Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 103 (2007) 434–443
Food Chemistry
www.elsevier.com/locate/foodchem

Induction of apoptosis by cinnamaldehyde from indigenous cinnamon *Cinnamomum osmophloeum* Kaneh through reactive oxygen species production, glutathione depletion, and caspase activation in human leukemia K562 cells

Tzou-Chi Huang [a], Hui-Yin Fu [b], Chi-Tang Ho [c], Di Tan [c], Yu-Ting Huang [d], Min-Hsiung Pan [d,*]

[a] *Department of Food Science, National Pingtung University, 912, Pingtung, Taiwan*
[b] *Department of Food Science and Technology, Tajen Institute of Technology, 907, Pingtung, Taiwan*
[c] *Department of Food Science, Rutgers University 65 Dudley Road, New Brunswick, NJ 08901-8520, USA*
[d] *Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung 811, Taiwan*

Received 7 June 2006; received in revised form 1 August 2006; accepted 22 August 2006

Abstract

The compositions of essential oils from leaves of two *Cinnamomum osmophloeum* clones (A and B) commercially cultivated by Taiwan Cinnamon Biotech Co. Ltd., in Taiwan were investigated. GC and GC–MS analyses showed that *Cinnamomum osmophloeum* clones A and B contain *trans*-cinnamaldehyde (91.15%) and cinnamyl acetate (46.39%), respectively, as the major component. This study demonstrated that cinnamaldehyde was able to induce apoptosis in a concentration-dependent manner. Cinnamaldehyde-induced cell death was characterized with changes in nuclear morphology, DNA fragmentation, and cell morphology. Furthermore, treatment with cinnamaldehyde caused a rapid loss of mitochondrial transmembrane potential, stimulation of reactive oxygen species (ROS) production, release of mitochondrial cytochrome *c* into cytosol, and subsequent induction of procaspase-9 and procaspase-3 processing. Taken together, these results suggest that ROS production and depletion of the glutathione that committed to cinnamaldehyde-induced apoptosis in K562 cells.

Keywords: *Cinnamomum osmophloeum*; Cinnamaldehyde; Apoptosis; ROS

1. Introduction

Cinnamon (*Cinnamomum osmophloeum*), commonly known as Canela, Sees (Ceylon cinnamon), Laurus Cinnamomum, and *Cinnamomum zeylanicum*, is native to Sri Lanka and India. It has also been cultivated in Brazil, Mauritius and Jamaica. *Cinnamomum osmophloeum* was used primarily in the flavor and fragrance industries for imparting a cinnamon flavor and/or fragrance to various types of foods, beverages, medical products, and perfumes.

Indigenous cinnamon *Cinnamomum osmophloeum* Kaneh is a major source of *trans*-cinnamaldehyde which are recognized as bioactive substances with potential health effects (Peters & Caldwell, 1994). Among selected essential oil components, carvacrol, (+)-carvone, thymol, and *trans*-cinnamaldehyde. *trans*-Cinnamaldehyde possessed the most inhibitory component toward *P. leiognathi*, *Escherichia coli* O157:H7 and *Salmonella typhimurium* (Chang, Chen, & Chang, 2001; Wang, Chen, & Chang, 2005). Cinnamaldehyde has been reported to have anti-platelet aggregating and vasodilatory action, inhibiting collagen-induced

* Corresponding author. Tel.: +886 7 361 7141x3623; fax: +886 7 361 1261.
E-mail address: mhpan@mail.nkmu.edu.tw (M.-H. Pan).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.08.018

platelet aggregation in a dose-dependent manner (Vander-Ende & Morrow, 2001).

Apoptosis is defined as a type of cell death, involving the concerted action of a number of intracellular signaling pathways, including members of the caspases family of cytsteine proteases, stored in most cells as zymogens or procaspases (Martin & Green, 1995). The two main apoptotic pathways that initiate apoptosis: the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, which can crosstalk to each other. The extrinsic pathway is triggered by the binding of ligands to their cell death receptors located on the plasma membrane (Gosslau & Chen, 2004). The crucial step in the mitochondria-mediated apoptosis is the change of mitochondrial membrane permeability and collapse of membrane potential (Balaban, Nemoto, & Finkel, 2005). Moreover, the mitochondrial pathway is regulated by the Bcl-2 family of proteins, including anti-apoptotic proteins such as Bcl-2 and Bcl-X_L and pro-apoptotic proteins such as Bad, Bid, Bim, Bax, and Bak (Li, Nijhawan, & Wang, 2004).

The mechanism of apoptosis by cinnamaldehyde in cultured leukemia cell lines K562 was examined and possible mitochondria involved investigated. The role of Bcl-2 family in the modulation of membrane potential was also studied.

2. Materials and methods

2.1. Samples

Fresh cinnamon leaves were purchased from Taiwan Sinnamon Company, Pingtung, Taiwan in the month of December 2004.

2.2. Extraction and concentration of volatiles

2.2.1. Simultaneous distillation-extraction (SDE)

A simple simultaneous distillation extraction apparatus (Pintung, Taiwan.) was used as previously described (Huang, Ho, & Fu, 2004). One hundred grams of chopped cinnamon leaves in 600 mL of water were extracted under atmospheric conditions for 2 h using ethyl ether (Sigma–Aldrich Co., St. Louis, MO, USA) as the extraction solvent. The extracts obtained were concentrated to approximately 0.5 mL using nitrogen then 2 μL of methyl benzoate added as internal standard and stored frozen at −18 °C for gas chromatographic analysis.

2.3. Analysis of volatiles

2.3.1. Gas chromatography

An Agilent 6890 System gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a fused silica column (60 m × 0.32 mm i.d., film thickness, 0.25 μm; HP-5 Agilent Technologies, Palo Alto, CA, USA) and a flame ionization detector was used to analyze the ethyl ether extracts. The operation conditions were as follows: injector and detector temperatures, 250 °C; extra pure helium carrier flow rate, 1.0 ml/min; temperature program, 70–180 °C at 4 °C/min.

