Anti-cancer activities of α- and γ-tocotrienol against the human lung cancer

Suphot Phutthaphadoong1, Supachai Yodkeeree1, Chaiyavat Chaiyasut2 and Pornngarm Limtrakul1*

1Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
2Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

Accepted 17 January, 2012

Lung cancer is the leading cause of cancer deaths in the world. To find a natural product that can be used in chemoprevention of lung cancer are the promising strategy for cancer prevention and therapy. In this study, we explore the contents of α-tocotrienol and γ-tocotrienol, a subgroup of vitamin E, in Thai rice and investigate their mechanisms underlying their anti-cancer activities on the human alveolar epithelial adenocarcinoma A549 cells. The results showed that Thai rice contained both α-tocotrienol and γ-tocotrienol, but the contents were found to be different when compared with rice varieties and areas of cultivation. Furthermore, γ-tocotrienol, but not α-tocotrienol, significantly inhibited A549 cell viability and proliferation in a dose- and time-dependent manner. γ-Tocotrienol treatment induces G0/G1 arrest and apoptosis induction in A549 cells as shown in the appearance of cell blebbing, increase of the poly (ADP-ribose) polymerase cleavage, and caspase-3 activation which were the signs of apoptosis. These apoptosis-associated phenomena were mediated via both intrinsic pathway (caspase-9 activation, overexpression of bax protein, and downexpression of bcl-xL protein) and extrinsic pathway (caspase-8 activation). Thus, our findings revealed that Thai rice contains both α-tocotrienol and γ-tocotrienol. Moreover, γ-tocotrienol but not α-tocotrienol has a potential to be the promising chemopreventive agent for human lung cancer.

Key words: α-Tocotrienol, γ-tocotrienol, A549 cell viability and proliferation.

INTRODUCTION

Cancer consists of a group of diseases characterized by uncontrolled cell division leading to the growth of abnormal tissue, and is a leading cause of death worldwide. In 2008, The International Agency for Research on Cancer (IARC) indicated that lung cancer was the most commonly diagnosed form of cancer and the most common cause of cancer deaths in males. Furthermore, it was the fourth most commonly diagnosed form of cancer and the second leading cause of cancer deaths in females (Jemal et al., 2011). There are many risk factors that have been identified for lung cancer such as smoking, air pollution, asbestos, and genetics (Alberg and Samet, 2003; Burch, 1986; Katanoda et al., 2011; Mossman et al., 2011; Yokota et al., 2010). Cancer is the result of a multistage carcinogenesis process, including initiation, promotion, and progression (Sugimura et al., 1992; Yokota and Sugimura, 1993). Since the carcinogenesis is the multistep process, arresting one or several of the steps may impede or delay the development of cancer. One approach to restrain the incidence of cancer incidence is cancer chemoprevention. The cancer chemoprevention uses natural, synthetic, or biologic substances to reverse, suppress, or prevent the development of cancer (Pan et al., 2011; Tsao et al., 2004). Several studies have reported that many of the chemopreventive agents can decrease tumor cell growth by induction of apoptosis in various animal models for carcinogenesis and in human chemoprevention trials (Galati et al., 2000; Sun et al.,...
Apoptosis is a multi-step, multi-pathway, and highly ordered process that controls the development and homeostasis of multicellular organisms. Furthermore, apoptosis induction is arguably the most potent defense measure against cancer. In mammals, apoptosis is initiated by two different pathways: Intrinsic (mitochondrial) and extrinsic (death receptor). In both pathways, the induction of apoptosis leads to the activation of the initiator caspases, and then the effector caspases. At last, the effector caspases, such as caspase-3, cleave the DNA damage repair protein called poly (ADP-ribose) polymerase or PARP to destroy its ability and eventually result in DNA fragmentation (ladder pattern) (Agarwal et al., 2009; Boulares et al., 1999; Burz et al., 2009). The intrinsic pathway of apoptosis depends on the permeabilization of the outer mitochondrial membrane and cytochrome c release from mitochondria. Mitochondrial integrity and the intrinsic pathway of the apoptosis are controlled by balancing the pro-apoptotic, with the anti-apoptotic-BCL-2-family members. During intrinsically-triggered apoptosis, antiapoptotic Bcl-2 family members (e.g., Bcl-2 and Bcl-xL) residing in the outer mitochondrial membrane can be destabilized by the induction of proapoptotic Bcl-2 family members (e.g., Bax, Bad, and Bak). In this scenario, the ratio of proapoptotic family members to the antiapoptotic family member becomes greater, which causes pores to form in the outer mitochondrial membrane, and lead to the release of cytochrome c. The released cytochrome c is associate with APAF1 and caspase-9 to form the apotosome, which activates caspase-3 and then induces apoptosis (Brenner and Mak, 2009; Khan et al., 2010; Lavrik, 2010). On the other hand, the extrinsic pathway is initiated when a death receptor ligand binds to its death receptor and promotes the recruitment of adapter molecules, which results in the cleavage of procaspase-8 to yield active caspase 8. After activation, caspase 8 triggered the activation of downstream effector caspases such as caspase-3, which executed the apoptosis (Mellier et al., 2010).

