Thesis for the Degree of Master of Science

Anticaner Activity of gamma-Tocotrienols on MCF-7 Human Breast Cancer Cells

February, 2006

Department of Food and Life Science

Graduate School

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감마 토코트라이에놀의 MCF-7 유방암 세포에 대한 항암성

Advisor : Prof. Hong-Soo Ryu

Min Soo Kim

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Anticaner Activity of gamma-Tocotrienols

on MCF-7 Human Breast Cancer Cells

A dissertation

by

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February 28, 2006

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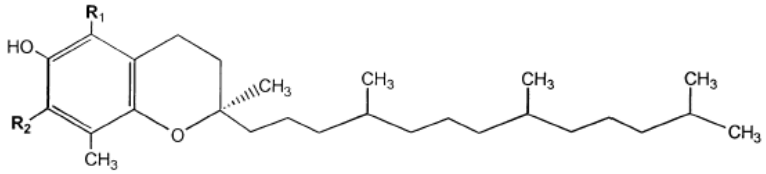
Abstract

Anticancer activity of γ-tocotrienols from rice bran oil was investigated using estrogen-responsive MCF-7 human breast cancer cell line. γ-Tocotrienols induced MCF-7 cells to undergo apoptosis, with concentrations for half-maximal response of 18 μg/ml. DNA fragmentation was induced by γ-tocotrienols treatment with dose- and time-dependent manner. The change of mRNA expression of MCF-7 breast cancer cells treated with γ-tocotrienols was evaluated with microarray analysis. Thirteen genes of MCF-7 cells were 2-fold over-expressed by γ-tocotrienols treatment and thirty-one genes were 0.5-fold down-expressed by same treatment, which provides an overview of the gene expression profiles during apoptosis of MCF-7 cells by γ-tocotrienols treatment. Among the over-expressed genes, JNK was selected to confirm change of mRNA expression level by RT-PCR. Western blot data of phospho-JNK revealed activation of JNK. Over-expression of JNK in MCF-7 cells treated with γ-tocotrienols was confirmed by dose- and dose-dependent pattern. Jun, which is a downstream gene of JNK, was also activated by γ-tocotrienols treatment, which suggests that apoptosis of MCF-7 cells by γ-

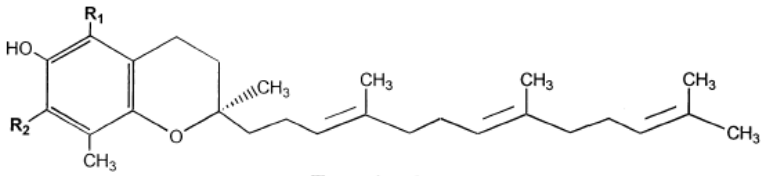
tocotrienols treatment was mediated through mitogen–activated protein kinase (MAPK) pathway.

1. INTRODUCTION

Tocopherols and tocotrienols have a structural similarity including a common chromanol head and a side chain at the C-2 position, that comprise eight compounds, α, β, γ, δ-tocopherols and α, β, γ, δ-tocotrienols (Fig. 1), totally called Vitamin E. Tocopherols and tocotrienols, as potent antioxidants, inhibit the peroxidation of lipids and scavenge the chain-propagnating peroxyradical (Guthrie et al., 1997). Their antioxidant activities are suggested to prevent cardiovascular diseases (CVD) and cancer by arresting free radical damage (Kamal et al., 1996; Meydani, 1995). Vitamin E inhibits mutagenesis and cell transformation mainly through its antioxidant function, eliminating oxygen free radicals and decreasing DNA damage (Maydani et al., 1995). Although they have potent antioxidant activities, the anticancer activity of those compounds is not dependent on their antioxidants (Packer et al., 1991). Interestingly, two studies that compared the effects of tocopherol and tocotrienols on human breast cancer cells showed that tocotrienols inhibited cell growth (Nesaretnam et al., 1995), whereas α-tocopherol had no anticancer activity (Yu et al., 1999). Therefore, tocotrienols apparently had



Tocopherol



Tocotrienol

	R_1	R_2
α-Tocotrienol/ Tocopherol	CH_3	CH_3
β- Tocotrienol/Tocopherol	CH_3	H
γ- Tocotrienol/Tocopherol	H	CH_3
δ– Tocotrienol/Tocopherol	H	H

Fig. 1. Structures of various homologs of tocopherols and tocotrienols. The four tocotrienols share a similar chromanol moiety with their corresponding tocopherols. Tocopherols have a saturated phytyl side-side chain and tocotrienols have an unsaturated prenylated side-chain.

anticancer activities to the human breast cancer cells without depending on their antioxidant activity, since α-tocopherol was unable to induce apoptosis. Their antioxidant activity to cancer are still controversy, it is obvious that they are working as effective inducers of arresting DNA synthesis in cancer cell. The order of inducing activity of DNA synthesis arresting is reported as follows: γ-tocotrienols > δ-tocotrienols > α-tocotrienols > δ-tocopherl > γ-tocopherol > α, β-tocopherol for MCF-7 cells and δ, γ-tocotrienol > δ-tocopherol > α-tocotrienol > α, β or γ-tocopherol for MDA-MB 435 cells (Guthrie et al., 1995; Nesaretnam et al., 1995).

Tocotrienols found in palm oil and rice bran oil are lipid-soluble compounds and a phytochemical class of isoprenoid molecules (Packer et al., 2001). A few cellular studies have been reported on characterization of the ability of tocotrienols to induce cancer cell apoptosis. Recent studies have shown that tocotrienols can exert direct inhibitory effects on cell growth in human breast cancer cell lines (Guthrie et al., 1995) and inhibitory effects occurred irrespective of estrogen receptor status of the cells (Nesaretnam et al., 1998). It was also reported that tocotrienols inhibit the growth of estrogen responsive ZR-75-1 breast cancer cell (Nesaretnam et al., 2000)

and both in the absence and in the presence of 10^{-8}M oestradiol. α, β, γ and δ–tocotrienols are potent inducers of apoptosis of estrogen-response and estrogen-nonresponse human breast cancer cell in culture (Yu et al**.,** 1999). Apoptosis induced by tocotrienol was demonstrated through DNA fragmentation in preneoplastic (CL-S1), neoplastic (-SA), and highly malignant (+SA) mammary ephithelial cells (Mcintyre et al., 2000), although the apoptotic mechanisms were fully characterized. γ-Tocotrienol induced apoptosis of malignant +SA mouse mammary ephithelial cells by increased level of cleaved active caspase-8 and caspase-3 (Shah and Sylvester, 2004). Also, γ-tocotrienols triggered the emergence of mitochondrial permeability transition (MPT) leading to eventual disruption of mitochondria in MDA-MB cells (Takahashi and Loo., 2004). Vitamin E succinate which have structural similarity with tocopherols, is a potent apoptic compound to breast cancer cells and its anticancer mechanism was related to Bax translocation for MDA-MB-435 (Yu et al., 2003), converting Fas resistant to Fas sensitive for MCF-7 (Yu et al., 1999), activation of MAPK and c-jun terminal kinase (Yu et al., 2001).

A diversity of extracellular stimuli derives cellular activities such as survival,

proliferation, differenciation and apoptosis through the activation of a family of mitogen-aciviated kinase (MAPKs) consisting of the extracellular signal-regulated kinases (ERKs), the p38MAPK, and the c-Jun N-terminal kinases (JNKs) (Davis, 2000). Each MAPK is activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) located in the activation loop (T-loop). The phosphorylation is mediated by a MAPK kinase (MAPKK) that is activated by phosphorylation of MAPKK kinase (MAPKKK) (Fig. 2). These MAPK are therefore activated by a kinase signaling cascade (Davis, 2000). The c-jun NH_2-termianl kinase (JNK), also known as stress-activated MAP kinases (SAPKs) serve as phosphorylation substrates for MAPK kinase kinases (MAPKKs) such as MAPKK4/7, which are activated in turn by phosphorylation via MAP kinase kinases (MAPKKs). JNK is activated in response to many different stress factors including heat shock, inflammatory cytokines, protein synthesis inhibitors, growth factor withdrawal, chemotheraputic drugs, and ultraviolet irradiation (Peng et al., 2004). JNK contains the dual phosphorylation motif Thr-Pro-Tyr (Davis, 2000). Biomedical studies led to the identification

