin and tamoxifen on the activation of Wnt/β-catenin signaling and the expression of vascular endothelial growth factor in the human breast cancer cells

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The Master's Thesis submitted to the Department of Medical Science,The Graduate School of Yonsei University In partial fulfillment of the requirements for the degree of Master of Medical Science

CHANG KI LEE

December 2004

This certifies that the Master's Thesis of

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December 2004

Acknowledgement

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Abstract

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(Directed by professor KWANG KYUN PARK)

Vascular endothelial growth factor (VEGF) is a key factor in promotion of tumor angiogenesis. Recent studies suggest that Wnt/β-catenin signaling pathway regulates vessel development in normal and pathological conditions. Activation of the Wnt signaling pathway is also a major feature of several human neoplasias and appears to lead to the cytosolic stabilization of β-catenin, which can regulate transcription of the cellular oncogenes such as

cyclin D1, c-myc and VEGF. Therefore, components of the Wnt/β-catenin pathway can be promising targets in the search of anticarcinogenic agents.

In the present study, we investigated that the effects of xanthorrhizol, curcumin and tamoxifen on the VEGF regulation through Wnt/β-catenin pathway in the presence or absence of 17β-estradiol(E_2) in estrogen receptor(ER)-positive and ER-negative breast cancer cells. The viabilities of MCF-7 cells and MDA-MB-231 cells were reduced by xanthorrhizol, curcumin and tamoxifen in a dose-dependent manner. The expression of Wnt signaling components (Dvl, GSK-3β, β-catenin) and VEGF was dose-dependently downregulated by xanthorrhizol, curcumin and tamoxifen in MCF-7 and MDA-MB-231 cells. We observed that the mRNA level of estrogen receptor-α(ER-α), VEGF and WNT-3/4/6 as well as the cell proliferation was increased by E2 in the MCF-7cells. However, in the MDA-MB-231 cells, the mRNA level of VEGF was not regulated by E2. Xanthorrhizol, curcumin and tamoxifen repressed the mRNA level of ER-α, VEGF and WNT-4.

Xanthorrhizol, curcumin and tamoxifen inhibit not only the growth of breast cancer cells but also angiogenesis by reducing the VEGF expression through Wnt/β-catenin pathway.

Key words: VEGF, Wnt/β-catenin, Xanthorrhizol, Breast cancer cells

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I. INTRODUCTION

Breast cancer is a worldwide epidemic among women, and one of the most rapidly increasing cancers[1]. Not only the incidence rate but also the death rate is increasing. Despite enthusiastic efforts in early diagnosis, aggressive surgical treatment and application of additional non-operative

modalities, its prognosis is still dismal[2]. Therefore, breast cancer chemoprevention is the subject of substantial research efforts to improve the health of women. Recently, attention has been focused on identifying dietary phytochemicals that have the ability to inhibit the processes of carcinogenesis[3]. A wide range of new breast cancer chemopreventative agents (e.g. curcumin, diallyl disulfide, garlic, resveratrol) are poised to be tested in clinical trials[4].

Tumor growth and metastasis are highly dependent upon angiogenesis, the formation of capillary spouts. Vascular endothelial growth factor(VEGF) is a key mediator of tumor angiogenesis and an important prognostic factor for many tumors, including breast cancer. In breast cancer tissue, VEGF mRNA expression is increased compared with adjacent normal breast tissue[5]. Moreover, high tissue VEGF levels appear to correlate with poor prognosis, and decrease overall survival for breast cancer patients. Recent studies have shown that 17β-estradiol (E_2) modulates VEGF expression by increasing gene transcription and mRNA stability in estrogen receptor positive MCF-7 cells[6]. However, the molecular mechanisms underlying up-regulation of VEGF by E_2 in breast cancer cells have not been elucidated.

Several reports have demonstrated that Wnt/β-catenin signaling regulates vessel development in normal and pathological conditions.

Wnt signaling plays a critical role in numerous processes of development and in adult tissues, and appears to be conserved across all animal taxa. β-Catenin is an intracellular transducer of canonical Wnt/β-catenin signaling and, thus, has a dual function: as a transcriptional factor[7] and, in a cadherin-bound form, as a regulator of cell adhesion and migration[8]. A recent report described VEGF as a β-catenin target gene in HeLa cells and colon cancer cells[9]. In addition, mutations that increase the stability of cytoplasmic β-catenin have been implicated in numerous malignant transformations and represented a leading cause of colorectal tumorigenesis[10].

Wnt proteins released from or presented on the surface of signaling cells act on target cells by binding to the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dvl), glycogen synthase kinase-3β (GSK-3β), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin. Cytoplasmic β-catenin levels are normally kept low through continuous phosphorylation-ubiquitination-coupled proteasomal degradation, which is controlled by a complex containing GSK-3β/APC/Axin. When cells receive Wnt signals, the degradation of β-catenin is inhibited, and consequently β-catenin accumulates in the cytoplasm and nucleus.

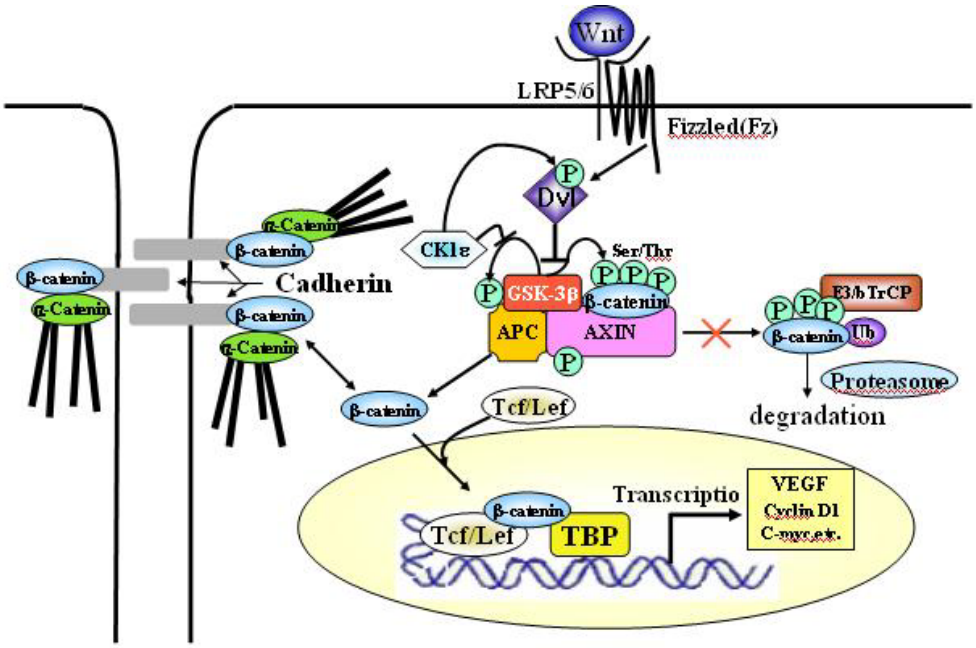


Fig. 1. Wnt/β-catenin signaling pathway.

Nuclear β-catenin interacts with transcription factors such as T cell factor/lymphoid enhancer factor (TCF/LEF) and leads to the activation of Wnt target genes such as VEGF, cyclin D1, c-myc and so on (Fig. 1). A large number of Wnt targets have been identified that include the members of Wnt signaling pathway itself, which provide feedback control during Wnt signaling[11].

Xanthorrhizol (Fig. 2) is a sesquiterpenoid compound isolated from the rhizomes of *Curcuma xanthorrhiza* Roxb.(Zingiberaceae) used in Indonesia as a rheumatic remedy and stomachics. In our previous study, xanthorrhizol has been shown a potent inhibition of COX-2 and iNOS activity in lipopolysaccharide-treated mouse macrophage cells, RAW 264.7[12]. Curcumin (diferuloylmethane) (Fig. 2), a major yellow pigment isolated from the ground rhizomes of the *Curcuma* species, has been reported to have multiple suppressive effects on human breast carcinoma cells[3,]. In chemoprevention testing program of American National Cancer Institute, curcumin is in phase I clinical trial to develop as a chemopreventive agent. A recent report on anti-angiogenic activity of curcumin showed that curcumin inhibits fibroblast growth factor (FGF)-induced vascularization but dose not have any effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced *VEGF* mRNA expression[13]. It has been also known that curcumin inhibits the expression of VEGF in colon