2.3.2. Gas chromatography–mass spectrometry

The extract (0.5 μL) was analyzed using a Agilent 6890 System with a Mass Selective Detector (5973 Network Agilent, Santa Clara, CA, USA). Mass spectra were obtained by electron ionization and chemical ionization at 70 eV and a source temperature of 250 °C. The filament emission current was 1 mA, and the spectra were recorded and analyzed with the HP G111034C MS Chemstation Software installed in an IBM Personal Computer. The operation conditions were the same as those used in the GC analysis described above.

Tentative identifications were made by matching the mass spectra of unknowns in the samples with those in the Wiley G 1035 A library. Positive identifications were based on comparison and match between the mass spectra and the retention indices of unknown compounds in the sample extracts with the authentic standards under the same experimental conditions (Kim & Ho, 1998).

2.4. DNA extraction and electrophoresis analysis

Human leukemia K562 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (Gibco, BRL), supplemented with 5 mM L-glutamine and 50 μg/mL of antibiotics (Penicillin/Streptomycin, Gibco, BRL) at 37 °C in a humidified 5% CO_2 incubator (Usta, Kreydiyyeh, Bajakian, & Nakkash-Chmaisse, 2002). The K562 human cancer cells were harvested, washed with phosphate-buffered saline (PBS), and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM tris(hydroxy methyl)aminomethan (pH 8.0), and 10 mM ethylenediaminetetraacetic acid (EDTA) at 56 °C overnight and treated with RNase A (0.5 μg/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) before loading and was analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris–borate/EDTA electrophoresis buffer (TBE). Approximately 20 μg of DNA were loaded in each well and visualized under UV light, and photographed.

2.5. Flow cytometry

The degree of DNA fragmentation in cinnamaldehyde treated K562 cells was determined by flow cytometry analysis. K562 cells were treated with 0.5% dimethyl sulphoxide (DMSO) as a control or with different concentration of cinnamaldehyde for 24 h. At each time point, cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol for at least 2 h at

−20 °C. Fixed cells were washed with PBS, incubated with 1 mL PBS containing 0.5 μg/mL RNase A and 0.5% Triton X-100 for 30 min at 37 °C, and then stained with 50 μg/mL propidium iodide. The stained cells were analyzed by FACScan laser flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

2.6. ROS production determination

ROS production was monitored by flow cytometry using dichlorofluorescin diacetate (DCFH-DA). This dye is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cells were treated with cinnamaldehyde (200 μM) for different time periods, and DCFH-DA (30 μM) was added into the medium for a further 30 min at 37 °C. To analyze the generation of superoxide radical (H_2O_2), an H_2O_2-sensitive probe DCFH-DA was used. DCFH-DA is deacetylated in a cell to a nonfluorescent compound 2′,7′-dichlorofluorescin, which remains trapped within the cell and is oxidized by H_2O_2 in the presence of endogenous peroxide to a highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) (Rota, Chignell, & Mason, 1999). The cells were harvested for 0, 2, 4, and 6 h after treatment with 60 μM of cinnamaldehyde. Briefly, K562 cells were exposed to cinnamaldehyde, and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6); (Molecular Probe, Eugene, OR, USA). The samples (10^4 events) were analyzed for fluorescence (FL1 detector, filter 430/30 nm band pass) using a FACScan flow cytometry (Becton Dickinson, San Jose, CA).

2.7. Western blotting

The nuclear and cytosolic proteins were isolated from K562 cells after treatment with 200 μM and total proteins were extracted via the addition of 100 μL of gold lysis buffer (50 mM Tris–HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10,000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 12% SDS–polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS–polyacrylamide gels. For electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris–HCl (pH 8.9), 192 mM glycine and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris–HCl and then immunoblotted with primary antibodies including anti-Bcl-2, anti-Bcl-X_L, anti-Bax, anti-β-actin (Santa Cruz Biotech.), anti-PARP (UBI Inc., Lake Placid, NY) (Transduction Laboratory, Lexington, KY) and anti-DFF45/inhibitor of caspase-activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h, followed by secondary antibody conjugated with horseradish peroxidase. Detection was achieved by measuring the chemiluminescence of the blotting agent (ECL, Amersham Corp., Arlington Heights, IL), after exposure of the filters to Kodak X-Omat films. The mitochondria and cytosolic fractions isolated from cells were used for immunoblot analysis of cytochrome *c* as described by Pan, Lin, Lin-Shiau, and Lin (1999). The cytochrome *c* protein was detected using anti-cytochrome *c* antibody (Research Diagnostic Inc., Flanders, NJ).

2.8. Activity of caspase

Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12,000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promeaga's CaspACE Assay System Corp., Madison, WI). Briefly, 50 μg of total protein, as determined by Bio-Rad protein assay (Bio-Rad Laboratories), was incubated with 50 μM substrate, Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3-specific substrate), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9-specific substrate) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (Hitachi, F2000).

2.9. Preparation of cell homogenate and GSH assay

Cells (1×10^6) were cultured in 100 mm culture dishes. After treated with cinnamaldehyde, they were scarped with a rubber policeman, collected, and homogenized in 0.5 ml cold 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, and centrifuged at 10,000*g* for 15 min at 4 °C and the supernatant was saved for GSH assay following the method of (Xu, Ma, & Purcell, 2003; Xu, Leo, & Lieber, 2003). Cell homogenate GSH was determined with the glutathione assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's specification.