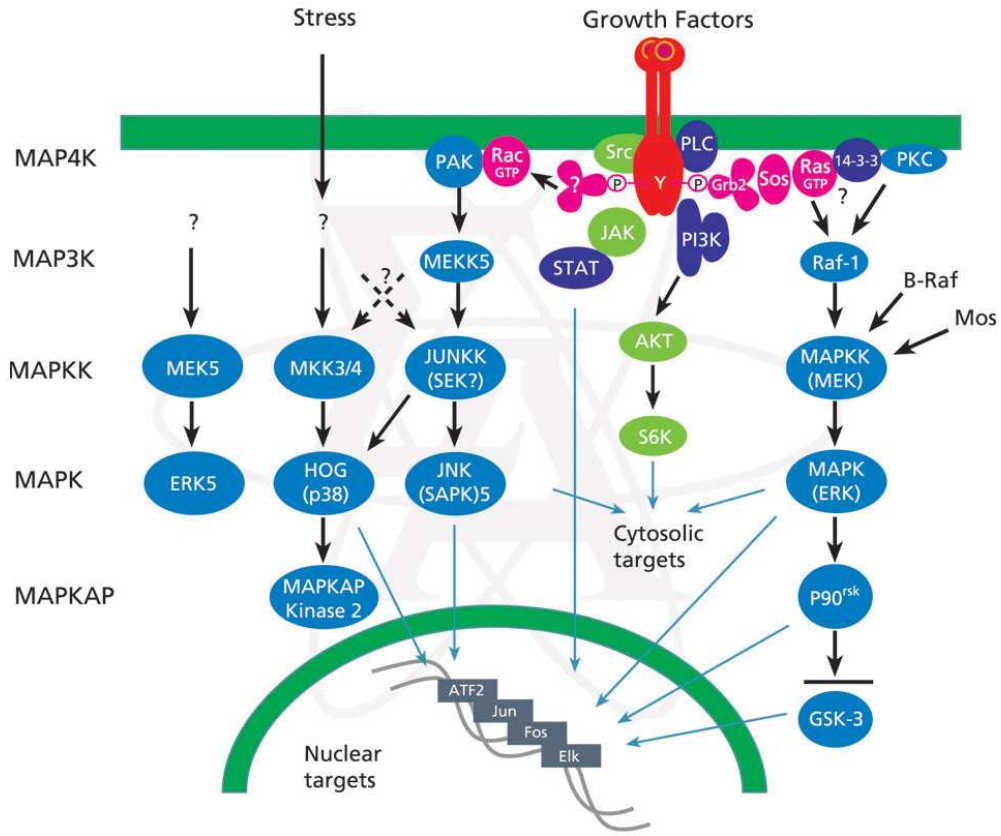


Fig. 2. The mitogen-activated protein kinase (MAPK) cascades. MAPK pathways operate through sequential phosphorylation events to phosphorylate transcription factors and regulate gene expression.

MAPK : The mitogen-activated protein kinase

MAPKK : The mitogen-activated protein kinase kinase

MAP3K : The mitogen-activated protein kinase kinase kinase

MAP4K : The mitogen-activated protein kinase kinase kinase kinase

MEK : MAP2K, also called MKK or MAP/ERK kinase

ERK : extracellular signal-regulated kinase

and purification of JNK as a “p54 microtubule-associated protein kinase” that was activated by cycloheximide (Kyriakis and Avruch, 1990). JNK was formed to bind the NH_2-terminal activation domain of c-jun (Alder et al., 1992; Hibi et al., 1993) and to phosphorylate c-jun on Ser-63 and Ser-73 (Pulverer et al., 1991). JNK also phosphorylates other AP-1 proteins, including Jun B, Jun D, and ATF2 (Ip and Davis, 1998). In each case, the sites of phosphorylation correspond to Ser/Thr-Pro motifs located in the activation domain of the transcriptional factor. Substrate recognition by JNK requires a docking site to tether the kinase to the subtrate (Hibi et al., 1993). A critical role for JNK appears to be the regulation of AP-1 transcription activity. This conclusion is supported by genetic analysis of Jun and JNK in *Drosophila* (Ip and Davis, 1998). The JNK protein kinases are encoded by three genes. The Jnk1 and Jnk2 genes are expressed ubiquitously. In contrast, the Jnk3 gene has more limited pattern of expression and is largely restricted to brain, heart, and testis. These genes are alternatively spliced to create ten JNK isoforms (Gupta et al., 1996). Transcripts derived from all three genes encode proteins with and without a COOH-terminal extension to create both 46 kDa and 55 kDa isoforms.

Recent advances in functional genomics have made possible new approaches to the diagnosis and management of a wide array of disorders. With new tool to explore gene expression and regulation, researchers can understand better the molecular basis of disease. Significant improvements in substrate materials, robotics, and signal detection have made possible miniaturization of arrays with the result that hundreds of thousands of oligonucleotides can be arrayed on a square-centimeter chip. This important feature makes it possible to study gene expression without specifying in advance which genes are to be studied. Thus, DNA microarrays permit systematic and comprehensive surveys of gene expression in an efficient manner (Kurella et al., 2001).

In this study, we screened gene expression profiles of MCF-7 cells treated with γ-tocotrienol and identify MAP kinase mediated apoptotic pathway from mRNA expression levels. Tocotrienols induced apoptosis of breast cancer cell, however, stress-activated MAP kinase modules have not been reported before. In this study, MAP kinase mediated apoptotic evidence was found on MCF-7 breast cancer cell lines and investigated its mRNA and protein expressions.

2. Materials and method

1) Material

RPMI 1640 for MCF-7 media and penicillin-streptomycin were purchased from Gibco BRL Life Technologies (CA, USA) and fetal bovine serum (FBS) purchased from Hyclone Laboratories Inc. (UT, USA). MTT, 1-(4,5-dimethylthiazol-2-yl)-, 5-diphenylformazan, trypsin, sodium bicarbonate and sodium pyruvate were obtained from Sigma Chemical Co. (MO, USA). TRizol reagent, from Promega Co. (WI, USA), for RNA isolation, RT-PCR PreMix kit from the Bioneer Co. (Daejeon, Korea), 100 bp DNA ladder from Takara Bio. (Shinga, Japan) and oligo primers from Bioneer Co. (Daejeon, Korea) were purchased. Agarose gel from Amresco Inc. (OH, USA) was used for electrophoresis.

2) Methods

Purification of γ-tocotrienol

Concentrated rice bran oil (Nutriene®) was obtained from the Eastman Chemical Co. (TN, USA). γ-Tocotrienol from the Nutriene® was purified using

normal phase HPLC with a silica column (Supelcosil LC-Si, 25 cm x 4.6 mm, Supelco, Bellefonte, PA). The mobile phase was hexane, ethyl acetate, and acetic acid (99, 0.5, and 0.5%, respectively). The flow rate was 2 ml/min. The excitation and emission wavelengths of the fluorescence detector were set at 290 and 330 nm, respectively. γ-Tocotrienol fraction was collected and used for this experiment.

Cell culture

MCF-7 cells, estrogen-responsive breast cancer cell line, obtained from Korea cell line bank (Seoul, Korea) and were grown in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin (10,000 U/ml penicillin and 10mg/mL streptomycin), The cells were cultured at 37^{o}C in a humidified incubator (95% air, 5% CO_2). The cells were subcultured every week at a ratio of 1:4, and cell culture medium was renewed two or three times a week.

Cell viability and proliferation

Cells were inoculated at a density of 10^5cells/well into 96-well plates and

cultured overnight before challenge with a series of concentrations of rice bran oil or γ-tocotrienols for dose-dependent of RBO and γ-tocotrienols. 0, 10, 25, 50 and 100 μg/ml of RBO and 0, 1, 10, 25 and 50 μg/ml of γ-tocotrienols were treated with MCF-7 cells for 24 hr. RBO and γ-tocotrienol were treated with MCF-7 cells for 1, 2, 4, 6, 12 and 24 hr for time-dependent experiment. MTT assay was applied to measure cell viability and proliferation. MTT stock solution of 5 mg/ml was prepared in dimethly sulfoxide (DMSO) and removed the small amount of insoluble reside. Ten microliters MTT solution (5 mg/ml) was mixed with 90 μl serum free RPMI-1640 media and added to each well of microplate. Cells were incubated in a humidified incubator at 37℃ for 4 h. After centrifugation at 3000 x g for 5 min, 100 μl DMSO was added to the well plate and shook for 20 min. Absorbance of each well was measured with ELISA reader at 540 nm.