The compounds of natural vitamin E are divided into two subgroups, tocopherols and tocotrienols, by their phytyl chain. Both subgroups have four isoforms, including alpha (α-), beta (β-), gamma (γ-), and delta (δ-) isoforms. The natural sources of tocopherols and tocotrienols are different. Tocopherols are components of nuts and common vegetable oils, but tocotrienols are primarily derived from oats, wheat germ, barley, rye, rice and palms. In rice, the most isoforms of tocotrienol are alpha (α-), and gamma (γ-) (Aggarwal et al., 2010; Sookwong et al., 2010). Both tocopherols and tocotrienols exhibit antioxidant and anticancer activities but differ in term of their isoforms. Various results indicate that γ- and δ-tocotrienol exhibit greater anticancer activity than α- or β-tocotrienol (Aggarwal et al., 2010). The results of several studies have indicated that the tocotrienols may have a stronger bioactivity than the tocopherols (Wada, 2011). Therefore, the objective of this study is to explore the concentration of α-tocotrienol and γ-tocotrienol, which are the most commonly found isoforms in rice (Aggarwal et al., 2010). They are present in various types of Thai rice and their chemopreventive effects on cell viability and apoptosis in the human alveolar epithelial adenocarcinoma A549 cell were investigated.

**MATERIALS AND METHODS**

The six lines of Thai rice including, Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1, RD31, RD41, and RD47, were collected from local markets in the northern and middle regions of Thailand. Purified α-tocotrienol, γ-tocotrienol, and ethidium bromide (EB) were purchased from Sigma (St. Louis, MO). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was purchased from invitrogen. Low melting point agarose was purchased from vivantis. While phenol/chloroform/isoamyl alcohol solution was purchased from Research Organics, Inc. Rabbit polyclonal antibodies specific to PARP (1:4,000), caspase-3 (1:1,000), and mouse monoclonal antibody specific to β-actin (1:5,000) were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific to bax (1:1,000), caspase-9 (1:1,000), rabbit monoclonal antibodies specific to bcl-xL, cyclin D1, and mouse polyclonal antibodies specific to caspase-8 were purchased from Cell Signaling Technology, Inc. Donkey anti-rabbit antibody (1:4,000) was bought from GE, Healthcare. Goat anti-mouse antibody (1:2,000) was purchased from Abcam (Cambridge, MA).

**Cell culture**

Human alveolar epithelial adenocarcinoma A549 cell line was purchased from the American Type Culture Collection (ATCC). A549 cells were cultured in DMEM (GIBCO) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO2 and 95% air. Cells were plated and allowed to grow to approximately 70 to 80% before experimentation.