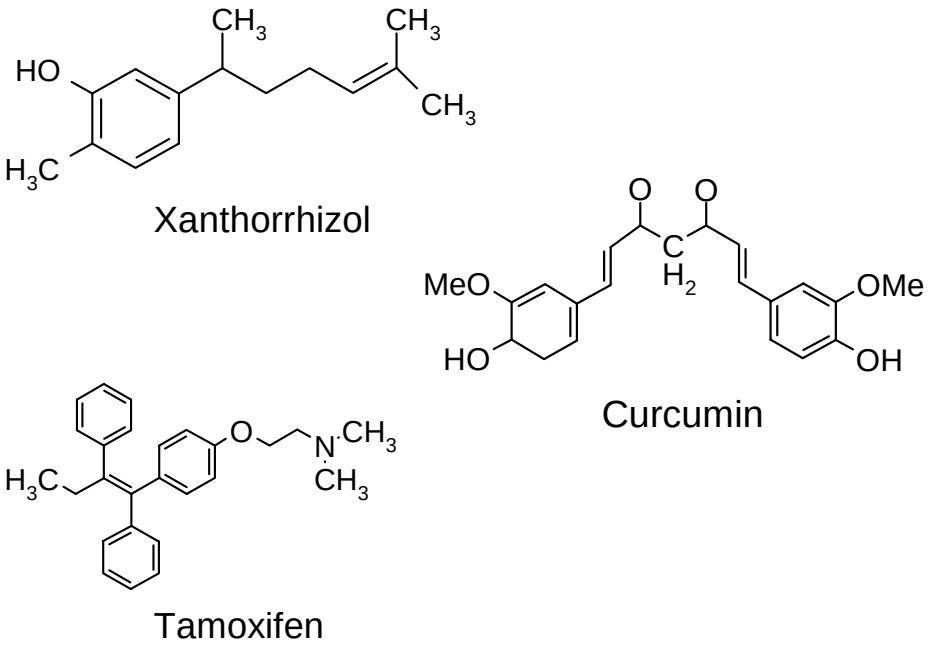


Fig. 2. Structure of xanthorrhizol, curcumin and tamoxifen

cancer[3] and breast cancer cells[8]. However, the inhibiting mechanism is still little known. Tamoxifen (Fig. 2), produced under the name Nolvadex, is the first chemopreventive agent against breast cancer approved by Food and Drug Administration, USA as well as the most commonly used drug for breast cancer therapy. Tamoxifen acts as an antagonist by competing with E_2 for binding to estrogen receptors (ER) and inhibits the effects of ER complexes on gene promoters[14].

In this study, to estimate the chemopreventive potential of xanthorrhizol on breast cancer, we investigated the effects of xanthorrhizol on the VEGF expression through Wnt/β-catenin pathway in both estrogen receptor (ER)-positive and ER-negative breast cancer cells. Furthermore, we estimated that the effect of xanthorrhizol on VEGF expression and Wnt signaling components compared with those of curcumin and Tamoxifen.

II. MATERIALS AND METHODS

1. MATERIALS

A. Chemicals

Xanthorrhizol was supplied from Professor Jae-Kwan Hwang of the Department of Biotechnology, Yonsei University (Seoul, Korea). Curcumin, 4-hydroxytamoxifen (tamoxifen), 17β-estradiol (E_2), sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF) and 0.4% trypan blue solution was obtained from Sigma (MO, USA). ICI182,780, antiestrogen, was purchased from Tocris (MO, USA). Dulbecco's Modified Eagle Medium (DMEM), phosphated-buffered saline (PBS), antibiotic-antimycotic and heat-inactivated fetal bovine serum (FBS) were obtained from Gibco BRL (NY, USA). Charcoal-dextran stripped FBS were obtained from Biotechnics (CA, USA). All other chemicals used were of analytical or the highest grade available.

B. Cell line

Human breast cancer cell lines, MCF-7 (estrogen receptor positive, p53 wild type) and MDA-MB-231 (estrogen receptor negative, p53 mutant type) cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic at 37℃ in a humidified 5% CO_2 atmosphere.

2. METHODS

A. Cell Culture

To assess the effects of xanthorrhizol, curcumin and tamoxifen on breast cancer cells, MCF-7 and MDA-MB-231 cells (1×10^5 cells/dish) were plated in 10cm dish, respectively and were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic at 37℃ in a 5% CO_2 atmosphere. After reaching 80% confluent growth, the indicated concentration of xanthorrhizol, curcumin and tamoxifen were added to each dish and cells were incubated for 24 hours.

To investigate the effects of E_2 on the breast cancer cells, and those of xanthorrhizol, curcumin and tamoxifen on E_2-stimulated breast cancer cells, MCF-7 and MDA-MB-231 cells (5×10^4 cells/dish), respectively, were cultured in 10cm culture dish including DMEM medium supplemented with 10% FBS for 24 hours. The media was changed to serum-free DMEM. 24 hours later, media was changed to DMEM with 10% dextran-charcoal stripped FBS and cells were treated by E_2 with or without ICI182,780, xanthorrhizol, curcumin and tamoxifen at the indicated concentration for 6 days.

B. Trypan blue exclusion assay

MCF-7 and MDA-MB-231 cells (1×10^5 cells/dish) were cultured in DMEM with 10% FBS, respectively. After incubation for the indicated duration, cells were centrifuged at 3,000 rpm for 5min and harvested. Cells were resuspended with the PBS and stained with 0.4% trypan blue solution. The number of unstained cells (viable cells) was counted with hematocytometer.

C. Western blotting

MCF-7 and MDA-MB-231 cells (1×10^5 cells/dish) were plated in 10 cm culture dish with DMEM containing 10% FBS. Xanthorrhizol, curcumin and tamoxifen were treated at the indicated concentrations after reaching 80% confluent growth and 24 hours later cells were harvested. Cells were washed twice with cold PBS and lysed in triple-detergent lysis buffer [50 mM Tris-HCl (pH8.0), 5 mM EDTA, 10% glycerol, 0.1% SDS, 0.2% Triton X-100, 1 mM PMSF] containing complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). The protein concentration was determined by using the BSA protein assay reagent (Pierce, PA, USA). The samples were boiled in sodium dodecyl sulfate (SDS) sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol 0.1% bromophenol blue] for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8～12% gel

and transferred to a polyvinylidene fluoride (PVDF) membrane (Pall Co., MI, U.S.A). The membrane was blocked with 5% skim milk-TBS [10 mM Tris (pH8.0), 150 mM NaCl] containing 0.1% Tween-20 (TBST) for 2 hours at room temperature and washed three times in TBST buffer for 5min. Then, it was incubated for 4 hours at room temperature with the primary antibodies diluted with the 3% skim milk-TBST. The following primary antibodies were used at the recommended dilution factor: mouse polyclonal anti-β-catenin (92kD) (BD Transduction Laboratories, CA, USA) was diluted by 1:2000, mouse polyclonal anti-GSK-3β (46kD) (BD Transduction Laboratories, CA, USA) was diluted by 1:1000, rabbit polyclonal phospho-GSK-3β (ser9) (46kD) (Cell signaling technology, MA, USA) was diluted by 1:1000, goat polyclonal anti-Dvl (Dishevelled) (96kD) (Santa Cruz Biotechnology, Inc, CA, USA) was diluted by 1:1000, rabbit polyclonal anti-VEGF (Santa Cruz Biotechnology Inc., CA, USA) was diluted by 1:1000, mouse monoclonal anti-β-tubulin (50kD) (Sigma, MO, USA) was diluted by 1:2000. After washed 3 times with TBST, the blots were incubated with horseradish peroxidase-conjugated anti-mouse, anti-goat or anti-rabbit IgG antibodies (Santa Cruz Biotechnology Inc, CA, USA) diluted by 1:1000 for 2 hours at room temperature, and then washed three times in TBST again. Transferred proteins were detected by an enhanced chemiluminescence detection method, immersing the blots for 1 min in a 1:1 mixture of chemiluminescence reagents A and B

(Santa Cruz Biotechnology Inc, CA, USA) and exposing to Kodak film for a few minutes.

D. RNA preparation

The harvested breast cancer cells were lysed using TRIzol reagent (Life technologies, Austria) and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complex. After the addition of 0.2 ml volume of chloroform (Sigma, MO, USA), samples were shaken vigorously for 15 seconds, incubated for 2-3 minutes, and centrifuged at 12,000 rpm for 15 minutes at 4℃. Total RNA in the upper aqueous phase was precipitated by mixing with an equal volume of isopropanol (Sigma, MO, USA). The mixtures were then incubated for 10 minutes at 4℃ and centrifuged at 12,000 rpm for 10 minutes at 4℃. The pellet was washed with 75% ethanol, dried and dissolved in RNase-free water. The concentration of total RNA was estimated by measuring the absorbance at 260 nm using a spectrophotometer (Pharmacia Biotech, Cambridge, England). Protein contamination of the RNA preparations was assessed by measuring the absorbance at 280 nm.

E. Primer design

To optimize the PCR amplification condition, the primers of the target gene were determined by the on-lined primer design program[15]. Table 1 shows the primer sequences used in this study.