3. Results and discussion

3.1. Volatile compounds identified in the essential oil of *Cinnamomum*

The essential oils obtained from leaves of *Cinnamomum osmophloeum* Kaneh, cultivated under commercial scale in Taiwan, were examined by GC and GC MS. The *Cinnamomum osmophloeum* clone A contains *trans*-cinnamaldehyde (91.15%) as the major component. Cinnamaldehyde is the main component of the essential oil, with the highest percentage found in the leaf oil (94%) (Chang et al., 2001). Interestingly, the same *Cinnamomum osmophloeum* clone B contains less *trans*-cinnamaldehyde (45.59%) but more cinnamyl acetate (46.39%) as compared with that of *Cinnamomum osmophloeum* clone A. Based on the chemical composition, nine types such as cassia, cinnamaldehyde, coumarin, linalool, eugenol, camphor, 4-terpineol, linalool-terpinenol, and mixed type of indigenous cinnamon in Taiwan have been classified by Hu, Lin, and Ho (1985). *Cinnamomum osmophloeum* clones A and B analyzed in this experiment were thus categorized as cinnamaldehyde type and mixed type, respectively. In addition to *trans*-cinnamaldehyde and cinnamyl acetate, at least 40 other compounds were characterized as shown in Table 1. Among the volatiles, benzenepropanal (1.39–1.52%), terpin-4-ol (1.98–2.94%), methylchavicol (1.45–2.11%), bornyl acetate (1.28–1.51%), and eugenol (1.47–2.03), were characterized as well.

Table 1
Compounds identified in the essential oil of *Cinnamomum*

Number	Compounds	KI[b]	A	B
			Concentration (%)[a]	
1	α-Thujene	926	0.48	0.54
2	α-Pinene	932	0.35	0.37
3	Camphene	947	0.16	0.13
4	Benzaldehyde	961	0.61	0.74
5	Sabinene	967	0.06	0.12
6	β-Pinene	975	0.01	0.22
7	β-Myrcene	991	0.02	0.03
8	α-Phellandrene	997	0.21	0.11
9	3-Carene	1004	tr	0.02
10	(+)-4-Carene	1016	0.26	1.01
11	*p*-Cymene	1025	0.11	2.25
12	D-limonene	1029	0.34	0.52
13	Eucalyptol	1042	0.60	0.42
14	(E)-Ocimene	1058	tr	0.03
15	γ-Terpinene	1066	0.01	0.04
16	*cis*-β-Terpineol	1072	0.01	tr
17	Terpinolene	1089	tr	0.04
18	Linalool	1106	0.17	0.23
19	Benzenepropanal	1168	1.52	1.39
20	Terpin-4-ol	1188	2.94	1.98
21	α-Terpineol	1193	0.05	1.04
22	Methylchavicol	1203	1.45	2.11
23	Decanal	1220	0.03	0.02
24	*cis*-Cinnamaldeyhde	1225	0.66	0.39
25	Neral	1228	0.04	0.98
26	Nerol	1235	0.02	0.04
27	*trans*-Cinnamaldeyhde	1284	91.15	45.59
28	Bornyl acetate	1328	1.51	1.28
29	Eugenol	1370	1.47	2.03
30	Copaene	1383	0.26	0.31
31	Geranyl acetate	1393	0.01	0.03
32	Methyl eugenol	1415	tr	0.04
33	Caryophyllene	1428	0.71	0.42
34	Cinnamyl acetate	1456	0.57	46.39
35	α-Caryophyllene	1461	0.10	0.21
36	Germacrene-D	1488	0.07	0.04
37	β-Humulene	1493	0.11	0.23
38	α-Muurolene	1512	0.03	0.08
39	α-Farnesene	1550	tr	0.03
40	Caryophyllene oxide	1595	0.40	tr

[a] Average peak areas of three replicates. tr: <0.01%.
[b] KI: Kovat's indices using a series of *n*-alkanes (C_8–C_{20}) on HP-5 MS column.

3.2. Cinnamaldehyde-induced DNA fragmentation in K562 cells

Previous studies has shown that cinnamaldehyde has biological properties on anti-proliferation and anticancer (Koh et al., 1998). In the present study, we investigated two structurally related compounds, cinnamaldehyde and cinnamyl acetate. The structures of these two compounds are illustrated in Fig. 1A. The effect of cinnamaldehyde and cinnamyl acetate on DNA fragmentation, a hallmark of apoptosis, was determined by incubating K562 cells with different concentrations of cinnamaldehyde and cinnamyl acetate for 24 h. DNA fragmentation appear at 200 μM

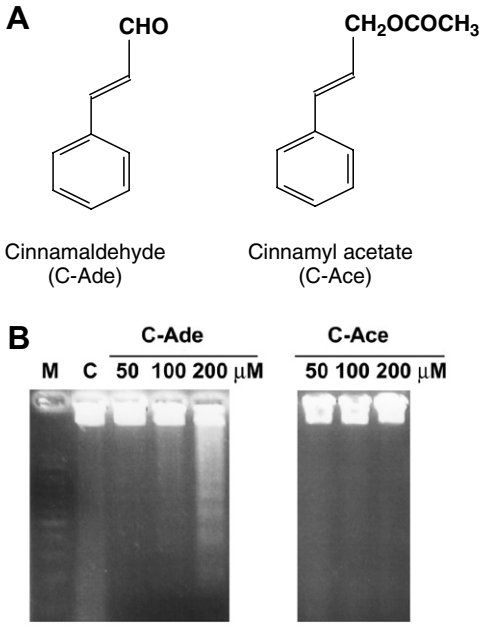

Fig. 1. Induction of DNA fragmentation by treating with various concentrations of cinnamaldehyde and cinnamyl acetate as indicated for 24 h. (A) Structures of cinnamaldehyde and cinnamyl acetate. (B) Internucleosomal DNA fragmentations were analyzed by agarose electrophoresis. M: 100 bp DNA ladder size maker.

of cinnamaldehyde treatment, and the DNA ladders response was dose-dependent from 50 to 200 μM treatment. When K562 cells were treated with 200 μM of cinnamaldehyde, DNA ladders were visible. However, cinnamyl acetate did not induced DNA fragmentation in K562 cells (Fig. 1B).