**High-performance liquid chromatography (HPLC) analysis**

Reversed-phase HPLC analysis for α-tocotrienol and γ-tocotrienol was modified from Britz et al. (2007). Briefly, the brown rice samples were ground and extracted three times in absolute ethanol (1:1) at 60°C for 15 min each time. The clear supernatants were collected, combined and then dried under N2. The brown rice extracts were resuspended by water and washed three times with hexane. The hexane supernatants were kept on ice, combined, and then dried under N2. Residues were dissolved in absolute ethanol and transferred in an amber vial immediately to HPLC (Agilent). The extracts were injected on a YMC C-80 reverse-phase (RP) column (4.6 × 250 mm, 5 μm) run at 25°C and 1.0 ml min⁻¹ with mobile phase solution (acetonitrile: methanol:water 85:12:3, v/v/v). Sample absorbance was monitored at 292 nm.

**Cell viability assay**

The effect of α-tocotrienol and γ-tocotrienol on the cell viability of A549 cells was determined by MTT assay. Briefly, A549 cells (3×10⁴ cells/well) were plated in 96-well microtiter plates. The next day, cells were treated with 200 μl of complete culture medium containing 25, 50, 75 and 100 μmol/L of α-tocotrienol with a negative control or 10, 20, 30, 40, 50, 60, and 70 μmol/L of γ-tocotrienol with a negative control and cultured for 12, 24, 36 and 48 h. α-Tocotrienol and γ-tocotrienol stocks were prepared in dimethylsulphoxide (DMSO) at 5×10⁻³ μmol/L.
concentration and mixed with fresh DMEM medium to achieve the desired final concentrations. DMSO was added to all treatment media such that the final DMSO concentration was the same in all groups within a given experiment and was always less than 0.4%. Each concentration of α-tocotrienol and γ-tocotrienol was repeated in five wells. At the end of the treatment, 20 µl of MTT (5 mg/ml in PBS) was added to each well, and the plates were incubated for 4 h at 37°C. After careful removal of the medium, 200 µl of DMSO was added to each well and they were shaken carefully. The absorbance was recorded on the Microplate Reader (EL311, BIO-TEK Instruments, Inc.) at a dual wavelength of 540/630 nm. The cell viability was assessed as the percent of cell viability where a negative control was taken as 100% cell viability. The half maximal inhibitory concentration or IC₅₀ value was calculated using the nonlinear regression model.

Cell proliferation assay
The effect of α-tocotrienol and γ-tocotrienol on the cell proliferation of A549 cells was determined by BrdU cell proliferation assay. Briefly, A549 cells (1×10⁵ cells/well) were plated onto 96 well microtiter plates. The next day, cells were treated with 100 µl of complete culture medium containing 40, 60, 80, and 100 µmol/L of α-tocotrienol or γ-tocotrienol with a negative control and cultured for 24 h. DMSO was added to all treatment media such that the final DMSO concentration was the same in all groups within a given experiment and was always less than 0.4%. Each concentration of α-tocotrienol and γ-tocotrienol was repeated in three wells. At the final 22 h of treatment, BrdU was added into each well of the microtiter plate. The cell proliferation was analyzed using BrdU Cell Proliferation Assay Kit (Millipore) according to the manufacturer’s instructions and the absorbance was recorded at dual wavelength of 450/550 nm. The effects of α-tocotrienol and γ-tocotrienol on cell proliferation were assessed as the presence of cell proliferation where a negative control was taken as 100% proliferation.

Flow cytometric analysis for cell cycle distribution
A549 cells were grown to about 70% confluency in a 100-mm Petri dish and then treated with 40, 60 and 80 µmol/L of γ-tocotrienol for 24 h with a negative control. After treatment, cells were harvested by trypsinization and centrifuged at 1500 rpm in a 15-ml tube. Harvested cells were washed twice with 1×PBS, resuspended and then fixed by adding 4 ml of cold 100% ethanol added in pulses of 1 ml each while being vortexed. Samples were stored -20°C for a minimum of 24 h and up to 1 month before analysis. In the step of analysis, samples were centrifuged and removed from the ethanol. Cell pellets were washed twice with 1×PBS and resuspended in 0.5 to 1 ml of propidium iodide (PI; 50 µg/ml) solution in PBS plus 500 µg/ml RNase, and incubated for 1 h at 37°C. Cell cycle distribution was analyzed by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA).