Table 1. Description of primers used in RT-PCR

primer	annealing temperature	Size(bp)	Sequence 5' → 3'	
ER-α	52	480	forward	AAGTTCAGGCACAATTGGATG
			reverse	CCCTGCATGACACTGATTACA
ER-β	60	395	forward	AGTCCGATGAATGTGCTTGCTCT
			reverse	CACATTTGGGCTTGTGGTCTGC
VEGF	60	97	forward	CTGGAGTGTGTGCCCACTGA
			reverse	TCCTATGTGCTGGCCTTGGT
WNT-1	60	245	forward	TGCACGCACACGCGCGTACTGCAC
			reverse	CAGGATGGCAAGAGGGTTCATG
WNT-2	52	378	forward	GCCACACGCTGCACCTAAAGC
			reverse	CAATTACCCTAAGGGTGGTAGC
WNT-3	60	223	forward	CTGCCAGGAGTGTATTCGCATC
			reverse	GAGAGCCTCCCCGTCCACAG
WNT-3A	56	326	forward	CAGGAACTACGTGGAGATCATG
			reverse	CCATCCCACCAAACTCGATGTC
WNT-4	56	400	forward	ACTGGTGCTGCTTCGTCAAGTG
			reverse	CTCCTTGTTACTCCACCTTAGG
WNT-5A	56	100	forward	AGGGCTCCTACGAGAGTGCT
			reverse	GACACCCCATGGCACTTG
WNT-6	60	411	forward	TGGTGCTGCGTAGTACAGTGC
			reverse	CCATCCTGTGGCCAGCAGTTC
WNT-7A	52	332	forward	AGATGTACACGTGCAAGTGAGC
			reverse	AATAAATTGTTAAATATTGCTGTGATG
WNT-7B	56	413	forward	ACTGGTGCTGCTTCGTCAAGTG
			reverse	CTTTGCTCTCTGGGATACAGGTG
WNT-8A	60	290	forward	GGGCGATGGGGAACCTGTTTATG
			reverse	CTCCAGCAGAGCTGATAGCATG
WNT-8B	56	310	forward	TTGCTAGGAGGAAGTAGGTCAG
			reverse	ATGTCTTTGGGGTTGGTTCCTAG
WNT-10A	54	290	forward	ATGTGGCTGCCTCAGCCATACAG
			reverse	TGTAAGCGGTGCAGCTTCCTAC
WNT-14	55	704	forward	TTTGAGCGCTGGAACTGCACG
			reverse	CTCAGCCCTTGCAGGTGTAG
WNT-14B	55	390	forward	GTGCAGGAGGAGCTTGTGTAC
			reverse	CCAGGAAGCAAGATGGAATAGC
WNT-16	52	200	forward	CAGGGACACAAGGCAGAGAATG
			reverse	GCTGGATGGAGTGGTTACTT
FZ-4	62	762	forward	TTCACAGTACTGACCTTCCTG
			reverse	ATGCCTGAAGTGATGCCCAC
FZ-5	52	200	forward	CCTACCACAAGCAGGTGTCC
			reverse	GGACAGGTTCTTCCTCGAAA
FZ-6	58	560	forward	AGTCTTCAGCGGCTTGTATCTTGT
			reverse	GCTCCGTCCGCTTTCACCTCT
FZ-7	63	572	forward	TTCGGTATGGCCAGCTCCATCTGGT
			reverse	CATGGTCATCAGGTACTTGATCAT
FZ-10	54	170	forward	ACACGTCCAACGCCAGCATG
			reverse	ACGAGTCATGTTGTAGCCGATG
GAPDH	52	420	forward	TGTGTGACCCAGGACTACCA
			reverse	ACTTGTTGAACATCCAGCCC

F. Reverse transcriptase - polymerase chain reaction (RT-PCR)

The mRNA expression of *ER-α/β*, *VEGF*, *WNT* family and *FRIZZLED* (*FZ*) receptor family was assessed by RT-PCR. The first-strand cDNA was synthesized with 1 ㎍ of the total RNA and 1μM of oligonucleotide primer (oligo-dT_{15}) using M-MLV Reverse Transcriptase (Promega, CO, U.S.A). Using the recombinant Taq DNA polymerase kit (Takara, Shiga, Japan), cDNA was amplified in 20 ㎕ of the final volume containing 0.5 ㎍ first-strand cDNA and 10 pmole/ml of the forward and reverse primers. The cDNA of the housekeeping gene glyceraldehydes-3 phosphate dehyrogenase (GAPDH) was used as the internal control for estimating the relative mRNA level of *ER-α/β*, *VEGF*, *WNT* family and *FZ* receptor family. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700, U.S.A) as follows: initial denaturation at 94℃ for 5minutes, 30 cycles of denaturation at 94℃ for 50 seconds, annealing at the temperature described in table 1 for 50 seconds, extension at 72℃ for 1 minutes and a final extention at 72℃ for 10 minutes. The amplification of GAPDH was carried out in replicates under the same PCR conditions. The amplification PCR products were electrophoresed on a 2% agaroge gel with 1 X TBE buffer (0.89M Tris-borate, 0.02M EDTA, pH8.3). After staining with 0.5 ㎍ /ml of ethidium bromide, the amplified samples were visualized by the UV transilluminator and photographed using Polaroid DS-34 Instant Camera system (Kodak, NY, USA).

The relative mRNA expression levels were normalized with that of *GAPDH* mRNA. Briefly, the net intensity of *ER-α/β*, *VEGF*, *WNT* family, *FZ* receptor family and *GAPDH* band was measured using image analysis program TINA (v.2.0). The intensity of the amplified GAPDH band was used as the baseline value.

G. Statistical analysis

The results are expressed as means ± standard error. Statistical evaluation of the data was done using Student's *t*-test.

III. RESULTS

Effects of xanthorrhizol, curcumin and tamoxifen on the growth of estrogen receptor-positive (ER+) MCF-7 human breast cancer cells

The proliferation of MCF-7 cells (Fig. 3A) was inhibited in a dose-related manner when treated by xanthorrhizol, curcumin and tamoxifen in the absence of 17β-estradiol (E_2) for 24 hours. IC_{50} of xanthorrhizol, curcumin and tamoxifen were 26μM, 24μM and 20μM in MCF-7 cells, respectively. The proliferation of MCF-7 cells was markedly increased after the treatment with 1 and 10nM E_2, compared with untreated cells (Fig. 3B). 10nM E_2 induced a 1.5-fold increase ($P < 0.01$) of the cell proliferation at 6 day and this effect was blocked to 42% or more by xanthorrhizol, curcumin and tamoxifen (Fig. 3C). The antiproliferative effect of xanthorrhizol on the ER+ MCF-7 cells in the presence of E_2 was more effective than that of antiestrogen, ICI182,780. However, xanthorrhizol showed a similar inhibitory effect to curcumin and tamoxifen on E_2-stimulated proliferation of MCF-7 cells.

Effects of xanthorrhizol, curcumin and tamoxifen on the expression of VEGF in MCF-7 cells

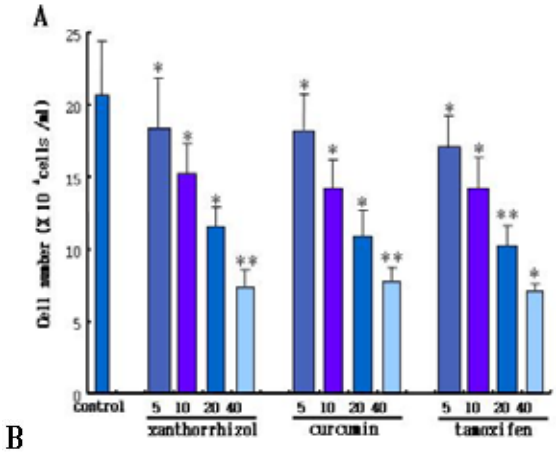
We investigated whether xanthorrhizol, curcumin and tamoxifen inhibit

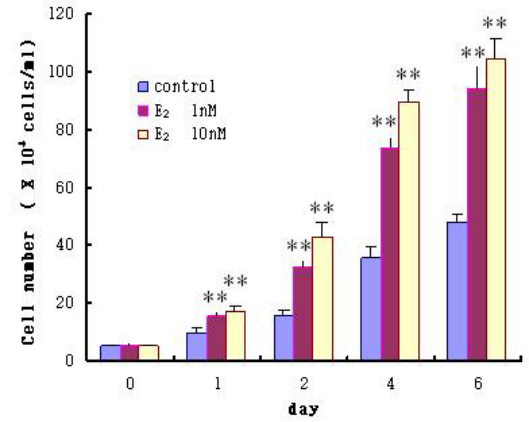
the expression of VEGF in MCF-7 cells cultured in media without or with E_2 (Fig. 4). In the absence of E_2 (Fig.4A), xanthorrhizol, curcumin and tamoxifen inhibited VEGF expression in a dose-related manner. In particular, xanthorrhizol showed a more significant reduction of VEGF expression in MCF-7 cells than curcumin and tamoxifen at concentrations of 20μM and 40μM.

In the experiment to assess the effect of these compounds on VEGF expression in E_2-treated MCF-7 cells, we found that the level of *VEGF* mRNA was increased by E_2 concentration- and time-dependently (Fig. 4B), but the enhanced *VEGF* mRNA level by E_2 was suppressed remarkably by xanthorrhizol, curcumin and tamoxifen as well as an estrogen antagonist ICI182,780 (Fig. 4C). Curcumin and tamoxifen, a selective estrogen receptor modulator, produced a greater inhibition than xanthorrhizol.