DNA fragmentation

Two hundered microliters of lysis buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, 0.5% (w/v) SDS) and 20 μg/ml DNase-free pancreatic RNase were added to

harvested cells and incubated at 38℃ for 1 hr. Five hundred microliters of lysis buffer and 2 μl of proteinase K (20mg/ml) were added to the tube. After incubation at 52℃ for 3 hr, equalilibrated phenol B 700 μl was added and centrifuged at 13,000 x g for 1 min. Six hundered microliters of supernatant were taken, PCI solution (phenol : chloroform : isoamylalcohol, 25 : 24 : 1) was added and centrifuged at 13,000 x g for 1 min. Five hundred microliters of supernatant were taken, 500 μl of chloroform : isoamlyalcohol (24 : 1) were added and then centrifuged at 13,000 x g for 1 min. Four hundred microliters of supernatant was taken, and 40 μl of 3 M sodium acetate (pH 5.2) was added. After ethanol precipitation, DNA was redissolved in 30 μl of TE buffer. Isolated DNA was separated on 0.7% agarose gel, and visualized with an ultraviolet transluminator.

RNA isolation

Cells were washed with cold PBS and collected by a cell scraper. The harvested cells were lysed with TRIzol reagent in microcentrifuge tubes. Tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2 to 3

minutes. Mixture was centrifuged at no more than 12,000 x g for 15 minutes at 2 to 8 ℃. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol was used to 1 ml of TRIzol reagent. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 2 to 8 ℃. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing. After centrifugation at 7,500 x g for 5 minutes at 2 to 8 ℃, the RNA pellet was briefly dried and dissolved into DEPC treated water.

Microarray Analysis

- DNA microarray fabrication

The experiments were performed on Platinum Biochip Human cancer 3.0K oligo microarrays (Genochek Co. Daejeon, Korea). A Platinum Biochip Human cancer 3.0K oligo microarray consists of 3096 oligo spot including Array_Ready Oligo (Operon, Germany) set, housekeeping genes, and Arabidopsis DNA as controls. The Oligo products were resuspended in 15 ㎕ of spotting solution

(Genocheck Co. Daejeon, Korea), and spotted onto CMT-GAPS Ⅱ silane slide glass (Corning Co. CA, USA) with a pixsys 5500 arrayer (Cartesian Technologies. CA, USA) using 24 Stealth Micro spotting pins. The printed slides were processed according to CMT-GAPS Ⅱ slide protocol. Briefly, the spots were rehydrated with SSC buffer (2×saline sodium citrate) for 1 min and then DNA linked using a UV cross linker (Stratagene Co. CA, USA). The slides were quickly transferred to 95% ethanol for 1 min and dried using a centrifuge at 3000 x g for 20 sec.

- Preparation of fluorescent DNA probe and hybridization

Total RNA was extracted from the treated and untreated MCF-7 cells after 24 hr using the TRIzol reagent. Fluorescence labeled cDNA probes were prepared form 40 ㎍ of total RNA by oligo $(dT)_{18}$-primed polymerization using SuperScript Ⅱ reverse transcriptase (Invitrogen Co. NY, USA) in a total reaction volume of 30 ㎕. The reverse transcription mixture included 400 U Superscript RNase H-reverse transcriptase (Invitrogen Co. NY, USA), 0.5 mM dATP, dTTP and dGTP, 0.2 mM dCTP and 0.1 mM Cy3 or Cy5 labeled dCTP (NEN Life Science, MA, USA). After reverse transcription, the RNA was degraded by adding 5 ㎕ of stop solution (0.5M

NaOH/50m EDTA) and incubating 65℃ for 10 min. The labeled cDNA mixture was concentrated using ethanol precipitation method. The concentrated Cy3 and Cy5 labeled cDNAs were resuspended in 20 ㎕ of hybridization solution (Genocheck Co. Daejeon, Korea). After two labeled cDNAs were mixed, the mixture was denaturized 95 ℃ for 2 min and then incubated in 45℃ water chamber for 20 min. The cDNA mixture was placed on the spotted slide position and covered by a cover slip (22 mm x 22 mm). The slides were hybridized for 12 hr at 62℃ hybridization chamber. The hybridized slides were washed in 2 x SSC containing 0.1 % SDS for 2 min, 1 x SSC for 3 min, and finally 0.2 x SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 sec to dry.

- Scanning and image analysis

Hybridized slides were scanned with the Axon Instruments GenePix 4000B scanner and the scanned images were analyzed with the software program GenePix Pro 5.1 (Axon Co. CA, USA) and GeneSpring 6.1 (Sillicongenetics Co. CA, USA), R package. In order to allow algorithm to eliminate all bad spots, no data points were eliminated by visual inspection from the initial GenePix image. For signal

normalization, positive control genes were spotted onto each slide. The signals of these spots were used for normalization. To determine the background signal intensity, the spotting solution was spotted on each slide. To filter out the unreliable data, spots with signal-to-noise (signal-background- background SD) below 100 were not included in the data. Data were normalized by Global, print-tip and scaled normalization for data reliability. Interested gene are sorted 2 fold changed gene between test and control sample.

RT-PCR analysis

One microliter of sample was added to 10 pmole of the reverse primers and heated at 70℃ for 5 min. Mixture of template and primer was put into ice bath and transferred the incubated mixture. Forward primers are added to Bionner AccuPower RT/PCR PreMix (Daejeon, Korea) tube and make up 20 μl of reaction volume with DEPC treated DW. Each cycle optimized for target genes was performed with a GeneAmp PCR system 2700 (Applied biosystems Co. CA, USA) at 42℃, for reverse transcription 60 min (cDNA synthesis)and 94℃ for 5 min (RTase

inactivation) and PCR was performed at 94℃ for 0.5 min, 59℃ for 0.5 min and 72℃ for 1 min and then 72℃ for 5 min at the end of procedure.

Table 1 shows the sequences of synthesized oligonucleotide primers used for RT-PCR of JNK (MAPK8) and Jun. Aftter PCR procedure, 10 μl sample aliquots were electrophoresed on 1% agarose gel and stained with ethidium bromide and photographed. GAPDH was used as a housekeeping gene.

Western blot analysis

MCF-7 cells treated with 18 μg/ml γ-tocotrienol were harvested after 0, 1, 2, 4, 6, 12 and 24 hr incubation. The total proteins were extracted by adding 200 μl of lysis buffer (50mM Tris-HCl (pH 7.4), 1% Triton ×100, 1 mM EDTA, 150mM NaCl) and protease inhibitor cocktail (Boehringer Mannheim, Germany) was added to cell pellets on ice, sonicated for 5 sec twice, followed by centrifugation at 8000 x g for 15 min at 4℃. Supernatant proteins were measured by Bradford assay with bovine serum albumin as a standard. Total cytosolic extracts (40 μg of protein) were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to

Table 1 Oligonucleotide primers for RT-PCR of JNK (Mapk-8) and Jun mRNA level.

Genes		Oligo	Product size	Annealing Tm (^{o}C)	Cycles
JNK (Mapk-8)	*F	**5'-GGGACTTAAAGCCCAGTAAT-3'**	382	59	28
	*R	**5'-TCAGGGAAGAGTTTCTCAAA-3'**			
Jun	F	**5'-GGAGGAAAAAGTGAAAACCT-3'**	476	59	25
	R	**5'-ATGTTAGGTCCATGCAGTTC-3'**			

***F; forward primer, *R; reverse primer**

nitrocellulose membrane (BIO-RAD lab. CA, USA). The membrane was blocked in 10% milk buffer (Dry milk, 20mM Tris-HCl, pH 7.5, and 136mM NaCl) for 20 min and incubated with overnight at 4℃ with indicated primary antibodies (1:500 dilution). After incubation with primary antibodies, the membrane was washed with washing buffer (0.1% Tween 20, 20mM Tris-HCl, pH 7.5 and 136mM NaCl) twice, incubated with HRP-labeled secondary antibody for 1 hr at room temperature, and washed with washing buffer twice. Final detection was performed with Western Lightning (PerkinElmer Life Sciences, MA, USA).