Scanning electron microscopy (SEM)
A549 cells were plated and treated with γ-tocotrienol at a concentration of 60 µmol/L for 12, 24 and 36 h. After treatment, cells were washed twice with PBS and fixed with 2.5% glutaraldehyde in phosphate buffer for 2 h at room temperature. Then, cells were washed twice with PBS and fixed with 2% OsO₄ in phosphate buffer for 1 h at room temperature. The cells were dehydrated in a series of ethanol rinses, followed by critical-point drying using CO₂ as the transitional fluid. Finally, the cells were mounted on stubs and were coated with platinum, examined with a scanning electron microscope (JEOL JSM-6610LV), and photographed.

Flow cytometric analysis for apoptosis
Apoptosis detection was performed using the ApopNexin FITC apoptosis detection kit for flow cytometry analysis (Chemicon). A549 cells were grown to about 70% confluency in a 100-mm Petri dish and then treated with 60 µmol/L of γ-tocotrienol for 24 and 48 h with a negative control. After treatment, cells were harvested by trypsinization and centrifuged at 1500 rpm in a 15-ml tube. Harvested cells were washed twice with 1×PBS and resuspended in 500 µl of binding buffer. Cell staining with annexin V and PI were performed according to the manufacturer’s instructions. FITC-labeled annexin V and PI were added to the cells, followed by 15 min incubation in the dark at room temperature. The apoptosis rate was immediately determined by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA).

Western blot analysis
A549 cells were treated with various concentrations of γ-tocotrienol for 24 h. After treatment, cells were washed with ice-cold PBS and harvested by scraping. Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250 mM NaCl, 20 mM HEPES, and 1% NP-40) with freshly added protease inhibitors (1 mM EGTA, 5 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, 2 µg/ml aprotinin, 5 µg/ml leupeptin, and 1% PMSF) on ice for 30 min. 50 µg protein was separated from 8 or 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 h at room temperature, incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 h. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad).

Statistical analysis
All data are expressed as means ± SD. ANOVA was used to evaluate the differences among multiple groups. If significance was observed between the groups, Dunnett’s test was used to compare the means of two specific groups, where p < 0.05 was considered to be significant.

RESULTS
Presence of α-tocotrienol and γ-tocotrienol in various types of Thai rice
To explore the concentration levels of α-tocotrienol and γ-tocotrienol in Thai rice, the six lines of Thai rice including, Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1, RD31, RD41, and RD47, were collected and analyzed by HPLC as shown in Figure 1. The quantitative data of both α-tocotrienol and γ-tocotrienol levels in Thai rice are shown in Table 1. All types of Thai rice contained both α-tocotrienol and γ-tocotrienol but the concentration of α-tocotrienol was higher than α-tocotrienol. Ranges of both α-tocotrienol and γ-tocotrienol in Thai rice were 1.88-5.87 and 15.32-54.81 mg/g. The contents of α-tocotrienol and γ-tocotrienol in rice were varied upon rice varieties. Even the same rice varieties, the contents of α-tocotrienol and γ-tocotrienol still varied depending on the cultivation area as compared with Khao Dawk Mali 105. Moreover, the rice that was cultivated in the northern region of Thailand (Maehongsorn, Sukhothai) contained higher contents of α-tocotrienol and γ-tocotrienol than rice cultivated in the middle part of Thailand (Suphanburi).
Figure 1. HPLC profile of standard tocotrienols and tocopherols monitored at 292 nm. Peak 1: α-tocotrienol; 2: γ-tocotrienol; 3: β-tocotrienol; 4: δ-tocotrienol; 5: α-tocopherol; 6: γ-tocopherol; 7: β-tocopherol; 8: δ-tocopherol.