Effects of xanthorrhizol, curcumin and tamoxifen on the expression of Wnt signaling components in MCF-7 cells

In MCF-7 cells without E_2 stimulation, xanthorrhizol, curcumin and tamoxifen suppressed the expression of β-catenin, GSK-3β and dishevelled (Dvl) and increased the phosphorylation of GSK-3β (ser9) in a dose-dependent manner (Fig. 5A). tamoxifen completely inhibited β-catenin expression at the concentration of more than 20μM.


A
Cell number (X 10 ⁴cells /ml)
control
5 10 20 40
xanthorrhizol
5 10 20 40
curcumin
5 10 20 40
tamoxifen


B
Cell number (X 10⁴ cells/ml)
control
E₂ 1nM
E₂ 10nM
day

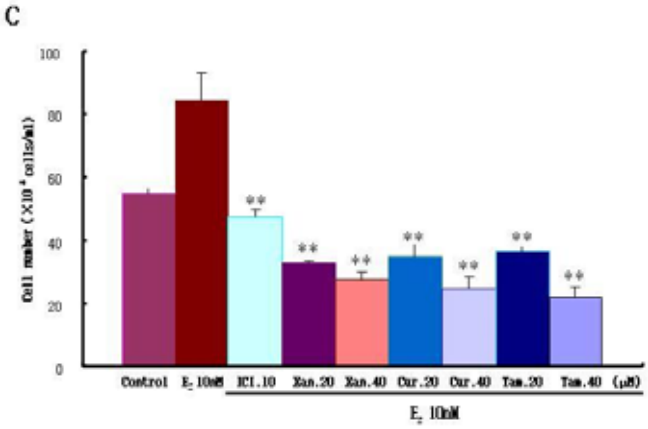

C
Cell number (X10⁴ cells/ml)
Control
E₂ 10nM
ICI.10
Xan.20
Xan.40
Cur.20
Cur.40
Tam.20
Tam.40 (μM)
E₂ 10nM

Fig. 3. Effects of xanthorrhizol, curcumin and tamoxifen on the viability of MCF-7 cells in the presence or absence of E_2. (A) MCF-7 cells (1×10^5cells/dish) were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic for 24 hours. When reaching 80% confluent growth, the indicated concentrations of xanthorrhizol, curcumin and tamoxifen were added to each dish with serum-free media. 24 hours later, cells were stained with 0.4% trypan blue and counted with hemacytometer. (B) MCF-7 cells (5×10^4 cells/dish) were incubated in DMEM medium supplemented with 10% FBS for 24 hours. The media was changed to serum-free DMEM to starve for 24 hours. And then media was changed to DMEM with 10% dextran-charcoal stripped FBS and cells were stimulated by 1 and 10 nM E_2. The number of MCF-7 cells treated by E_2 was counted at 0, 1, 2, 4 and 6 days. (C) The number of E_2-stimulated MCF-7 cells treated by xanthorrhizol (xan.), curcumin (cur.), tamoxifen (tam.) and ICI182,780 (ICI) were counted at 6 days. * $P < 0.01$, ** $P < 0.005$

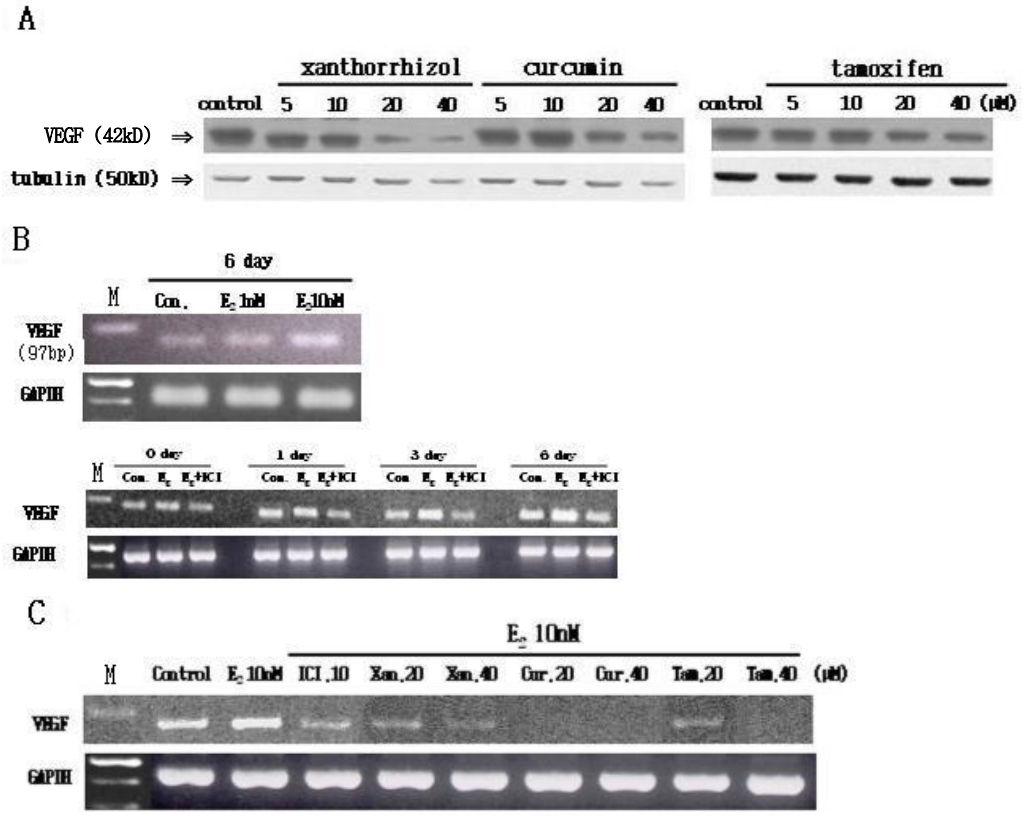


Fig. 4. The expression of vascular endothelial growth factor (VEGF) in MCF-7 cells treated with xanthorrhizol, curcumin and tamoxifen in the presence or absence of E_2. (A) Cells were incubated in serum-free DMEM media containing various concentrations of xanthorrhizol, curcumin and tamoxifen. 24 hours later, the expression of VEGF was shown by western blotting as described in Materials and Methods. (B) The mRNA expression levels of *VEGF* (97bp) were evaluated by RT-PCR in MCF-7 cells treated with E_2 (1 and 10nM) alone for 6 days, and with E_2 (10nM) and ICI182,780 (10μM) for 0, 1, 3 and 6 days. (C) The mRNA expression levels of *VEGF* (97bp) were evaluated in MCF-7 cells treated with E_2 alone and co-treated with ICI182,780 (ICI), xanthorrhizol (xan.), curcumin (cur.) and tamoxifen (tam.) for 6 days by RT-PCR. M : marker

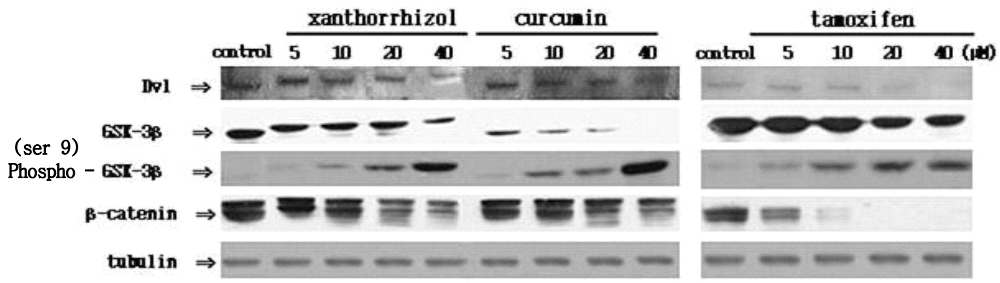


Fig. 5. The expression of Wnt signaling components in MCF-7 cells treated with xanthorrhizol, curcumin and tamoxifen in the presence or absence of E_2. (A) MCF-7 cells were treated with the indicated concentrations of xanthorrhizol, curcumin and tamoxifen for 24 hours. (B) MCF-7 cells were treated with E_2 alone and co-treated with ICI182,780, xanthorrhizol, curcumin and tamoxifen for 6 days. Harvested cells were lysed with triple-detergent lysis buffer. Cytosolic protein concentration was determined by using the BSA protein assay reagent. Wnt signaling components was determined by western blotting analysis.