3.3. Cinnamaldehyde causes dose-dependent increasing in leukemia cell apoptotic ratio

A fluorometric method using DAPI was applied to quantify the amount of fragmented DNA. Similar with the results of DNA fragmentation, cinnamaldehyde

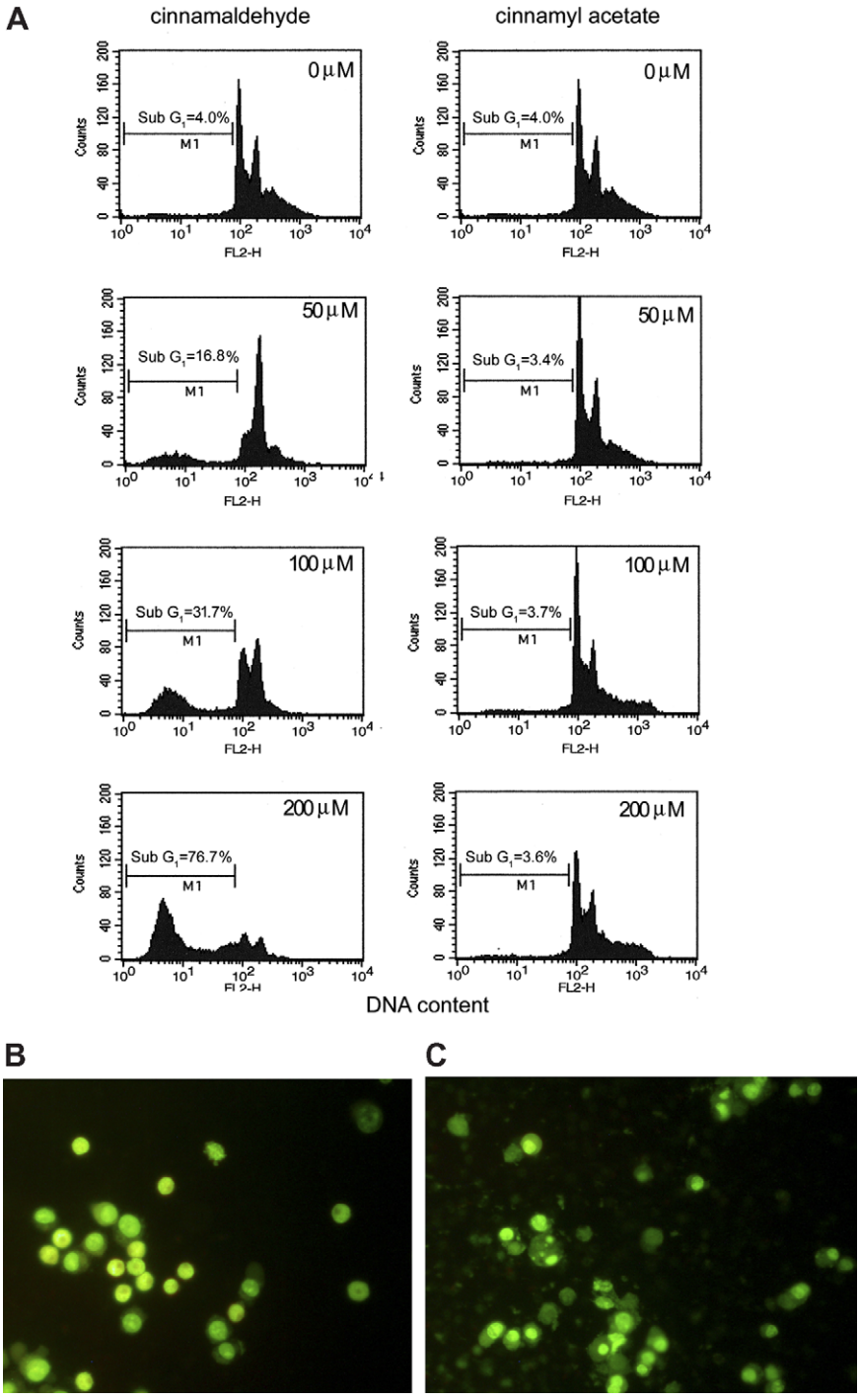

Fig. 2. Determination of sub-G1 and chromatin condensation in cinnamaldehyde- and cinnamyl acetate-treated K562 cells by (A) flow cytometry and acridine orange staining. (B) Untreated cells; (C) cells treated with cinnamaldehyde (50 μM) for 24 h. The condensed chromosomes are seen as spots in the nucleus. AP (apoptotic peak) represents apoptotic cells with a lower DNA content. Data is from three independent experiments.

showed a dose dependent apoptotic effect on K562 cells. Sub G1 peak represents apoptotic cells with a lower DNA content. After 48 h of treatment, a sub G1 (sub 2N) DNA peak, which has been suggested to be the apoptotic DNA, was detected, and the percentages of apoptotic K562 cells was 4.0, 16.8, 31.7, and 76.7% after 0, 50, 100, and 200 μM of incubation with cinnamaldehyde, respectively, and 4.0, 3.4, 3.7, and 3.6% after incubation with 0, 50, 100, and 200 μM cinnamyl acetate, respectively (Fig. 2A). Cinnamaldehyde appeared to be a potent apoptosis-inducing agent for K562 cells, and the apoptotic effect was found to be dose-dependent in the range up to 200 μM.

3.4. Effects of cinnamaldehyde on morphological changes in K562 cell

K562 cells were treated with 0 μM and 50 μM of cinnamaldehyde for 24 h. Cells were harvested and washed with ice-cold PBS, and was examined by fluorescence microscopy. Fig. 2B showed the representative morphological changes of K562 cells exposed to cinnamaldehyde (50 μM) for 24 h. Under control conditions, K562 cells appeared normal and the nuclei were round and homogeneous. After being treated with cinnamaldehyde, the cells exhibited characteristic features of apoptosis including plasma membrane blebbing and cell shrinkage as shown in Fig. 2C. These results indicate that cinnamaldehyde induced apoptosis in K562 cells.

3.5. ROS production involves in cinnamaldehyde-induced apoptosis

The effect of cinnamaldehyde on intracellular superoxide radical level was studied. Fig. 3A shows the typical histograms of superoxide radical in control and cinnamaldehyde-treated cells. There was an increase in ROS. The assay measures an increase in mean fluorescence intensity.