Table 1. The content of α-tocotrienol and γ-tocotrienol in 9 Thai rice samples.

<table>
<thead>
<tr>
<th>No</th>
<th>Lines</th>
<th>Cultivated area in Thailand (Region/province)</th>
<th>Concentration (mg/g of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-Tocotrienol</td>
</tr>
<tr>
<td>1</td>
<td>Khao Dawk Mali 105</td>
<td>Northern/Maehongson</td>
<td>3.41</td>
</tr>
<tr>
<td>2</td>
<td>Khao Dawk Mali 105</td>
<td>Northern/Sukhothai</td>
<td>3.11</td>
</tr>
<tr>
<td>3</td>
<td>Khao Dawk Mali 105</td>
<td>Middle/Suphanburi</td>
<td>1.88</td>
</tr>
<tr>
<td>4</td>
<td>Suphan Buri 1</td>
<td>Middle/Suphanburi</td>
<td>3.34</td>
</tr>
<tr>
<td>5</td>
<td>Pathum Thani 1</td>
<td>Middle/Pathumthani</td>
<td>5.41</td>
</tr>
<tr>
<td>6</td>
<td>RD31</td>
<td>Middle/Suphanburi</td>
<td>5.62</td>
</tr>
<tr>
<td>7</td>
<td>RD31</td>
<td>Middle/Pathumthani</td>
<td>4.48</td>
</tr>
<tr>
<td>8</td>
<td>RD41</td>
<td>Middle/Suphanburi</td>
<td>5.87</td>
</tr>
<tr>
<td>9</td>
<td>RD47</td>
<td>Middle/Suphanburi</td>
<td>2.66</td>
</tr>
</tbody>
</table>

Decrease of cell viability of A549 cells by γ-tocotrienol

To investigate the inhibitory effects of α-tocotrienol and γ-tocotrienol on the cell viability of A549 cells, we determined the cell viability by MTT assay. The A549 cells were treated with various concentrations of α-tocotrienol (25, 50, 75 and 100 μmol/L) and γ-tocotrienol (10, 20, 30, 40, 50, 60 and 70 μmol/L) for 12, 24, 36 and 48 h, respectively. As shown in Figure 2, γ-Tocotrienol significantly decreased cell viabilities of A549 cells in a dose- and time-dependent manner. The half maximal
Figure 2. Effects of α-tocotrienol (A) and γ-tocotrienol (B) on cell viability of human alveolar epithelial adenocarcinoma A549 cells. A549 cells were treated with α-tocotrienol (0 to 100 μmol/L) or γ-tocotrienol (0 to 70 μmol/L) for 12 h (dot line), 24 h (small dash line), 36 h (large dash line), and 48 h (black line). Each concentration was repeated in five wells. The cell viability was determined by MTT assay and expressed relative to the negative control group. The results represent the mean of at least three independent experiments.

Inhibitory concentration (IC$_{50}$) of γ-tocotrienol, which is the concentration that reduces the viability of A549 cells by 50%, at 24 and 48 h were 35.19±0.98 and 31.54±0.49 μmol/L, respectively. In addition, α-tocotrienol treatment had no significant effect on the cell viabilities of A549 cells. Furthermore, we analysed the effects of α-tocotrienol and γ-tocotrienol on the cell proliferation of A549 cells by BrdU cell proliferation assay. As shown in Figure 3, γ-tocotrienol significantly inhibited the cell proliferation of A549 cells in a dose-dependent manner. The half maximal inhibitory concentration (IC$_{50}$) of γ-tocotrienol for the inhibition of A549 cell viability at 24 h was 55.63±0.56 μmol/L. Nevertheless, α-tocotrienol had no effect on the cell proliferation of A549 cells.