Identification of *WNT* and *FRIZZLED* (*FZ*) family expressed in MCF-7 cells

To determine *WNT* and *FZ* family triggering Wnt signaling in MCF-7 cells, we investigated the mRNA expression of *WNT* and *FZ* family using RT-PCR in E_2-treated MCF-7 cells and untreated cells (Fig. 6A). *WNT1, WNT3, WNT3A, WNT4, WNT6, WNT7B, WNT8B, WNT10A* and *WNT16* mRNA were identified and *FZ4, FZ5, FZ6* and *FZ7* mRNA also detected in MCF-7 cells cultured without E_2, whereas *WNT2, WNT5, WNT5A, WNT7A, WNT8A, WNT14, WNT14B* and *FZ10* mRNA was not detected. In E_2-treated MCF-7 cells, the expression of *WNT1, WNT3, WNT4, WNT6* and *WNT16* mRNA were upregulated in proportion to E_2 concentration. In contrast, *WNT3A* and *WNT10A* mRNA expression were downregulated by E_2. The mRNA levels of *WNT7B* and *WNT8B* were not affected by E_2 treatment. *FZ4, FZ5, FZ6* and *FZ7* mRNA levels were enhanced by E_2 treatment.

Moreover, the time-dependent increase of *WNT3, WNT4, WNT6* and *FZ4* mRNA levels by E_2 was blocked by 10μM of ICI182,780 (Fig. 6B).

Effects of xanthorrhizol, curcumin and tamoxifen on the mRNA expression of ER in MCF-7 cells

MCF-7 cells expressed *ERα* and *ERβ* mRNA. The mRNA level of *ERα* was increased by E_2, but the level of *ERβ* mRNA did not change (Fig. 7A). In E_2-treated MCF-7 cells, the elevated mRNA level of *ERα* was suppressed very strongly at 40μM curcumin and tamoxifen, not xanthorrhizol (Fig. 7B).

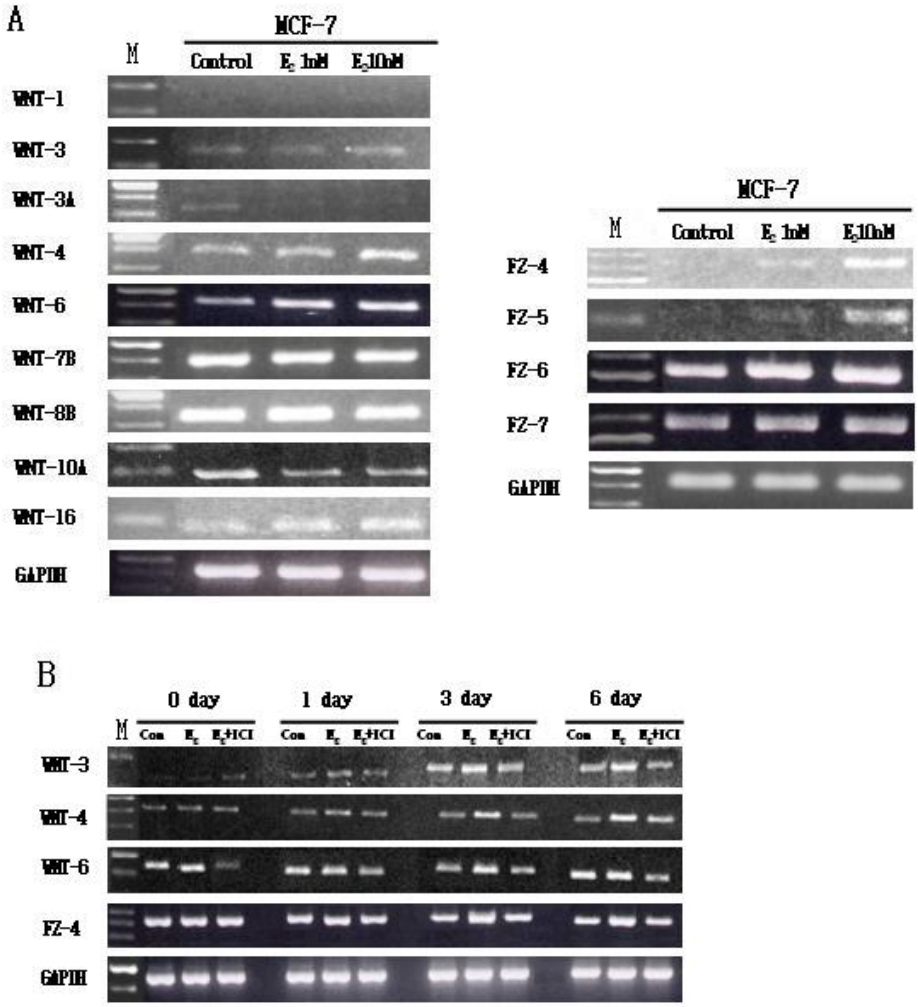


Fig. 6. The mRNA expression of WNT and FRIZZLED family in MCF-7 cells. The mRNA expression levels of *WNT*s and *FZ*s were evaluated by RT-PCR in MCF-7 cells without or with E_2 (1 and 10nM) for 6 days (A), and with E_2 (10nM) and ICI172,780 (10μM) for 0, 1, 3 and 6 days (B). M : marker

Effects of xanthorrhizol, curcumin and tamoxifen on the mRNA expression of *WNT* family in MCF-7 cells in the presence or absence of E_2.

We investigated the alteration of mRNA expression of *WNT* family by xanthorrhizol, curcumin and tamoxifen in MCF-7 cells. MCF-7 cells in untreated by E_2, *WNT1* and *WNT4* mRNA levels were upregulated by xanthorrhizol, curcumin and tamoxifen. On the contrary, *WNT6* mRNA level was downregulated by xanthorrhizol and curcumin tamoxifen did not change the mRNA expression of *WNT6* (Fig. 8A). Furthermore, the increase of *WNT4* mRNA levels by E_2 was inhibited by xanthorrhizol, curcumin and tamoxifen, especially by curcumin and tamoxifen at 40μM. The increased *WNT6* mRNA level was reduced remarkably by 40μM of xanthorrhizol and tamoxifen, respectively (Fig. 8B).

Effects of xanthorrhizol, curcumin and tamoxifen on the growth of ER-negative (ER-) MDA-MB-231 human breast cancer cells

The proliferation of MDA-MB-231 cells was inhibited in a dose-related manner by the treatment of xanthorrhizol, curcumin and tamoxifen for 24 hours in the absence of E_2 (Fig. 9A). IC_{50} of xanthorrhizol, curcumin and tamoxifen was 23μM, 23μM and 19μM, respectively. The proliferation of MDA-MB-231 cells was remarkably increased by 1nM E_2 (1.2-fold increase, $P < 0.05$) but was decreased by 10nM E_2 (0.8-fold increase, $P < 0.01$) at 6 day, compared with untreated cells (Fig. 9B)

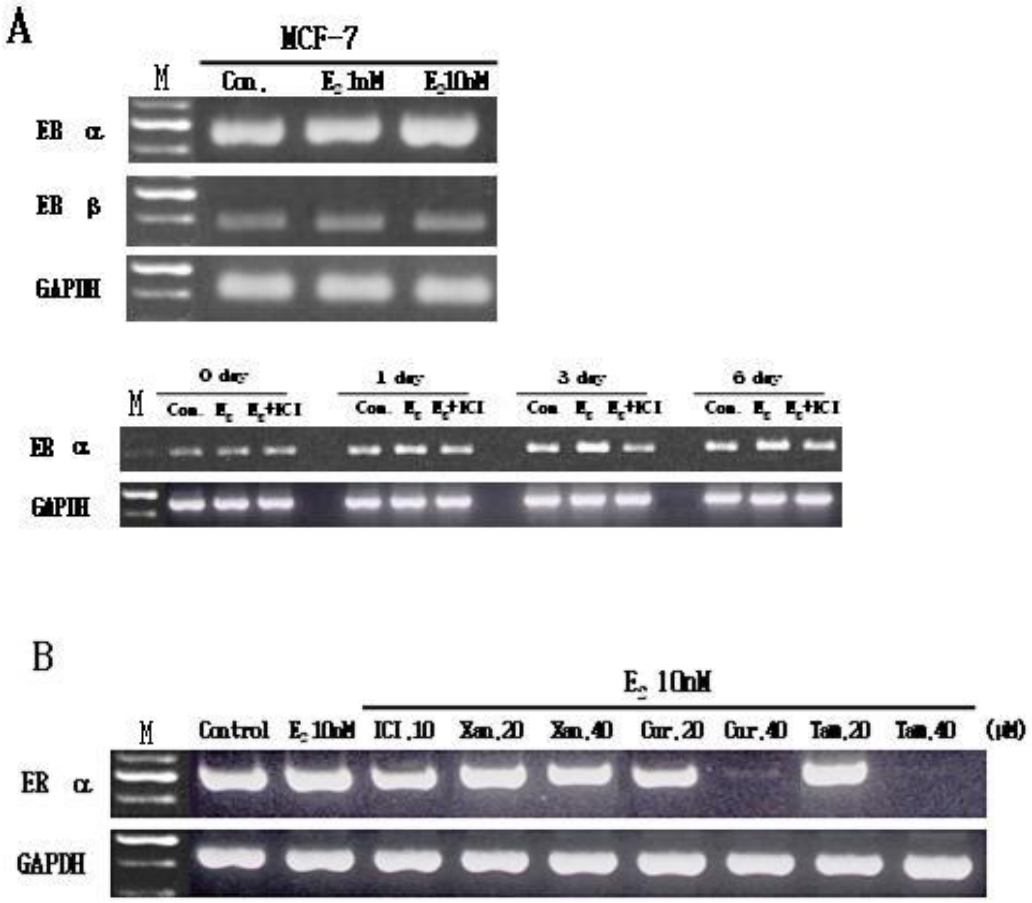


Fig. 7. The effects of xanthorrhizol, curcumin and tamoxifen on mRNA expression of *ER*s in MCF-7 cells treated with E_2. The mRNA expression levels of *ERα* (480bp) and *ERβ* (395bp) were evaluated by RT-PCR in MCF-7 cells treated with E_2 alone for 6 days (A), and with E_2 (10nM) and ICI182,780 (10μM), xanthorrhizol (xan.), curcumin (cur.), and tamoxifen (tam.) for 0, 1, 3 and 6 days (B). M : marker