3. Results and Discussion

Chromatogram of rice bran oil and γ-tocotrienol

Rice bran oil is a rich natural source of vitamin E, containing up to 300 mg/kg (Shin et al., 1997). Figure 3 shows chromatogram of concentrated rice bran oil (A) and purified γ-tocotrienol (B) by normal phase HPLC with a silica column. From Fig. 3A, concentrated rice bran oil contains 25-30% of γ-tocotrienols.

Effect of tocotrienol on cell vialbility

IC_{50} (concentration causing 50% cell death) of RBO was estimated to be 126 (±12) μg/ml for MCF-7 from Fig. 4 and 6. IC_{50} of γ-tocotrienols was determined to be 18 (±1.2) μg/ml for MCF-7 from Fig. 5 and 7. Our result showed that rice bran oil has anticancer activities on estrogen-responsive human breast cancer cells and isolated γ-tocotrienols showed much higher anticancer activity to breast cancer cells compared to rice bran oil. The anticancer activity of γ-tocotrienols in rice bran oil to human breast cancer cells would be an interesting candidate for further characterization of its anticancer effects in vivo for possible chemotherapeutic use.

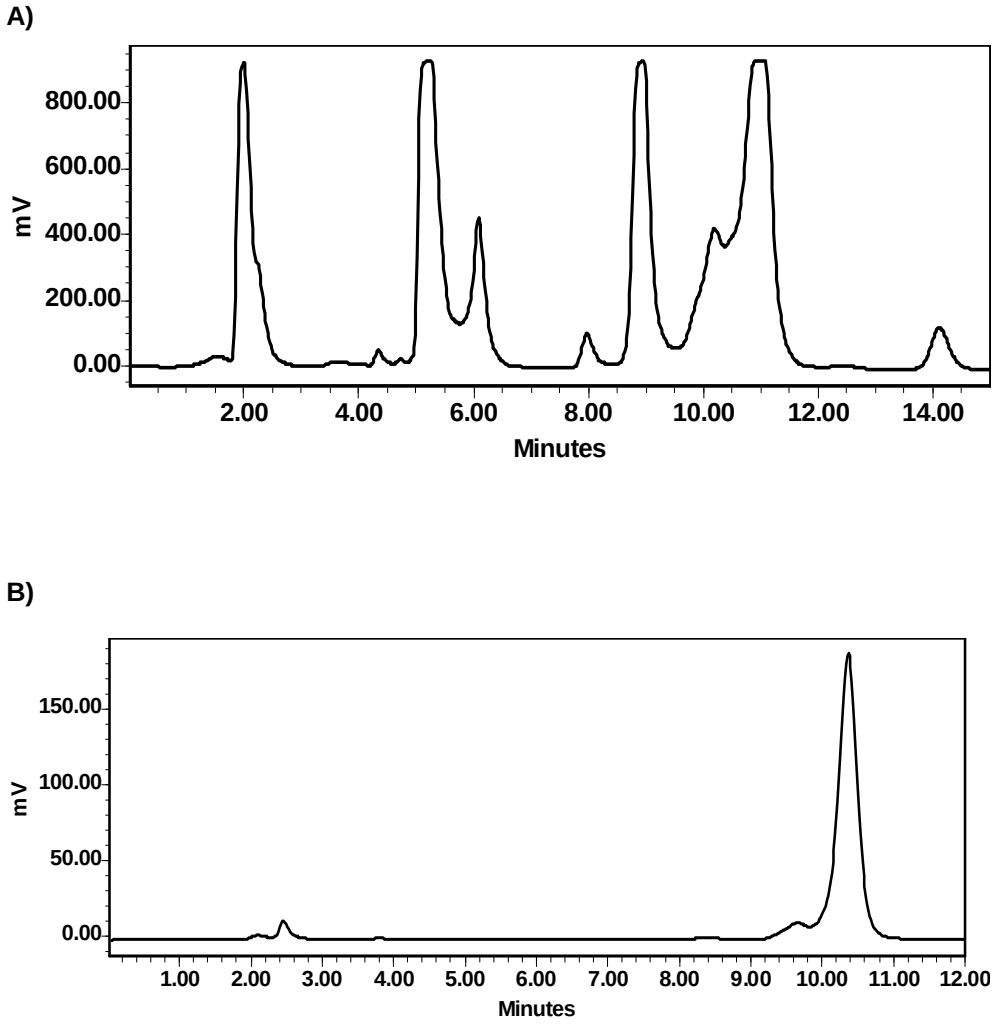


Fig. 3. Chromatogram of concentrated rice bran oil (A) and purified γ-tocotrienol (B)

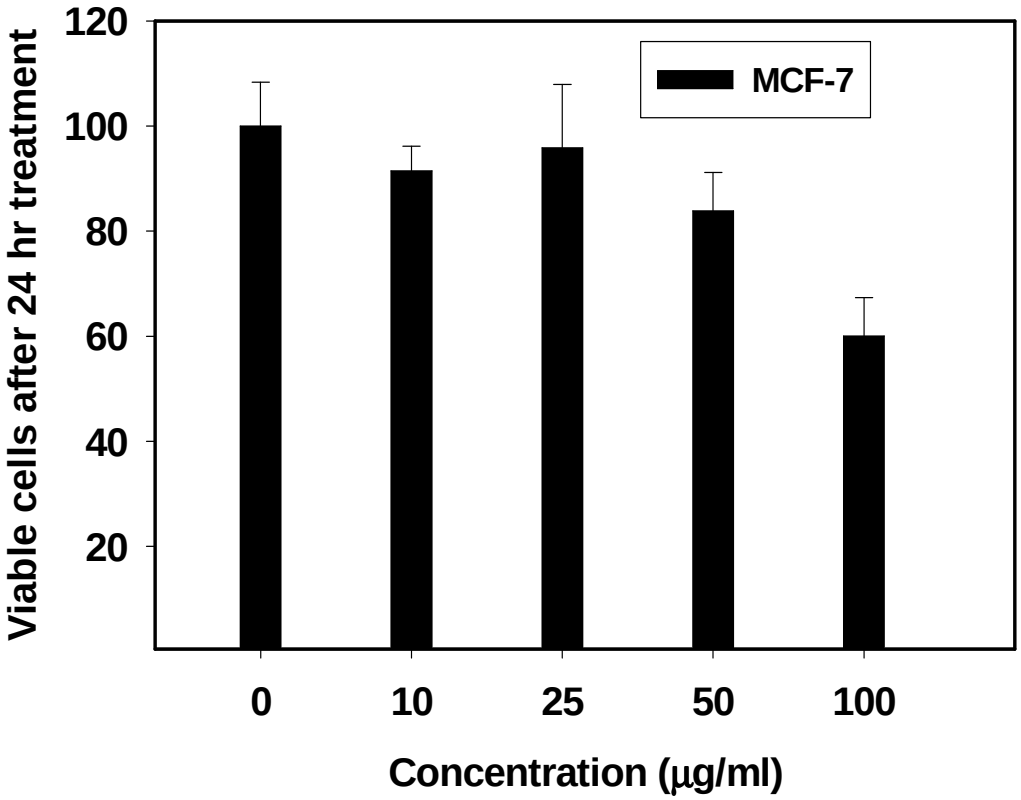


Fig. 4. Effect of various concentrations of rice bran oil on viability of MCF-7 cells. Dose-dependent effect on exponentially growing cells treated either with vehicle (E-OH) or different concentrations of rice bran oil for 24 hrs.