Induction of G0/G1 arrest by γ-tocotrienol in A549 cells

To determine whether the inhibitory effect γ-tocotrienol on the cell proliferation of A549 cells was due to the cell cycle arrest activity, we determined the cell cycle
Figure 3. Effects of α-tocotrienol and γ-tocotrienol on cell proliferation in A549 cells. A549 cells were treated with α-tocotrienol or γ-tocotrienol (0, 40, 60, 80 and 100 μmol/L) for 24 h. Each concentration was repeated in three wells. The cell proliferation was determined by BrdU cell proliferation assay and the values were expressed relative to the negative control group. *, significantly different from 0 μmol/L (p < 0.05), **, significantly different from 0 μmol/L (p < 0.001).

Figure 4. Effect of γ-tocotrienol on cell cycle distribution in A549 cells. A549 cells were treated with 0, 40 and 60 μmol/L of γ-tocotrienol for 24 h and analyzed by flow cytometry. The results represent the mean of at least three-independent experiments. *, Significantly different from 0 μM (p < 0.005).

distribution in A549 cells. As shown in Figure 4, γ-tocotrienol at 60 μmol/L significantly increased G0/G1 phase population and decreased M-phase population in A549 cells after 24 h of treatment, but γ-tocotrienol at 40 μmol/L had no significant effect on G0/G1 cell cycle arrest.

The cyclin D1/cyclin-dependent kinase (Cdk) holoenzymes have been identified as the G1 phase cell cycle regulatory proteins. The downregulation of cyclin D1 protein expression leads to G0/G1 cell cycle arrest and decreases the proliferation rate. Therefore, we further investigated the effects of γ-tocotrienol on cyclin D1 protein expression. The treatment of γ-tocotrienol decreased the cyclin D1 protein expression in a dose-response manner as shown in Figure 5. These results indicated that γ-tocotrienol induced G0/G1 cell cycle arrest due to the downregulation of cyclin D1 protein expression in A549 cells.
\(\gamma\)-Tocotrienol treatment (\(\mu\)mol/L)

<table>
<thead>
<tr>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
</table>

**Cyclin D1**

**\(\beta\)-actin**

*Figure 5.* Effect of \(\gamma\)-tocotrienol on the protein expressions of cyclin D1. A549 cells were treated with \(\gamma\)-tocotrienol at 0, 20, 40, 60, and 80 \(\mu\)mol/L for 24 h and analyzed by western blot analysis (Three-independent experiments).

*Figure 6.* Scanning electron microscopy of \(\gamma\)-tocotrienol-induced apoptosis. Human alveolar epithelial adenocarcinoma A549 cells was incubated with \(\gamma\)-tocotrienol at a concentration of 60 \(\mu\)mol/L for the indicated periods of time. Samples were proceed for scanning electron microscopy and analyzed by JEOL JSM-6610LV. A-D, \(\gamma\)-tocotrienol-treated cells for 0, 12, 24 and 36 h, respectively; Magnification, \(\times 3,000\); bar indicates 5 \(\mu\)m. E-G, Magnification of \(\gamma\)-tocotrienol-induced apoptosis, \(\times 10,000\). E, untreated A549 cells. F, nuclear condensation and segregation. G, cell blebing.

**\(\gamma\)-Tocotrienol induced apoptosis in A549 cells**

To determine whether the inhibitory effect \(\gamma\)-tocotrienol on cell viability of A549 cells was due to the induction of apoptosis, we detected the morphological and specific biochemical changes in the cell surface membrane. For the detection of the morphological change in \(\gamma\)-tocotrienol-treated A549 cells, we observed the cells by using the scanning electron microscopy (SEM) technique. As shown in Figure 6, after the treatment of \(\gamma\)-tocotrienol at 60 \(\mu\)mol/L for 12 h, the morphology of A549 cells were changed. The condensation of the nucleus and the loss of microvilli appeared. After 24 h, the cell became spherical as its cytoskeleton, which holds the cell shape, was digested. Moreover, the nucleus condensed completely and segregated into several fragments. After 36 h, the blebs or vesicles were formed on its surface. Finally, the cell broke into several vesicles, now known as apoptotic bodies.