3.6. Treatment with cinnamaldehyde caused the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$)

The cytogram in Fig. 3B shows a typical result of the increase of $\Delta\Psi_m$ in cinnamaldehyde induced apoptosis in K562 cells. During cinnamaldehyde induced apoptosis in K562 cells, dissipation of $\Delta\Psi_m$ led to leakage of DiOC6 (Jayaprakasha, Jagan Mohan, & Sakariah, 2000) from the mitochondrial matrix, which can be measured by flow cytometry as a decrease in the fluorescence intensity of the dye from 116.71 to 62.42. It was found that cinnamaldehyde was able to decrease mitochondrial membrane potential. It is well established that the change of mitochondrial membrane potential ($\Delta\Psi_m$) causes the disruption of the outer mitochondrial membrane and contributes to the release of cytochrome *c* (Zhuang & Cohen, 1998). These observations suggest that an apoptosis-inducing mechanism via mitochondria pathway is triggered by cinnamaldehyde in K562 cells.

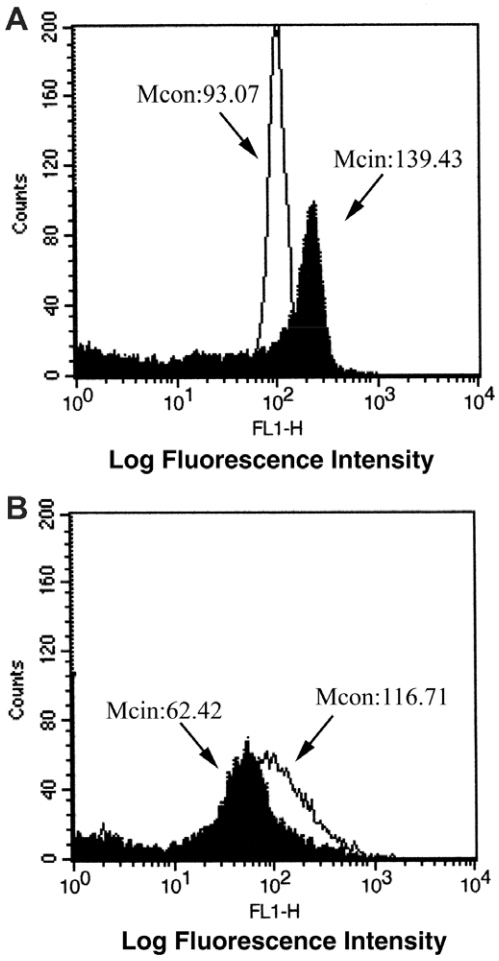

Fig. 3. Induction of ROS generation and mitochondrial dysfunction in cinnamaldehyde-treated cells. Cells were treated with 200 μM cinnamaldehyde for 6 h. (A) Typical histograms showing the increase of intracellular superoxide radical in cinnamaldehyde-treated K562 cells. (B) The Mcon represented the profile of control cells, and the Mcin indicated the treated cells. The numbers represented the mean of the relative fluorescent intensity.

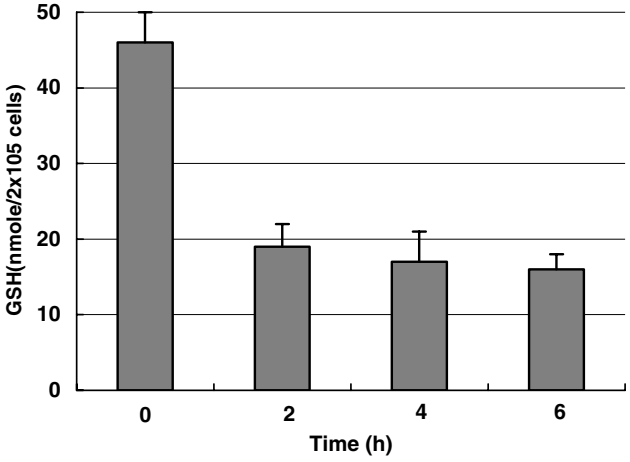

Fig. 4. Effects of cinnamaldehyde on the levels of intracellular glutathione in K562 cells.

3.7. Treatment with cinnamaldehyde caused glutathione depletion

To investigate the influences of cinnamaldehyde treatment on the level of intracellular glutathione, K562 cells were treated with cinnamaldehyde (200 μM). Harvested cells were treated with 5% TCA and GSH was determined by enzymatic method using glutathione reductase. Our results confirmed that cinnamaldehyde is a powerful GSH depletor. The level of intracellular glutathione in K562 cells decreased from 45 mg to 24 mg in 2 h after cinnamaldehyde treatment as shown in Fig. 4.

3.8. Involvement of release of cytochrome c from mitochondria to cytosol, and cspase-9 and caspase-3 activation in cinnamaldehyde-induced apoptosis

The effects of cinnamaldehyde on the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of mitochondrial cytochrome *c* into cytosol were also evaluated. As shown in Fig. 5A, the release of mitochondrial cytochrome *c* into the cytosol was detected at 3 h in cinnamaldehyde-treated K562 cells. Fig. 5B shows that exposure of K562 cells to cinnamaldehyde causes the down-regulation of DNA fragmentation factor 45 (DFF-45) and poly (ADP-ribose) polymerase (PARP). Furthermore, the activation of caspase-9 and caspase-3 by fluorogenic peptide substrate was examined. Cinnamaldehyde induced a significantly increase in caspase-9 and caspase-3 activities in K562 cells, approximately 2.8- and 4.2-fold at 12 h, respectively (Fig. 5C).