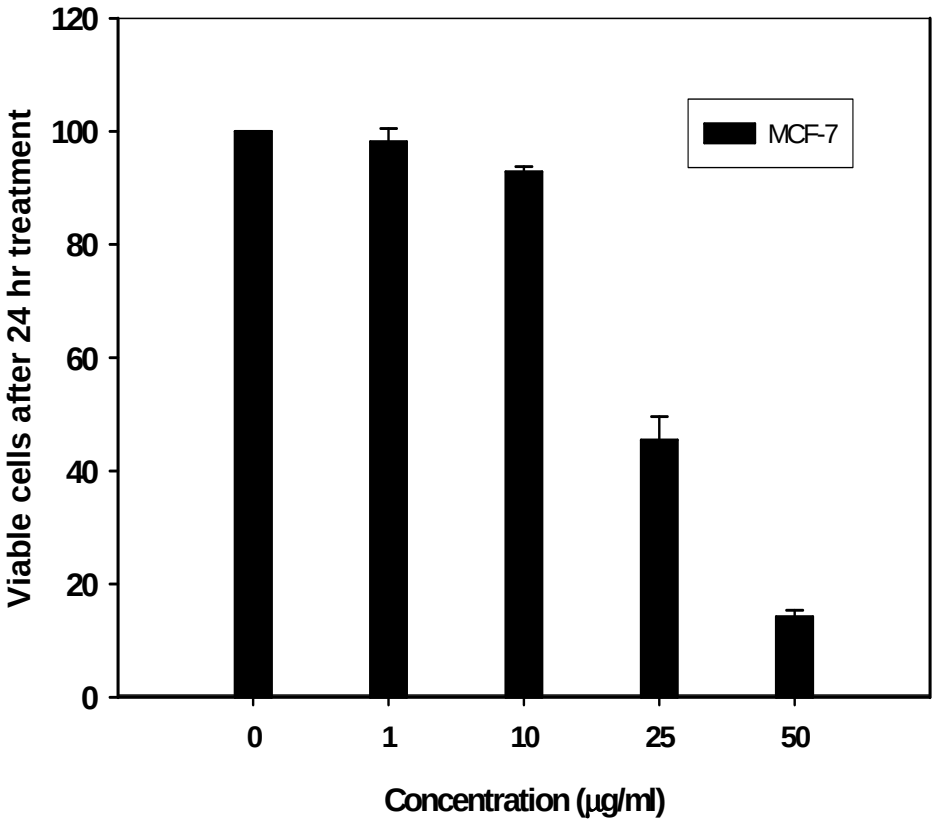


Fig. 5. Effect of various concentrations of γ-tocotrienol on viability of MCF-7 for 24 hr.

Table 2. Comparison of anticancer activities of concentrated rice bran oil and purified γ-tocotrienol. IC_{50}, effective concentration for inducing 50% reduction in viable cell number, was compared with control after 24 hr exposure.

	MCF-7
	IC50 (μg/ml)
Rice bran oil	126 (±12)
γ-Tocotrienol	18 (±4.55)

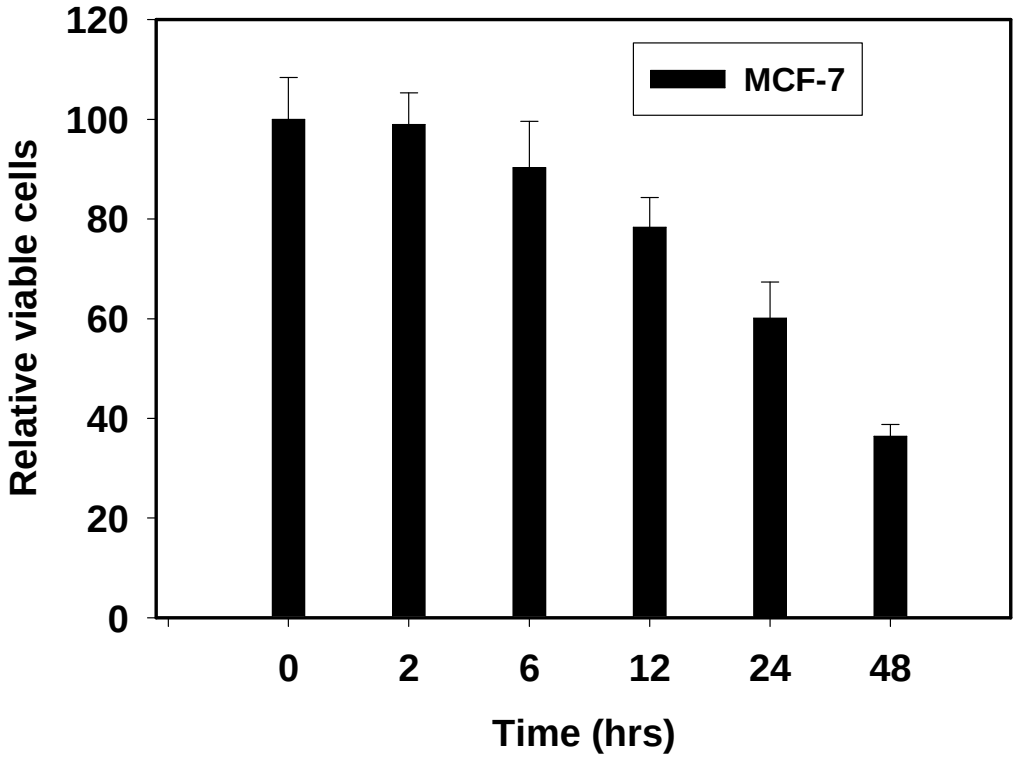


Fig. 6. Effect of incubation time on the inhibition of breast cancer cells. MCF-7 were treated with 126 μg/ml of rice bran oil for indicated time intervals.

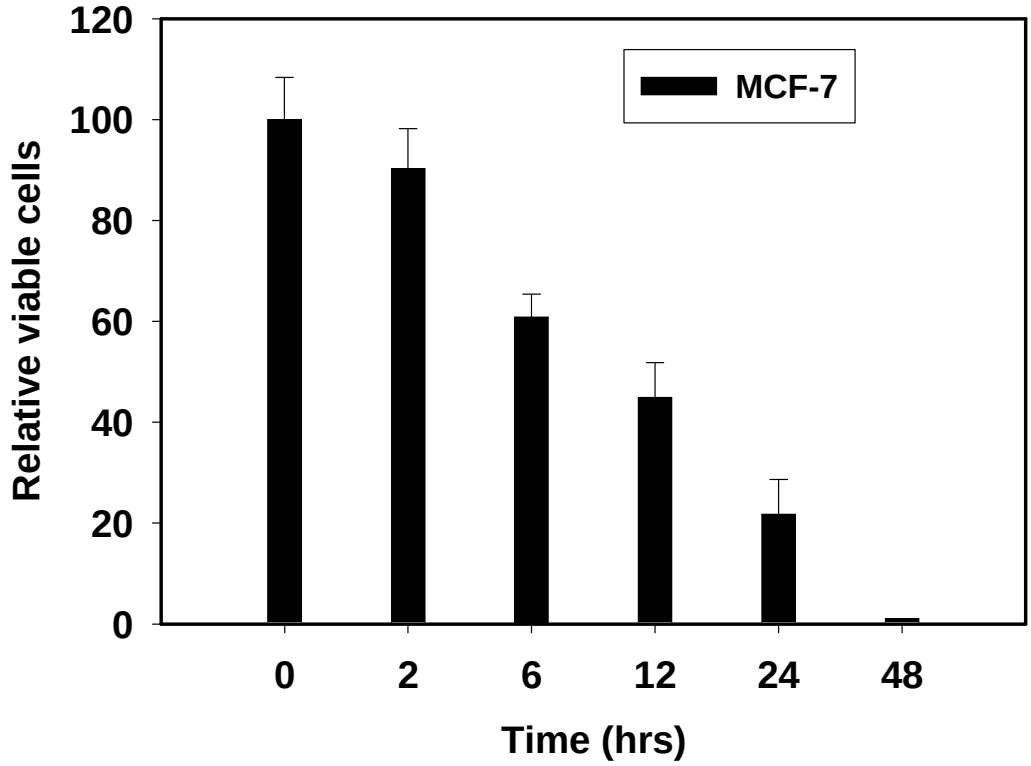


Fig. 7. Effect of incubation time on the inhibition of breast cancer cells. MCF-7 were treated with 18 μg/mlof γ-tocotrienol for indicated time intervals.

Fig. 5 shows that cell viability was significantly decreased at high concentration of tocotrienols over 10 μg/ml.