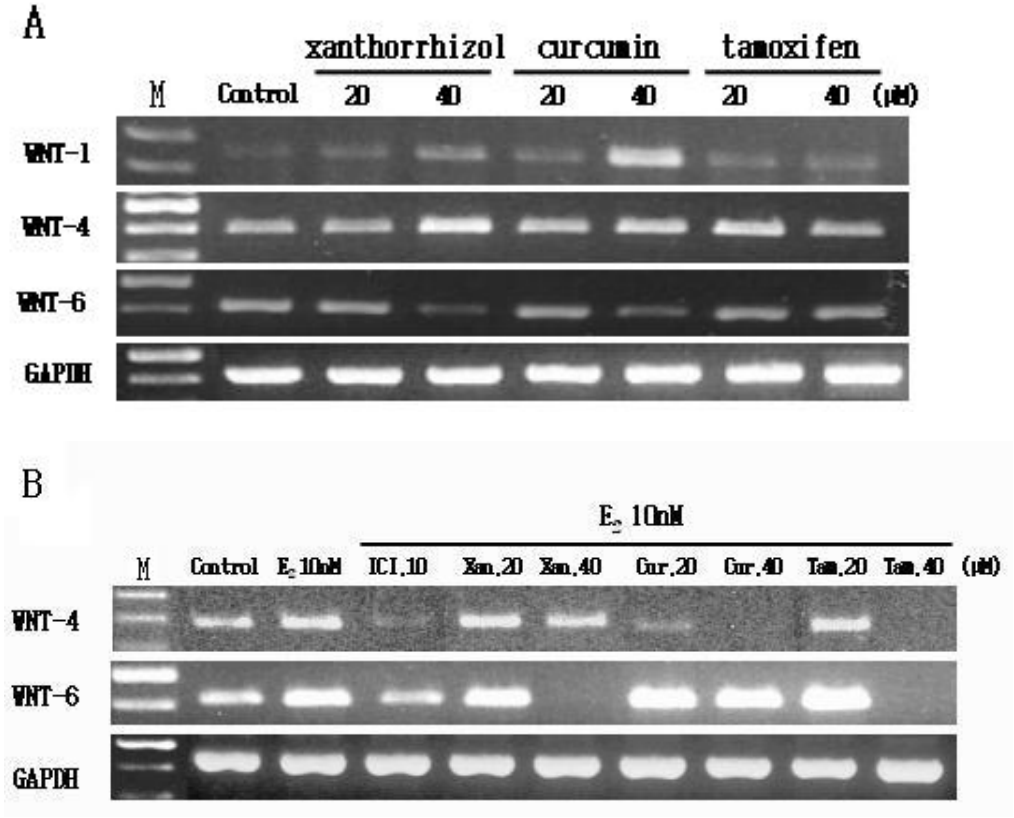


Fig. 8. The effects of xanthorrhizol, curcumin and tamoxifen on the mRNA expression of *WNT* family in MCF-7 cells. The mRNA expression levels of *WNT*s were evaluated by RT-PCR in E_2-umtreated MCF-7 cells for 24hours (A). The mRNA expression levels of *WNT-4* (400bp) and *WNT-6* (411bp) were evaluated in MCF-7 cells treated with E_2 alone and co-treated with ICI182,780, xanthorrhizol (xan.), curcumin (cur.) and tamoxifen (tam.) for 6 days by RT-PCR (B). M : marker

Effects of xanthorrhizol, curcumin and tamoxifen on the expression of VEGF in MDA-MB-231 cells

Xanthorrhizol and tamoxifen suppressed VEGF expression at 40μM in E_2-untreated cells, whereas VEGF expression scarcely reduced by curcumin (Fig. 10A). In addition, the level of *VEGF* mRNA did not change by E_2 stimulation (Fig. 10B).

Effects of xanthorrhizol, curcumin and tamoxifen on the expression of Wnt signaling components in MDA-MB-231 cells

In MDA-MB-231 cells, the expression of β-catenin, GSK-3β and Dvl was significantly downregulated and phosphor-GSK-3β (ser9) upregulated by xanthorrhizol and curcumin (Fig. 11). Xanthorrhizol showed a more significant inhibition on β-catenin and Dvl expression than curcumin, whereas curcumin relatively highly affected the expression and phosphorylation of GSK-3β in comparison with xanthorrhizol. The expression of Wnt signaling components did not modulate significantly by tamoxifen.

Identification of *WNT* and *FRIZZLED* (*FZ*) family expressed in MDA-MB-231 cells

To determine *WNT* and *FZ* family initiating Wnt signaling in MDA-MB-231 cells, we investigated mRNA expression of *WNT* and *FZ* family using RT-PCR in

E_2-treated MDA-MB-231 cells and untreated cells (Fig. 12). *WNT1, WNT3, WNT4, WNT6, WNT7B, WNT8B, WNT10A* and *WNT16* mRNA were identified and *FZ4, FZ5, FZ6* and *FZ7* mRNA also detected in E_2-untrated MDA-MB-231 cells, whereas *WNT2, WNT3A, WNT5, WNT5A, WNT7A, WNT8A, WNT14, WNT14B* and *FZ10* mRNA was not detected. In E_2-treated MDA-MB-231 cells, the expression of *WNT3, WNT4, WNT8B, WNT10A* and *WNT16* mRNA were upregulated by E_2. However, the mRNA levels of *WNT1, WNT6* and *WNT7B* were not affected by E_2 treatment. The mRNA levels of *FZ4, FZ5* and *FZ6* not *FZ7*, were also enhanced by E_2 treatment.

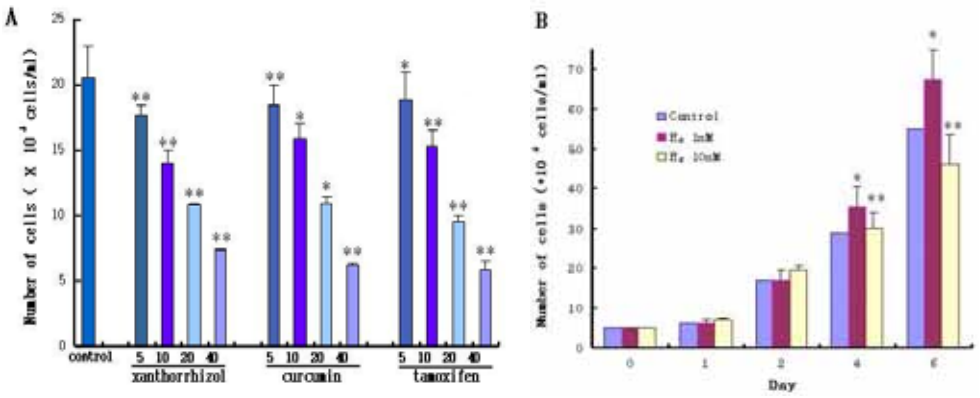


Fig. 9. The effects of xanthorrhizol, curcumin and tamoxifen on the viability of breast cancer cells in the presence or absence of E_2. (A) MDA-MB-231 cells (1×10^5 cells/dish) were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic for 24 hours. After reaching 80% confluent growth, xanthorrhizol, curcumin and tamoxifen were added to each dish at the indicated concentration. 24 hours later, cells were harvested and counted by trypan blue exclusion method. (B) MDA-MB-231 cells (5×10^4 cells/dish) were cultured in DMEM medium supplemented with 10% FBS for 24 hours. The media was changed to serum-free DMEM to starve for 24 hours. Then, media was changed to DMEM with 10% dextran-charcoal stripped FBS and cell were stimulated by 1 and 10 nM E_2. Cells were counted by trypan blue exclusion assay at 0, 1, 2, 4 and 6 days. * $p < 0.01$, ** $p < 0.005$