For the detection of the specific biochemical changes in the cell surface membrane, we analyzed the translocation of phosphatidylserine to the outer membrane surface, which is a signature event of early apoptosis. The results showed that \(\gamma\)-tocotrienol at 60 \(\mu\)mol/L significantly increased the percentage of cells in early apoptosis cells and significantly decreased the percentage of viable cells in time-dependent manner (24 and 48 h) compared with the untreated cells (Figure 7). Then, to further confirm whether \(\gamma\)-tocotrienol induced apoptosis in A549 cells, we analyzed the expression of apoptosis-related proteins,
Figure 7. Effect of γ-tocotrienol on the translocation of phosphatidylinerine in A549 cells. Early apoptosis induction in A549 cells treated with 60 μmol/L of γ-tocotrienol for 24 and 48 h was analyzed by flow cytometry. The results represent the mean of at least three independent experiments. *, Significantly difference from 0 h (p < 0.01).

Figure 8. Effect of γ-tocotrienol on the induction of apoptosis in A549 cells. The cleavage of PARP protein and protein expressions of cleaved, procaspase-3, procaspase-8, procaspase-9, bcl-XL, and bax in A549 cells treated with γ-tocotrienol.

γ-Tocotrienol induced apoptosis in A549 cells via both extrinsic and intrinsic pathways

To explore the apoptotic pathway of A549 cells induced by γ-tocotrienol, protein levels of pro-caspase-8, pro-caspase-9, pro-apoptotic protein bax, and anti-apoptotic poly(ADP-ribose) polymerase (PARP) and procaspase-3 by western blotting analysis. γ-Tocotrienol treatment represented a decrease of pro-caspase-3 expression and an increase of cleaved-PARP expression (Figure 8), which confirmed that γ-tocotrienol induced apoptosis in A549 cells.
protein bcl-xL were determined by western blotting analysis. As shown in Figure 8, γ-tocotrienol treatment decreased protein levels of pro-caspase-9, and bcl-xL, but increased the protein level of bax in the dose-response manner, which were the patterns of the intrinsic (mitochondria-associated) apoptosis pathway. Pro-caspase-8 (a marker of extrinsic pathway of apoptosis) also decreased by the treatment of γ-tocotrienol in a dose-response manner.

**DISCUSSION**

Thai rice is one of the most popular and economic rice varieties, worldwide. This major staple food contains many compounds that can be considered, chemopreventive agents, but most of them were lost during the rice processing. Therefore, in this study, we highlighted the new economic properties of Thai rice in chemoprevention. Thai rice contains tocotrienols, a subgroup of vitamin E, which has been suggested to be one of the promising chemopreventive agents (Das, 2011; Kannappan et al., 2011; Sylvester et al., 2010; Sylvester et al., 2011). In this study, we founded that all Thai rice samples contained both α-tocotrienols and γ-tocotrienols, but their content varied widely between lines and cultivated areas. There are many factors such as temperature, soil-quality, and rice varieties, that influence the concentrations of the natural compounds present, such as tocotrienol (Britz et al., 2007). The content of γ-tocotrienol in all the Thai rice samples was higher than α-tocotrienol. This result is similar to the results found in Japan (Sookwong et al., 2010). Moreover, the contents of both α-tocotrienol and γ-tocotrienol in Thai rice are relatively high compared with other rice (Britz et al., 2007; Sookwong et al., 2010).