Tocotrienols belong to a phytochemical class of isoprenoid molecules, which have been shown to exhibit anticarcinogenic activities. Isoprenoids including tocotrienols also suppressed the growth of murine B16 melanomas in vitro and in vivo (He et al., 1997). Tocotrienol might exert antiproliferative activities by interfering with signal transduction events involving protein kinase C (Packer et al., 2001). The tocotrienol-rich fraction (TRF) of palm oil showed antiproliferative effects in human breast cancer cell lines (Nesaretnam et al., 2000; Guthrie et al., 1997). TRF inhibited the proliferation of estrogen-nonresponsive human breast cancer cell, whereas α-tocopherol had no effect on the growth of the cell line (Nesaretnam et al., 1995). TRF also inhibited growth of MCF-7 cells in both the presence and absence of estradiol with a nonlinear dose-dependent response. However, the inhibition of the growth of MDA-MB-231 cells by TRF showed a linear dose-dependent pattern (Nesaretnam et al., 1998). γ-tocotrienol and δ-tocotrienol evidenced higher inhibitory activities on the proliferation of both breast cancer cell lines; complete

inhibition of MCF-7 cell growth was achieved at 6 μg/ml of γ-tocotrienol and δ-tocotrienol in the absence of estradiol and 10 μg/ml of δ -tocotrienol in the presence of estradiol, whereas complete suppression of MDA-MB-231 cell growth was not achieved even at concentration of 10 μg/ml of δ-tocotrienol (Nesaretnam et al., 2000; 1998). In addition to the anticancer effect of tocotrienols for human breast cancer cells, high concentration of γ-tocopherol induced antiproliferative activities in both MCF-7 and MDA-MB-435 cells, and The IC_{50} values for γ-tocotrienols was 15 and 28 μg/ml in MCF-7 and MDA-MB-435 cells (Yu et al., 1999). Treatment with 20 μM γ-tocotrienol resulted in approximately 65% of the +SA cells displaying positive TUNEL staining, indicating that the majority of cells in this treatment group are actively undergoing apoptosis and 20 μM γ-tocotrienol inducing approximately 80% cell death (Shah and Sylvester. 2004). He et al. (1997) reported IC_{50} of d-γ-tocotrienol was 20±3 μmol/L, determined by 0.4% trypan blue for 48 hr incubation.

DNA fragmentation induced by γ-tocotrienol in MCF-7

As a biochemical hallmark of apoptotic cell death, DNA fragmentation was determined. To investigate the ability of γ-tocotrienols to induce apoptosis of cancer

cells, MCF-7 cells were treated with time and dose dependent. After treatments, isolated DNA from cells was subjected to 0.7% agaorse gel elctrophoresis. As shown in Fig. 8, DNA isolated from untreated controls showed little fragmentation or laddering in MCF-7 cells. However, treatments with time and dose dependent had effect on DNA fragmentation. A dose- and time- responsive increase in cellular levels of these compounds was observed. The technique used for DNA ladders examined apoptotic DNA fragments in the supernatant after removal of nonapoptotic intact nuclei by centrifugation. By use of this technique, virtually all the apoptotic DNA fragments are recovered from the supernatant, whereas intact nuclei from nonapoptotic cells are pelleted and discareded. Thus the levels of DNA added to each lane for DNA laddering analyses vary, depending on the level of apoptosis (Yu et al., 1999). Mcintyre et al. (2000) showed DNA fragmentation of 5 μM treatments produced detectable levels of ɣ-tocotrienols in CL-S1, -SA and +SA cells. MCF-7 and MDA-MB-435 cells were cultured with 20 μg/ml of ɣ-tocotrienols for 3 days. Evidence of DNA fragmentation was determined by DNA laddering (Yu et al., 1999). When any concentrations of ɣ-tocotrienols was treated on MCF-7, DNA

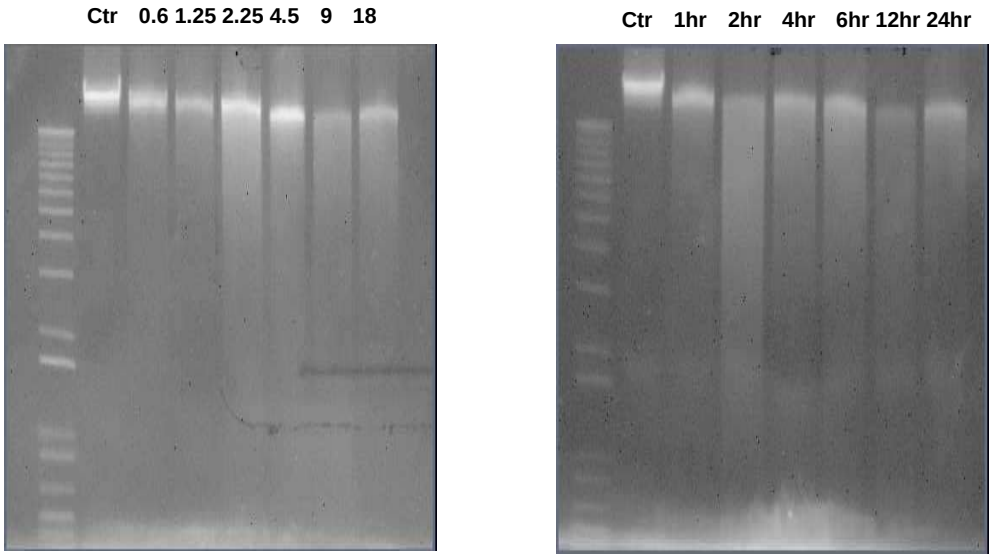


Fig. 8. Evidence of γ-tocotrienols induction of apoptosis via DNA laddering analyses. MCF-7 cells was cultured with 0, 0.6, 1.25, 2.25, 4.5, 9, 18 μg/ml for 24 hr and 0, 1, 2, 4, 6, 12 and 24 hr at 18 μg/ml. Evidence of DNA fragmentation was obviously observed at any manners.

fragmentation was obviously observed at any conditions. It means that specific cellular accumulation of γ-tocotrienols induces MCF-7 to cell death.

Microarray analysis

Fig. 9 represented fold changes of gene expression level as a result of microarray analysis and showed over-expressed thirteen genes and down-expressed thirty genes in range of the average fold changes of differentially expressed genes**.** This result is thoroughly low numerical values, comparing to microarray of rat fetal brain treated vitamin E, denoted from 20 to -10 of competitive change (Roy et al., 2002). Table. 3 and 4 listed the up-regulated (>2.0) genes and down regulated (<2.0) of MCF-7 by γ-tocotrienol treatment.

48 genes (3%) of total were over 1.5-fold up-regulated and 13 of them increased by a magnitude of two-fold or more. On the other hand, 104 genes (5%) of total were down-regulated and 30 of them lowered by two-fold or more. Over-expressed 2 genes, JNK (MAPK8) and Jun, have been known as proapoptotic genes mediated MAP kinase pathway.

In apoptotic pathways, JNK (MAPK8) induces the phosphorylation of Jun (Davis, 2000). Table 3 shows that JNK (3.61-folds) and Jun (2.57-folds) were over-expressed by γ-tocotrienol treatment, which suggests anticancer effect of