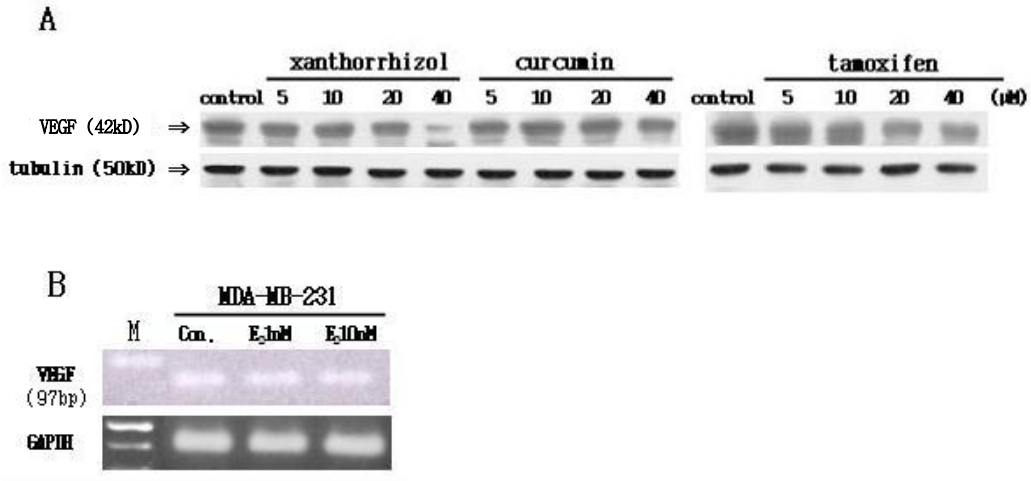


Fig. 10. The expression of vascular endothelial growth factor (VEGF) in MDA-MB-231 cells treated with xanthorrhizol, curcumin and tamoxifen in the presence or absence of E_2. (A) Cells were incubated in serum-free DMEM media containing various concentrations of xanthorrhizol, curcumin and tamoxifen. 24 hours later, the expression of VEGF was shown by western blotting as described in Materials and Methods. (B) The mRNA expression levels of *VEGF* (97bp) were evaluated by RT-PCR in MDA-MB-231 cells treated with E_2 alone for 6 days. M : marker

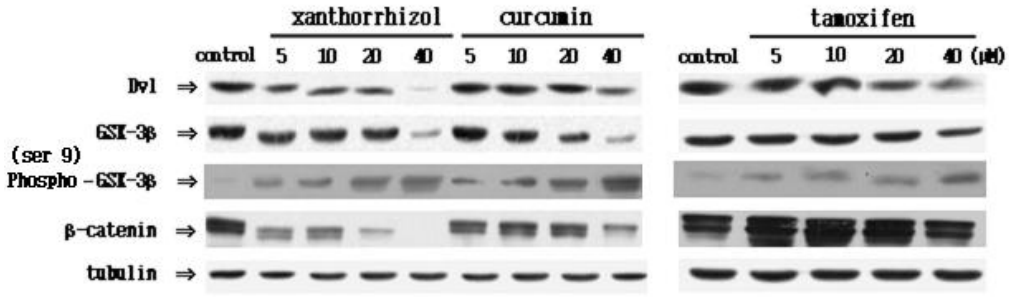


Fig. 11. The expression of Wnt signaling components in MDA-MB-231 cells treated with xanthorrhizol, curcumin and tamoxifen. MDA-MB-231 cells were incubated with the indicated concentrations of xanthorrhizol, curcumin and tamoxifen. After 24 hours, harvested cells were lysed with triple-detergent lysis buffer. Cytosolic protein concentration was determined by using the BSA protein assay reagent. Wnt signaling components was determined by western blotting analysis.

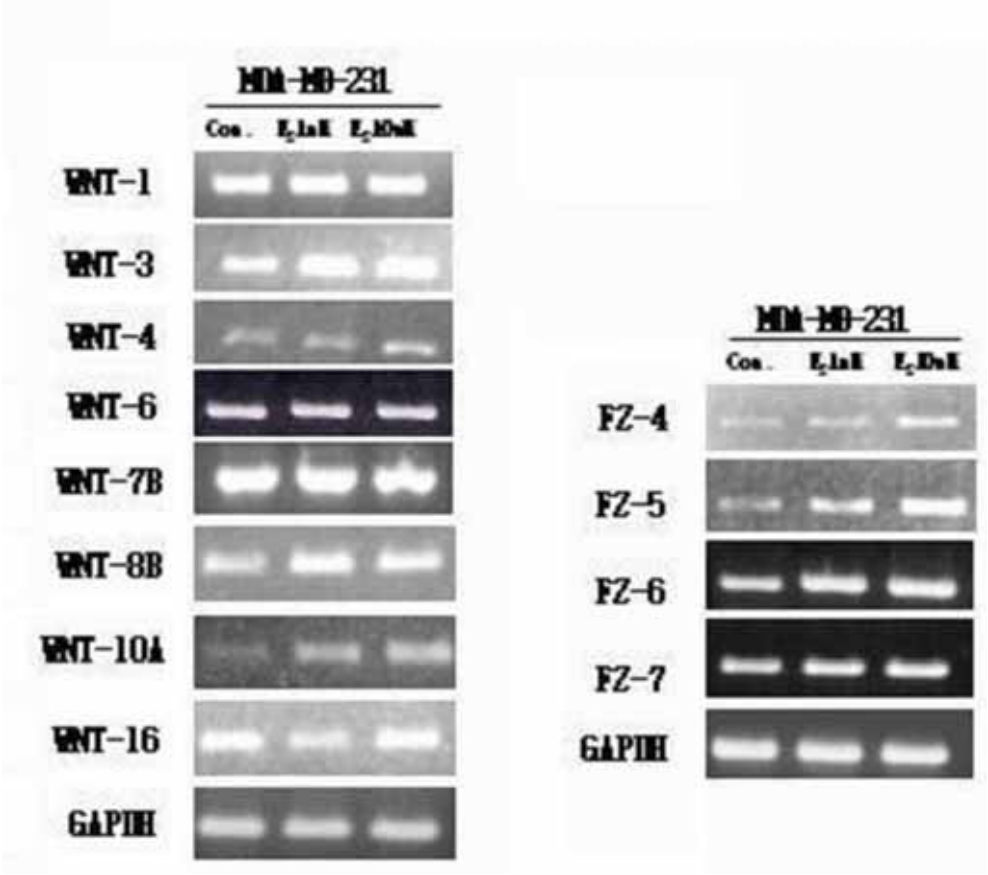


Fig. 12. The mRNA expression of *WNT* and *FRIZZLED* family in MDA-MB-231 cells. The mRNA expression levels of WNTs and *FRIZZLED* (FZs) were evaluated by RT-PCR in MDA-MB-231 cells without or with E_2-stimulation for 6 days.

Ⅳ. Discussion

Angiogenesis has become an attractive target for the development of novel anti-cancer drugs, based on its important roles in tumor growth, invasion, and metastasis. In particular, VEGF, not only a major angiogenic factor but also a survival factor for normal and cancer cells including breast cancer cells[16], is a potential target for antiangiogenic therapy because its overexpression has been associated with tumor vascularity, poor prognosis, and aggressive disease in many malignancies. Therefore, understanding the mechanism(s) driving the regulation of VEGF expression will be crucial in designing effective therapeutic strategies targeting VEGF to contol tumor growth and metastasis.

Although the mechanisms that upregulate VEGF in cancerous states are complex, it has been known that VEGF expression can be modulate by transcription factors (e.g. HIF-1, AP-1, NF-κB, Sp1, estrogens), agents influencing transcription factors (e.g. nitric oxide, prostaglandins, interleukin 1β, transforming growth factor α and β, tumor necrosis factor α) through different cellular signaling pathways, and the mutations of oncogenes (e.g. ras, erbB2, v-src) and tumor suppressor genes (e.g. von Hippel-Lindau). Recent reports also described that VEGF is a β-catenin target gene in HeLa

cells and colon cancer cells[17], and VEGF expression was reduced strikingly when colon cancer cells with elevated β-catenin levels were treated with *β-catenin* antisense oligonucleotides[18].

Through developmental, genetic and biochemical studies, a preliminary outline of Wnt signaling has been proposed[19,20]. Secreted Wnt ligands form complexes with receptors of the Frizzled family[21,22]. The signal is then transduced through Dvl, a negative regulator of the Wnt pathway inhibiting GSK-3β, The process by which the signal is propagated from dishevelled to GSK-3β is not understood. In the absence of Wnt signaling, GSK-3β phosphorylates β-catenin, targeting it for degradation[23]. In contrast, the Wnt-dependent inhibition of GSK-3β alleviates this negative regulation and increases β-catenin levels. β-catenin then binds to the Lef/Tcf family of transcription factors and, the complex translocates to the nucleus and binds directly to the promoters of Wnt-target genes.

In the present study, we first evaluated the suppressive effects of xanthorrhizol, curcumin and tamoxifen on cell growth and VEGF expression in ER+ MCF-7 cells and ER- MDA-MB-231 cells. Xanthorrhizol, curcumin and tamoxifen inhibited with a similar potency the growth of E_2-stimulated and untreated MCF-7 cells and MDA-MB-231 cells, respectively. VEGF was expressed highly in both ER+ and ER- breast cancer cells. While xanthorrhizol, curcumin

and tamoxifen in MCF-7 cells reduced in a dose-related manner the increased *VEGF* expression by E_2 stimulation as well as *VEGF* expression of E_2-unstimulated cells, xanthorrhizol reduced markedly VEGF expression at 40μM concentration and tamoxifen and curcumin showed a weak inhibition in MDA-MB-231 cells.