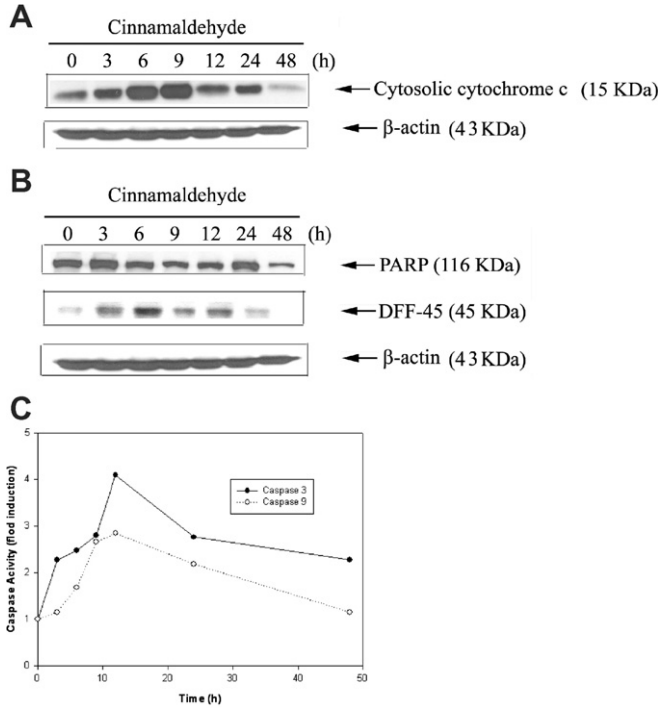

Fig. 5. Induction of cytochrome *c* release following caspase-9 and caspase-3 activation in cinnamaldehyde-induced apoptosis. (A) K562 cells were treated with 200 μM cinnamaldehyde at indicated periods. (B) Time-dependent down-regulation of PARP and DFF-45 in cinnamaldehyde-treated cells. (C) Kinetics of caspase-9 and caspase-3 activation, cells were treated with 200 μM cinnamaldehyde for different time periods or treated with 0.05% DMSO as vehicle control. Data represent means ± SE for three determinations.

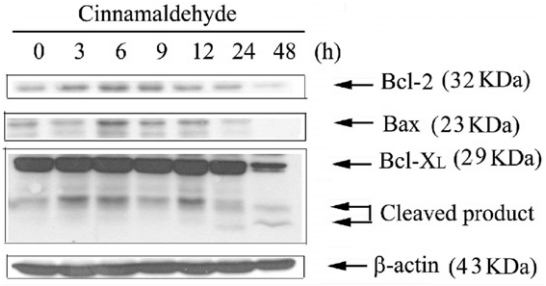

Fig. 6. Effect of cinnamaldehyde on Bcl-2 family protein expression in K562 cells. K562 cells were treated with 200 μM cinnamaldehyde for indicated time point. Expression of Bcl-2, Bax, and Bcl-X_L was analyzed by Western blotting as described in Section 2. This experiment was repeated three times with similar results.

3.9. Effect of cinnamaldehyde on the expression of Bcl-2 family in K562 cells

Several gene products are known to be important in controlling the apoptotic process. The imbalance of expression of anti- and pro-apoptotic protein after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in apoptotic process. The expression of pro-apoptotic protein, Bax, which inserts into the outer member of mitochondria and forms a large channel, allowing the release of cytochrome *c* was examined; this process can be prevented by Bcl-2 or Bcl-X_L (Antonsson et al., 1997). Fig. 6 shows a marked decrease of Bcl-2 and increase of Bax protein in cinnamaldehyde-treated K562cells. The expression of anti-apoptotic proteins, Bcl-X_L of cinnamaldehyde treatment was also tested. A time increase of the cleaved product of Bcl-X_L was observed in cinnamaldehyde-treated cells.

4. Conclusions

Cinnamaldehyde causes dose-dependent increase in leukemia cell apoptotic ratio, whereas low inhibition was observed in the investigation of cinnamyl acetate. A number of *in vitro* and *in vivo* experiments have demonstrated that cinnamaldehyde targets the thiols of cysteine residues on protein molecules (Dornish, Pettersen, & Oftebro, 1989). The aldehyde group may play an important role in anti-proliferative activity against human leukemia cell lines. Cinnamaldehyde has been found to be cytotoxic to L1210 mouse cells. The degree of cytotoxicity of cinnamaldehyde was found to be proportional to the amount of the compound added to the cell culture medium. The mechanism by which cinnamaldehyde inhibits L1210 mouse cell growth was

examined by studying the effect of cinnamaldehyde on RNA, DNA and protein synthesis as well as its effect on glycolysis. Cinnamaldehyde was found to inhibit the growth of L1210 cells by blocking protein synthesis through a direct interaction with sulphydryl-containing amino acids. A more recent study supports the theory that the formation of a cinnamaldehyde–protein conjugate in the skin is via cinnamaldehyde binding sites on the protein that appear to be predominantly the thiol groups of cysteine residues (Smith, Moore, Elahi, Smart, & Hotchkiss, 2000).

Cinnamaldehyde exhibited a concentration-dependent dissipation of the membrane potential producing a 50% increase in the fluorescence intensity. NADH-oxidase (complex I) and succinate dehydrogenase (complex II) constitute, respectively, the main entry site of reducing equivalents NADH and FADH2 into respiratory chain, where oxidation occurs leading ultimately to phosphorylation of ADP into ATP (Usta et al., 2002). Cinnamaldehyde exhibited a concentration-dependent inhibition on NADH oxidation. Among the well established sites where electron transfer occurs coupled to proton release into the cytosol is complex-I (NADH-oxidase). A major consequence of the inhibition of cinnamaldehyde on NADH oxidase is a decrease in ATP production with subsequent accumulation of oxidized dysfunction proteins that will ultimately impair the mitochondrial function leading to cell death (Usta et al., 2002). The cinnamaldehyde effect on membrane potential and stimulation in ATPase activity are indicative of their possible influence on proton redistribution across the membrane. We postulated that cinnamaldehyde may inhibit NADH oxidase, a proton pumping site, ultimately resulting in a decline in ATP level in the treated K562 cells.