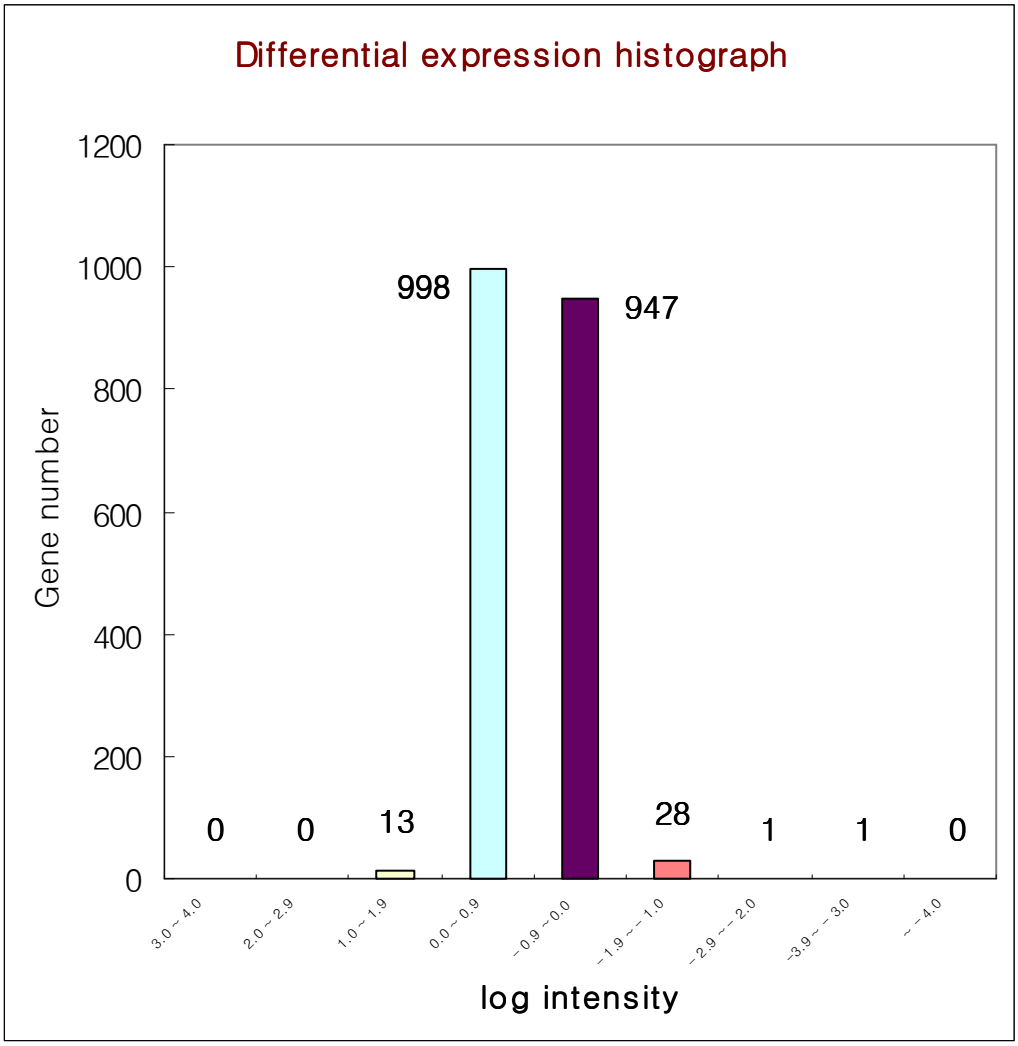


Fig. 9. Differential expression histogram. Range of the average fold changes of differentially expressed genes. Average fold changes were calculated for both over and down expressed genes in response to tocotrienol.

Table 3. The up-regulated(>2.0) genes in MCF-7 treated with gamma-tocotrienol

Accession no.	Gene symbol	Fold change	Description
NM_003260	TLE2	2.00	transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)
NM_003295	TPT1	2.01	tumor protein, translationally-controlled 1
NM_012396	PHLDA3	2.04	pleckstrin homology-like domain, family A, member 3
NM_003745	SOCS1	2.05	suppressor of cytokine signaling 1
NM_004864	GDF15	2.06	growth differentiation factor 15
NM_004324	BAX	2.32	BCL2-associated X protein
U18018	ETV4	2.34	ets variant gene 4 (E1A enhancer binding protein, E1AF)
NM_000932	PLCB3	2.57	Phospholipase C, beta 3 (phosphatidylinositol-specific)
NM_002228	JUN	2.57	v-jun sarcoma virus 17 oncogene homolog (avian)
NM_014330	PPP1R15A	2.75	protein phosphatase 1, regulatory (inhibitor) subunit 15A
NM_002586	PBX2	2.97	pre-B-cell leukemia transcription factor 2
NM_002750	MAPK8	3.61	mitogen-activated protein kinase 8
NM_004636	SEMA3B	3.82	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B

Table. 4. The down-regulated(<2.0) genes in MCF-7 treated gamma-tocotrienol

Accession no.	Gene symbol	Fold change	Description
NM_003045	SLC7A1	0.12	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
NM_003032	SIAT1	0.25	sialyltransferase1(beta-galactosidealpha-2,6-sialyltransferase)
NM_003036	SKI	0.28	v-ski sarcoma viral oncogene homolog (avian)
NM_014916	LMTK2	0.29	lemur tyrosine kinase 2
NM_018556	SIRPB2	0.31	signal-regulatory protein beta 2
NM_004176	SREBF1	0.33	sterol regulatory element binding transcription factor 1
NM_004810	GRAP2	0.38	GRB2-related adaptor protein 2
NM_005312	RAPGEF1	0.39	Rap guanine nucleotide exchange factor (GEF) 1
NM_002287	LAIR1	0.41	leukocyte-associated Ig-like receptor 1
NM_001739	CA5A	0.41	carbonic anhydrase VA, mitochondrial
U43586	KSR	0.41	kinase suppressor of ras
NM_024016	HOXB8	0.43	homeo box B8
NM_005247	FGF3	0.44	fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)
NM_005845	ABCC4	0.45	ATP-binding cassette, sub-family C(CFTR/MRP),member 4
NM_005188	CBL	0.46	Cas-Br-M (murine) ecotropic retroviral transforming sequence
NM_006480	RGS14	0.46	regulator of G-protein signalling 14
NM_002011	FGFR4	0.46	fibroblast growth factor receptor 4
NM_000174	GP9	0.46	glycoprotein IX (platelet)
NM_001242	TNFRSF7	0.47	tumor necrosis factor receptor superfamily,

			member 7
NM_002995	XCL1	0.47	chemokine (C motif) ligand 1
NM_002776	KLK10	0.47	kallikrein 10
NM_004104	FASN	0.47	fatty acid synthase
NM_004283	RAB3D	0.48	RAB3D, member RAS oncogene family
AF263541	DYRK4	0.48	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4
NM_004932	CDH6	0.48	cadherin 6, type 2, K-cadherin (fetal kidney)
NM_000118	ENG	0.49	Endoglin (Osler-Rendu-Weber syndrome 1)
NM_005860	FSTL3	0.50	follistatin-like 3 (secreted glycoprotein)
NM_004229	CRSP2	0.50	cofactor required for Sp1 transcriptional activation, subunit 2, 150kDa
NM_003682	MADD	0.50	MAP-kinase activating death domain
NM_002344	LTK	0.50	leukocyte tyrosine kinase

γ-tocotrienol follows MAP kinase apoptotic module. Therefore, anticancer activity of γ-tocotrienol on MCF-7 breast cancer cells might be caused by MAP kinases mediated apoptotic pathway

through JNK and Jun. In addition, this is very interesting result, because there has not known that γ-tocotrienol induces to programmed cell death by MAP kinase mediated apoptosis before, although, Takahashi and Loo (2004) reported that γ-tocotrienols induce Bax tranlocation to mitochondria and release of cytochrome C in MDA-MB-231 human breast cancer cell. c-Jun N terminal kinase (JNK) signaling pathway plays a critical role in ischemic brain injury (Guan et al., 2005). CD40-activation induced caspase-3 dependent cholangiocyte apoptosis and 3-fold increases in JNK/ERK phosphorylation (concomitant with increased AP-1 binding activity) and 4-fold increases in pSTAT3, which were sustained for up to 24 h. Protein levels of c-Jun, c-Fos and pSTAT3 confirmed the up-regulation (Ahmed-Choudhury et al., 2006). c-Jun N-terminal kinase JNK signaling pathway plays a critical role in ischemic brain injury. But the downstream mechanism that accounts for the proapoptotic actions of JNK during cerebral ischemia/reperfusion still

remains to be investigated in detail. DP5, one of the mammalian BH3-only proteins, was cloned as a neuronal apoptosis-inducing gene (Guan et al., 2006). The activation of a p38 MAPK pathway, but not that of ERK1/2 or JNK, plays an essential role in the apoptosis-like cell death induced by low concentrations of NO (Yamamoto et al., 2006). Dynamic JNK activation may play a key role in neuritis outgrowth during neuronal development (Xiao et al., 2006). Sb_2O_3 activates JNK and its downstream target, AP-1. In fibroblasts with a genetic deletion in SEK1, an upstream regulator of JNK, Sb_2O_3-induced growth inhibition as well as JNK activation was decreased. These data suggest roles for ROS and the SEK1/JNK pathway in the cytotoxicity associated with Sb_2O_3 exposure (Mann et al., 2006).

Among down expression genes, ABCC4, the multidrug resistance-associated protein, functions as adenosine triphosphate (ATP)-dependent export pumps (Trauner and Boyer. 2003). It was recently found to be the first transporter that supported efflux of nucleoside monophosphate analogs like 9-(2-phosphonylmethoxyethyl) adenine and azidothymidine monophosphate from mammalian cells (Schuetz et al., 1999). Obstructive cholestasis in the rat results in

up-regulation of ABCC4 (MRP4) in liver but down-regulation in kidney. This increase in hepatic ABCC4 expression is consistent with an adaptive response that provides an alternative pathway to ameliorate hepatocellular overloading with bile acids in cholestasis (Denk et al., 2004).