Furthermore, we estimated the effects of xanthorrhizol, curcumin and tamoxifen on Wnt signaling pathway as a possible mechanism involved in regulating VEGF expression in both ER+ and ER- breast cancer cells. Xanthorrhizol, curcumin and tamoxifen decreased the expression of Dvl, GSK-3β and β-catenin and increased the phosphorylation of GSK-3β in a dose-related manner in MCF-7 cells. However, in MDA-MB-231 cells, the level of Dvl and GSK-3β inhibited at 40μM of xanthorrhizol, curcumin and tamoxifen, while β-catenin expression was reduced and the phosphorylation of GSK-3β was elevated by xanthorrhizol and curcumin in a dose-dependent manner. Tamoxifen did not affect β-catenin expression.

Based on our data demonstrating the expression of Wnt signaling components in both ER+ and ER- breast cancer cells, to determine Wnt ligands triggering the signal cascade, the mRNA expressions of Wnt family were investigated in MCF-7 and MDA-MB-231 cells, respectively. In two breast cancer cells which were not stimulated by E_2, *WNT1*[24], *WNT3*, *WNT4*, *WNT6*, *WNT7B*[25],

WNT8B, *WNT10A*[26] and *WNT16* mRNA expressions were identified but *WNT2*[27], *WNT5*, *WNT5A*[28], *WNT7A*, *WNT8A*[29], *WNT14* and *WNT14B* mRNA were not observed. *WNT3A* mRNA was detected only in MCF-7 cells. In E_2-stimulated MCF-7 cells, *WNT1*, *WNT3*, *WNT4* and *WNT6* mRNA levels were upregulated and *WNT3A*[30] and *WNT10A*[31] mRNA levels were downregulated. These results are in accord with the previous studies reported by other researchers.

WNT1 and *WNT4* mRNA levels were elevated by xanthorrhizol, curcumin and tamoxifen in MCF-7 cells, whereas *WNT6* mRNA levels was reduced by xanthorrhizol and curcumin and not influenced by tamoxifen. Interestingly, in E_2-treated MCF-7 cells, the increase of *WNT4* mRNA level was blocked by xanthorrhizol, curcumin and tamoxifen, especially by 40μM of curcumin and tamoxifen. The increased *WNT6* mRNA level by E_2 stimulation was completely inhibited 40μM of xanthorrhizol and tamoxifen, but curcumin rarely inhibited the *WNT6* mRNA expression. These results demonstrate that xanthorrhizol and tamoxifen blocked the signaling cascade by *Wnt4* and *Wnt6* and curcumin by *Wnt4*, resulting in the decrease of *VEGF* expression in E_2-stimulated MCF-7 cells. Moreover, the elevated mRNA levels of *WNT4* and *WNT6* by E_2 treatment were downregulated by ICI182,720, an synthetic estrogen antagonist. Therefore, *WNT4* and *WNT6* mRNA expression seem to be upregulated through ER-dependent pathway. As expected, the enhanced *ERα* mRNA level in E_2-treated MCF-7 cells

was decreased by xanthorrhizol, curcumin and tamoxifen, especially to lower level compared with untreated cells by 40μM of curcumin and tamoxifen.

Ⅴ. Conculsion

In the present study, xanthorrhizol, curcumin and tamoxifen inhibited with a similar potency the growth of E_2-stimulated and untreated MCF-7 cells and MDA-MB-231 cells, respectively. Xanthorrhizol, curcumin and tamoxifen in MCF-7 cells reduced in a dose-related manner the increased VEGF expression by E_2 stimulation as well as VEGF expression of E_2-unstimulated cells. However, xanthorrhizol reduced markedly VEGF expression at 40 μM concentration and tamoxifen and curcumin showed a weak inhibition in MDA-MB-231 cells.

Furthermore, we estimated the effects of xanthorrhizol, curcumin and tamoxifen on Wnt signaling pathway involved in regulating VEGF expression in both ER+ and ER- breast cancer cells. Xanthorrhizol, curcumin and tamoxifen decreased the expression of Dvl, GSK-3β and β-catenin and increased the phosphorylation of GSK-3β in a dose-related manner in MCF-7 cells. However, in MDA-MB-231 cells, the level of Dvl and GSK-3β inhibited at 40 μM of xanthorrhizol, curcumin and tamoxifen, while β-catenin expression was reduced and the phosphorylation of GSK-3β was elevated by xanthorrhizol and curcumin in a dose-dependent manner. Tamoxifen did not affect β-catenin expression.

WNT1 and *WNT4* mRNA levels were elevated by xanthorrhizol, curcumin and tamoxifen in E_2-unstimulated MCF-7 cells, whereas *WNT6* mRNA levels was reduced by xanthorrhizol and curcumin and not influenced by tamoxifen.

Interestingly, in E_2-treated MCF-7 cells, the increase of *WNT4* mRNA level was blocked by xanthorrhizol, curcumin and tamoxifen, especially by 40μM of curcumin and tamoxifen. The increased *WNT6* mRNA level by E_2 stimulation was completely inhibited by 40μM of xanthorrhizol and tamoxifen, but curcumin scarcely inhibited the *WNT6* mRNA expression. Our results demonstrate that xanthorrhizol and tamoxifen block the signaling cascade by *Wnt4* and *Wnt6* and curcumin by *Wnt4*, resulting in the decrease of VEGF expression in E_2-stimulated MCF-7 cells.

Additionally, the enhanced *ERα* mRNA level in E_2-treated MCF-7 cells was decreased by xanthorrhizol, curcumin and tamoxifen, especially by 40μM of curcumin and tamoxifen.

Taken together, xanthorrhizol exerts a greater inhibitory effect on VEGF production in ER+ and ER- human breast cancer cells despite a similar potency on the cell growth, compared with curcumin and tamoxifen. The downregulation of VEGF results from the inhibition of Wnt signaling components and *ERα* mRNA expression by xanthorrhizol. Therefore, xanthorrhizol can be a beneficial chemopreventive agent in hormone-responsive tumors.

Ⅵ. References

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국문 요약

유방암 세포 주에서 vascular endothelial growth factor 의 발현과 Wnt/β-catenin 신호전달의 활성화에 따른 xanthorrhizol, curcumin 과 tamoxifen 의 효과

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Vascular endothelial growth factor(VEGF)은 종양 신생혈관을 촉진하는 요소이다. 최근 연구에서 Wnt/β-catenin 신호 전달 체계가 일반적인 이거나 병적인 상태에서 혈관 발달을 조절한다고 보고하고 있다. Wnt 신호전달 체계의 활성화는 여러 인간의 종양 형성의 중요한 특징 이며, 또한 cyclin D1, c-myc and VEGF 와 같은 세포 내의 암유전자 전사를 조절하는 β-catenin 의 세포 내의 세포질에 안정화를 이끄는 것으로 보여진다. 그럼으로, 항 발암성의 약제 조사에 있어 Wnt/β-catenin 신호전달 체계의 구성요소가 전도 유망한 표적이 될 수 있을 것 이다.

Xanthorrhizol, curcumin 과 tamoxifen 이 VEGF 조절에 대한 효과는 estrogen receptor (ER)-positive and ER-negative 형태의 유방암 세포들에서 17β-estradiol (E_2)의 유무에 따른 Wnt/β-catenin 신호체계를 통한다고 생각한다. MCF-7 cell 과 MDA-MB-231 cells 의 생존율은 xanthorrhizol, curcumin 과 tamoxifen 의 농도 의존적으로 감소 되었다. MCF-7 cells 과 MDA-MB-231 cells 에서 Wnt 신호전달 체계의 구성요소(Dvl(disheveled), GSK-3β(normal and phosphor-form), β-catenin)와 VEGF 의 발현은 xanthorrhizol, curcumin 과 tamoxifen 에 의해서 농도의존적으로 감소되었다. 우리는 MCF-7 cells 에서 ER-α, VEGF, WNT-3, WNT-4 와 WNT-6 의 mRNA level 뿐만 아니라, 세포 증식도 E_2 에 의해서 증가하는 것을 발견하였다. 그러나, MDA-MB-231 cells 에서는 VEGF mRNA 의 level 이 E_2 에 의해서 증가 되지 않았다. Xanthorrhizol, curcumin 과 tamoxifen 은 MCF-7 cells 에서 E_2 로 유도된 ER-α, VEGF, WNT-4 의 mRNA level 을 감소시켰다. 그럼으로 xanthorrhizol, curcumin 과 tamoxifen 은 유방암 세포의 성장뿐만 아니라 Wnt/β-catenin 신호체계를 통한 VEGF 발현의 감소에 의한 신생혈관 생성을 억제 한다.

핵심 되는 말: xanthorrhizol, VEGF, Wnt/β-catenin 신호전달, 유방암세포,