Structural changes of cells associated with oxidative stress, such as increased vacuolization and membrane blebbing, were found to be closely correlated with glutathione depletion (Ault & Lawrence, 2003). A high GSH-to GSSG ratio is maintained within cells, which has been shown to be important in the structural integrity and functional processes of membranes, the maintenance and polymerization of microtubules, the conformation of proteins and modulation of their activities (Kosower & Kosower, 1978). Maintaining a high GSH-to GSSG ratio contributes to the redox homeostasis of a cell, which provides an antioxidant defense mechanism against ionizating radiation, reactive oxygen species, free radicals and toxic xenobiotics (Arrick & Nathan, 1984). Cinnamaldehyde has been reported to be a potent depletor of hepatic GSH *in vivo* (Boyland & Chasseaud, 1970). Previous studies have demonstrated that cinnamaldehyde displays a novel property of inducing apoptosis in rat Leukemia L1210 cells through rapid depletion of intracellular thiols (Moon & Pack, 1983). The overall findings thus suggest that mitochondrial permeability transition resulted from intracellular thiol depletion is a critical event in cinnamaldehyde-induced apoptosis.

Recently, the molecular mechanisms of apoptosis have been actively investigated in various *in vitro* and *in vivo* models, and numerous studies have demonstrated that mitochondria play a pivotal role in transducing a variety of pro-apoptotic stimuli (Mignotte & Vayssiere, 1998). It seems that various pro-apoptotic stimuli provoke alteration of the permeability of the apoptotic proteins such as cytochrome *c* (Green & Kroemer, 1998). Ka et al. (2003) postulated that cinnamaldehyde can act as a cytostatic agent for human promyelocytic leukemia HL-60 cells by modulating the mitochondrial permeability transition. The anti-proliferative activity effect of cinnamaldehyde was proposed to be the result of a ROS- and caspase-induced apoptosis. The cytotoxicity of cinnamaldehyde was thus suggested to be the consequence of increased covalent binding of cinnamaldehyde to critical proteins. The cytotoxic activity of cinnamaldehyde on human leukemia cell line K562 could be attributed to follow a similar mechanism. A nonspecific Schiff base reaction may occur between the unsaturated aldehyde group of cinnamaldehyde and either sulphydryl group or amino group of a protein leading to the inactivation of related enzymes.

The disruption of mitochondrial membrane potential results from mitochondrial permeability transition which is due to the permeability transition pore opening. The permeability transition pore is a multi-protein complex. The opening pore has been shown to possess redox-sensitive sites of critical vicinal thiols which are in redox equilibrium with matrix glutathione (Yang, Shen, & Ong, 2000). These thiols are suggested to be a key factor of apoptotic signaling pathways. Therefore, depletion or oxidation of these

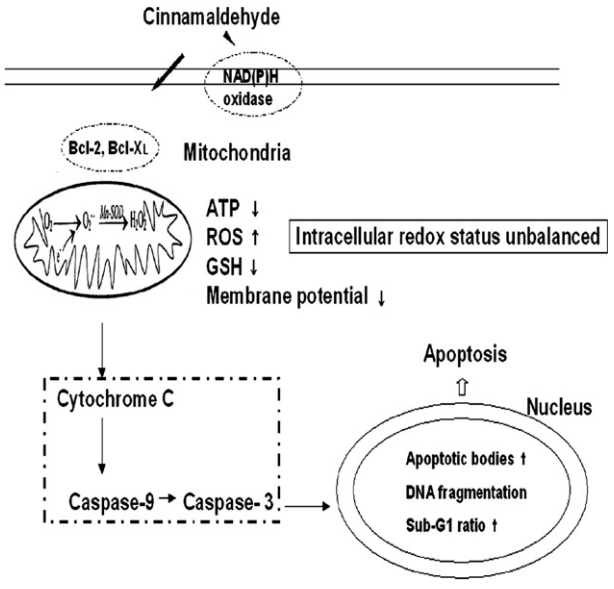

Fig. 7. Schematic representation of action mechanism by cinnamaldehyde induced apoptosis in K562. The initial event induced by cinnamaldehyde may be induce GSH depletion and ROS production further dissipates $\Delta\Psi_m$, resulting in cytochrome *c* release, and leading to consequent activation of caspase-9, and caspase-3 by triggering the translocation of pro-apoptosis Bcl-2 family, Bax, to mitochondria or directly perturbing mitochondria.

thiols or glutathione can facilitate the membrane potential transition (Marchetti et al., 1997). The α,β-unsaturated carbonyl group in cinnamaldehyde is responsible for the potent depletory of these thiols or glutathione. Cinnamaldehyde is conjugated across the carbon–carbon double bond with reduced glutathione (Iersel et al., 1996). Whereas, no α,β-unsaturated carbonyl structure exists in the cinnamyl acetate molecule which seemed to be ineffective in the inhibition of K562 cell growth.

In the present study, it was further demonstrated that mitochondrial permeability transition results from intracellular thiol depletion which is a critical event in cinnamaldehyde induced apoptosis. Thus we postulate that the exerted effects on membrane potential of cinnamaldehyde may be attributed to the release of protons into the matrix or to interaction with sulphydryl groups of membrane proteins. Based on the results in this paper, we propose the signaling pathway of cinnamaldehyde-induced apoptosis in K562 cells as shown in Fig. 7. The possible mechanism acted on the induction of apoptosis by cinnamaldehyde was mitochondria-mediated pathway, activation of caspase cascades responsible for apoptosis occurred following the loss of $\Delta\Psi_m$ and release of cytochrome *c* into cytosol, partially initiate by the imbalance of Bcl-2 family of proteins.

Acknowledgement

This study was supported by the National Science Council NSC 93-2321-B-022-001, NSC 93-2313-B-022-004, and NSC 94-2321-B-022-001.

References

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., et al. (1997). Inhibition of Bax channel-forming activity by Bcl-2. *Science, 277*, 370–372.

Arrick, B. A., & Nathan, C. F. (1984). Glutathione metabolism as a determinant of therapeutic efficacy: a review. *Cancer Research, 44*, 4224–4232.

Ault, J. G., & Lawrence, D. A. (2003). Glutathione distribution in normal and oxidatively stressed cells. *Experimental Cell Research, 285*, 9–14.

Balaban, R. S., Nemoto, S., & Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell, 120*, 483–495.

Boyland, E., & Chasseaud, L. F. (1970). The effect of some carbonyl compounds on rat liver glutathione levels. *Biochemical Pharmacology, 19*, 1526–1528.