The loss of Fas, one of the down-expression genes, expression is very interesting, because Fas is a cell surface receptor and belongs to the tumor necrosis factor receptor superfamily (Nagata and Golstein, 1995). It has recently been reported that Fas is implicated in the induction of apoptosis during mammary gland involution (Song et al., 2000). Defects in the Fas/Fas L apoptotic signaling pathway provide a survival advantage to cancer cells and may be implicated in tumorigenesis. Indeed, expression of Fas L by breast cancer cells is associated with the loss of Fas expression, thus eliminating the possibility of self-induced apoptosis (Chopin et al., 2002). In this reason, down-regulation of Fas expression has many possibilities to investigate for cell death by apoptosis in human breast cancer cells and is necessary to need more experiments about Fas down-regulation in future.

Analysis of JNK and Jun by RT-PCR

γ-Tocotrienol sensitive genes identified by the microaray analysis were verified by conventional RT-PCR using primers for JNK and Jun. As treated with 0, 0.8, 1.6, 2.25, 4.5, 9.0, and 18 μg/ml of γ-tocotrienol, expression levels of JNK and Jun mRNA were increased with dose-dependent pattern (Fig. 10) and Fig. 11 shows on the up-regulation of JNK and Jun with progress of incubation time by 18 ug/ml of tocotrienol treatment. GAPDH was used as reference gene.

mRNA expression of JNK and Jun are explained from other reports about affects of chemicals on cells. Synthesized 9-hydroxypheophorbide (9-HPbD) alpha, a new chlorophyll-derived photosensitizer induced involvement of JNK signaling on MCF-7 cells. After photosensitization (5 μM 9-HPbD and 20 kJ/m^2), JNK and p53 were phosphorylated form-specific antibodies (Choi et al., 2004). The activation of JNK contributes tumor cells apoptosis induced by paclitaxel on MCF-7. After treatment of cells with 1 μM paclitaxel for 18 h, the activities of ERK1 and JNK1 were determined by immunocomplex kinase assay (Lee et al., 2003). The activation of melatonin receptors can stimulate the activity of the JNK subgroup of MAPKs via

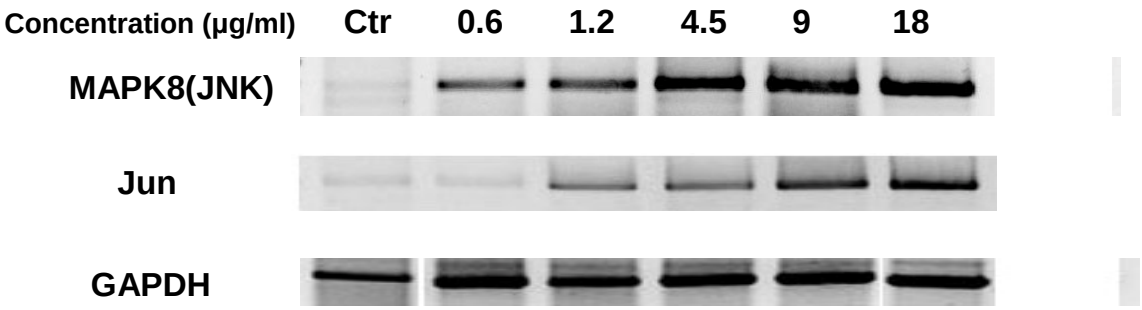


Fig. 10. Dose-dependent change of the expression of JNK and Jun by γ-tocotrienol treatment. MCF-7 cells were exposed for 24 hr with indicated concentration of γ-tocotrienol. GAPDH was used for a house keeping gene.

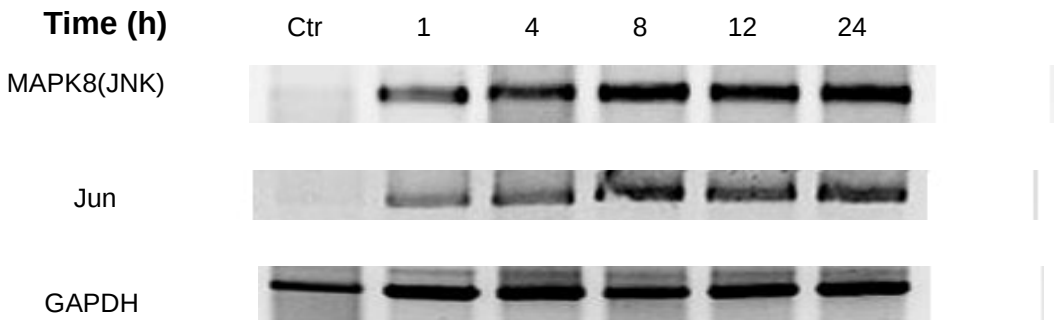


Fig. 11. Time-dependent change of the expression of JNK and Jun by γ-tocotrienol treatment. Cells were treated with 18 μg/ml of γ-tocotrienol for indicated time. GAPDH was used for a house keeping gene.

PTX-sensitive G_1 protein. Stimulation of the endogenous melatonin receptor in MCF-7 cells resulted in activation of both JNK and ERK (Chan et al., 2002). Signal transduction pathways involved in glucose deprivation-induced oxidative stress were investigated in MCF-7/Adr. In MCF-7/ADR, glucose deprivation-induced prolonged activation of c-Jun N-terminal kinase (JNK1) as well as cytotoxicity and the accumulation of oxidized glutathione (Lee et al., 1999).

γ-Tocotrienols induce JNK activation

Fig. 12 shows protein expression of phospho-JNK at time dependent manner. JNK contains the dual phosphorylation motif Thr-Pro-Tyr (Davis, 2000). Increase of phospho-JNK expression level means that JNK is activated by phosphorylations. The appearance of JNK phosphorylation by γ-tocotrienols has a similar that of DNA fragmentation. Thus, γ-tocotrienols-induced apoptosis might be through the JNK activation pathway.

Expression of phosho-JNK is a critical role of MAPK signal transduction, Peng et al. (2004) said that exposure to paraquat for 12-18 hr resulted in a progressive increase in immunoreactivity for phospho-JNK, whereas antibody against total JNK showed no paraquat-induced change in expression (Peng et al., 2004). The similar tendency of increasing JNK phosphorylation has been reported, 100 μM delphindin stimulates JNK phosphoryaltion in HepG2 cells and then, gradually increased JNK phosphorylation levels through the 24h treatment (Yeh and Yen, 2005). JNK phosphorylation is activated by MAP2 kinase, MAPKK4 (MKK4) and MAPKK7 (MKK7). In mammalian cells, MKK4 and MKK7 are the two MAP2K family

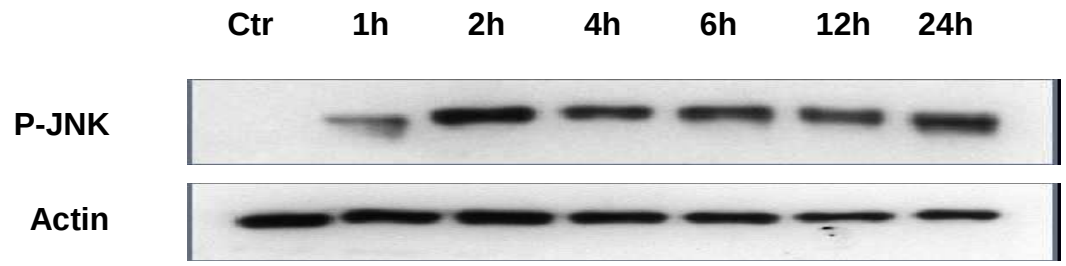


Fig. 12. γ-Tocotrienols induces JNK phosphorylation. MCF-7 cells were treated with 18 μg/ml γ-Tocotrienols for 0, 1h, 2h, 4h, 6h, 12h and 24h. Lysate protein 40 μg was applied on 10% SDS-PAGE.

members that directly phosphorylate JNK based on extensive biochemical studies and gene disruption: they may have discrete biological functions or act simultaneously in JNK activation (Chen et al., 2002). Signal transduction of both the p38 MAP kinase and JNK pathways as an essential component in pX-mediated apoptosis. pX expression in less differentiated hepatocytes mediates the sustained activation of both the JNK and p38 MAP kinase pathways (Wang et al., 2004). The activation of melatonin receptors can stimulate the activity of the JNK subgroup of MAPKs via PTX-sensitive G_1 protein. Stimulation of the endogenous melatonin receptor in MCF-7 cells resulted in activation of both JNK and ERK (Chan et al., 2002).

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