In this study, we suggest that γ-tocotrienol but not α-tocotrienol has the chemopreventive effects on lung cancer. γ-Tocotrienol inhibited cell viability of the human alveolar epithelial adenocarcinoma A549 cells. The IC₅₀ values were 43.19±0.11, 35.19±0.98, 33.43±0.02 and 31.54±0.49 µmol/L (approximately 3.55, 2.89, 2.75, and 2.59 µg of γ-tocotrienol, respectively) at 12, 24, 36 and 48 h, respectively. From the data shown in Table 1, we founded γ-tocotrienol content in various Thai rice in the range of 15 to 50 mg/g of rice. Therefore, daily brown rice intake should provide enough γ-tocotrienol for chemopreventive effect against human lung cancer. Although, tocotrienols share common structural features of a chromanol ring, and a side-chain at the C-2 position, and unsaturated isoprenoid sidechain, they differ in their number and position of the methyl group on the chromanol ring. These differences may influence their anticancer properties. Moreover, only γ-tocotrienol, but not α-tocotrienol, inhibited A549 cell proliferation. The anti-proliferative activity of γ-tocotrienol is involved with G0/G1 cell cycle arrest. Apoptosis plays an important role in the maintenance of homeostasis and in eliminating damaged cells. It is well known that many chemopreventive agents induce apoptosis in cancer cells (Burz et al., 2009; Galati et al., 2000; Khan et al., 2010; Sun et al., 2004; Tsao et al., 2004). The treatment of γ-tocotrienol can induce apoptosis in the human alveolar epithelial adenocarcinoma A549 cells. After the cleavage of poly (ADP-ribose) polymerase (PARP), caspase-3 activation, and cell blebbing, all signs of apoptosis were presented in γ-tocotrienol-treated A549 cells. Finally, the treatment of γ-tocotrienol led to apoptosis via both intrinsic and extrinsic pathways upon its induction of caspase-8 and caspase-9 activation, and an increase in the bax/bcl-xL ratio (Brenner and Mak, 2009; Mellier et al., 2010). Although, we only detected the protein expressions of pro-caspase 3, 8 and 9, which were pro-forms or inactive forms of caspase enzymes, the active forms of caspase 3, 8, and 9 were not detected. However, we suggest that the apoptosis occurred as the cleaved PARP increased, along with the upregulation of Bax protein, and an increase in the bax/bcl-xL ratio, all of which were the signs of apoptosis. It is well known that the activation of caspase-8 can cleave the BH3-interacting domain death agonist (Bid), a pro-apoptotic member of the B-cell CLL/lymphoma 2 (Bcl-2) family. This cleaved or truncated Bid (tBid) engages the intrinsic apoptotic pathway by binding itself to the Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK), resulting in their oligomerization and translocation to the mitochondrial outer membrane. BAX and BAK oligomers then promote a decrease in the mitochondrial membrane potential and subsequent/concomitant formation of pores leading to the outer membrane permeabilization (Mellier et al., 2010). Therefore, an increase of the bax and caspase-9 activation may result from the activation of caspase-8. To prove that the γ-tocotrienol directly induced the intrinsic pathway of apoptosis, further studies with the caspase-8 inhibitor, for example Z-IETD-FMK, should be conducted. Furthermore, γ-tocotrienol has been reported for its ability to induce apoptosis in other cancers, such as gastric cancer (Sun et al., 2009), T cell lymphoma (Wilankar et al., 2011), and in leukemic cells (Inoue et al., 2011). The sensitivity for γ-tocotrienol- induced apoptosis is different among these types of cancer. From our results, lung cancer is more sensitive than gastric cancer but less sensitive than T cell lymphoma, and leukemic cells. In conclusion, we highlight that Thai rice contains both α-tocotrienol and γ-tocotrienol. γ-Tocotrienol exhibits the chemopreventive activities by the inhibition of the proliferation and induction of apoptosis in the human alveolar epithelial adenocarcinoma A549 cell line via both the intrinsic and extrinsic pathways.

**ACKNOWLEDGEMENTS**

This research was supported by grants from the National Research Council of Thailand (NRCT) and the Royal Council of Thailand (NRCT).
Golden Jubilee PhD Program of Thailand. Suphot Phuthaphadoong is a PhD student under the Royal Golden Jubilee PhD Program of Thailand.

REFERENCES