Chang, S. T., Chen, P. F., & Chang, S. C. (2001). Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. *Journal of Ethnopharmacology, 77*, 123–127.

Dornish, J. M., Pettersen, E. O., & Oftebro, R. (1989). Modifying effect of cinnamaldehyde and cinnamaldehyde derivatives on cell inactivation and cellular uptake of *cis*-diamminedichloroplatinum(II) in human NHIK 3025 cells. *Cancer Research, 49*, 3917–3921.

Gosslau, A., & Chen, K. Y. (2004). Nutraceuticals, apoptosis, and disease prevention. *Nutrition, 20*, 95–102.

Green, D., & Kroemer, G. (1998). The central executioners of apoptosis: caspases or mitochondria? *Trends in Cell Biology, 8*, 267–271.

Hu, T. W., Lin, Y. T., & Ho, C. K. (1985). Natural variation of chemical components of the leaf oil *Cinnamimum osmphloeum* Kaneh. *Bulletin Taiwan Formosa Research Institue English, 78*, 18.

Huang, T. C., Ho, C. T., & Fu, H. Y. (2004). Inhibition of lipid oxidation in pork bundles processing by superheated steam frying. *Journal of Agricultural and Food Chemistry, 52*, 2924–2928.

Iersel, M. L., Ploemen, J. P., Struik, I., van, A. C., Keyzer, A. E., Schefferlie, J. G., et al. (1996). Inhibition of glutathione *S*-transferase activity in human melanoma cells by α,β-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and *trans*-2-hexenal. *Chemico–Biological Interactions, 102*, 117–132.

Jayaprakasha, G. K., Jagan Mohan, R. L., & Sakariah, K. K. (2000). Chemical composition of the flower oil of *Cinnamomum zeylanicum* blume. *Journal of Agricultural and Food Chemistry, 48*, 4294–4295.

Ka, H., Park, H. J., Jung, H. J., Choi, J. W., Cho, K. S., Ha, J., et al. (2003). Cinnamaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. *Cancer Letters, 196*, 143–152.

Kim, Y. S., & Ho, C. T. (1998). Formation of pentylpyridines in an oil medium. *Journal of Agricultural and Food Chemistry, 46*, 644–647.

Koh, W. S., Yoon, Y., Kwon, B. M., Jeong, T. C., Nam, K. S., & Han, M. Y. (1998). Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. *International Journal of Immunopharmacology, 20*, 643–660.

Kosower, N. S., & Kosower, E. M. (1978). The glutathione status of cells. *International Review of Cytology, 54*, 109–160.

Li, P., Nijhawan, D., & Wang, X. (2004). Mitochondrial activation of apoptosis. *Cell, 116*, S57–S59, 2.

Marchetti, P., Decaudin, D., Macho, A., Zamzami, N., Hirsch, T., Susin, S. A., et al. (1997). Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. *European Journal of Immunology, 27*, 289–296.

Martin, S. J., & Green, D. R. (1995). Protease activation during apoptosis: death by a thousand cuts? *Cell, 82*, 349–352.

Mignotte, B., & Vayssiere, J. L. (1998). Mitochondria and apoptosis. *European Journal of Biochemistry, 252*, 1–15.

Moon, K. H., & Pack, M. Y. (1983). Cytotoxicity of cinnamic aldehyde on leukemia L1210 cells. *Drug and Chemical Toxicology, 6*, 521–535.

Pan, M. H., Lin, J. H., Lin-Shiau, S. Y., & Lin, J. K. (1999). Induction of apoptosis by penta-*O*-galloyl-beta-D-glucose through activation of caspase-3 in human leukemia HL-60 cells. *European Journal of Pharmacology, 381*, 171–183.

Peters, M. M., & Caldwell, J. (1994). Studies on *trans*-cinnamaldehyde. The influence of dose size and sex on its disposition in the rat and mouse. *Food and Chemical Toxicology, 32*, 869–876.

Rota, C., Chignell, C. F., & Mason, R. P. (1999). Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. *Free Radical Biology and Medicine, 27*, 873–881.

Smith, C. K., Moore, C. A., Elahi, E. N., Smart, A. T., & Hotchkiss, S. A. (2000). Human skin absorption and metabolism of the contact allergens, cinnamic aldehyde, and cinnamic alcohol. *Toxicology and Applied Pharmacology, 168*, 189–199.

Usta, J., Kreydiyyeh, S., Bajakian, K., & Nakkash-Chmaisse, H. (2002). In vitro effect of eugenol and cinnamaldehyde on membrane potential and respiratory chain complexes in isolated rat liver mitochondria. *Food and Chemical Toxicology, 40*, 935–940.

VanderEnde, D. S., & Morrow, J. D. (2001). Release of markedly increased quantities of prostaglandin D2 from the skin in vivo in humans after the application of cinnamic aldehyde. *Journal of the American Academy of Dermatology, 45*, 62–67.

Wang, S. Y., Chen, P. F., & Chang, S. T. (2005). Antifungal activities of essential oils and their constituents from indigenous cinnamon (Cinnamomum osmophloeum) leaves against wood decay fungi. *Bioresource Technology, 96*, 813–818.

Xu, J., Ma, M., & Purcell, W. M. (2003). Biochemical and functional changes of rat liver spheroids during spheroid formation and main-

tenance in culture: II. nitric oxide synthesis and related changes. *Journal of Cellular Biochemistry, 90*, 1176–1185.

Xu, Y., Leo, M. A., & Lieber, C. S. (2003). Lycopene attenuates alcoholic apoptosis in HepG2 cells expressing CYP2E1. *Biochemical and Biophysical Research Communications, 308*, 614–618.

Yang, C. F., Shen, H. M., & Ong, C. N. (2000). Ebselen induces apoptosis in HepG(2) cells through rapid depletion of intracellular thiols. *Archives of Biochemistry and Biophysics, 374*, 142–152.

Zhuang, J., & Cohen, G. M. (1998). Release of mitochondrial cytochrome *c* is upstream of caspase activation in chemical-induced apoptosis in human monocytic tumour cells. *Toxicology letters*, 121–129.