The Essential Oils from *Zanthoxylum schinifolium* Pericarp Induce Apoptosis of HepG2 Human Hepatoma Cells through Increased Production of Reactive Oxygen Species

Soon-Young PAIK,^{a,1)} Kyung-Hee KOH,^{b,1)} Sung-Mok BEAK,^c Seung-Hwan PAEK,^c and Jung-Ae KIM^{*,c}

^a College of Medicine, The Catholic University of Korea; 16–1 Dongsomoon-ro, Jongno-gu, Seoul 137–701, Korea:

^b Department of Food Science and nutrition, The Catholic University of Korea; San 43–1 Yeogoug 2 dong, Bucheon 420–743, Korea: and ^c College of Pharmacy, Yeungnam University; 214–1 Dae-dong, Gyongsan 712–749, Korea:

Received October 22, 2004; accepted February 2, 2005

The volatile extract from dried pericarp of *Zanthoxylum schinifolium* that was obtained by simultaneous distillation with dichloromethane and water was composed of 29.9% geranyl acetate, 15.8% citronella, 15.4% sabinene and the minor volatile components included β -myrcene, linalool, (–)-isopulegol, citronellyl acetate, 1,4-dimethyl pyrazole, α -terpinene, 3-methyl-6-(1-methylethyl)-2-cyclo-hexene-1-ol and trans-geraniol. The volatile extract decreased the cell viability and induced apoptotic death in HepG2 human hepatoma cells in a concentration- and time-related manner. In addition, the volatile extract increased the production of reactive oxygen species in a dose-dependent manner. Pretreatment of the cells with Trolox, a well-known antioxidant, significantly suppressed the generation of reactive oxygen species and cell death induced by the extract. However, caspase-3 activity was not changed in the extract-treated cells, suggesting that the extract-induced apoptosis of HepG2 cells is caspase-3 independent. Furthermore, in nude mice inoculated with Huh-7 human hepatoma cells, the extract significantly inhibited tumor development. These results suggest that the volatile extract from *Zanthoxylum schinifolium* pericarpium is a good candidate for hepatocellular carcinoma (HCC) therapy and that reactive oxygen species are the key signaling molecules in the volatile extract-induced cell death in HepG2 cells.

Key words *Zanthoxylum schinifolium*; apoptosis; HepG2; reactive oxygen species; caspase-3

Essential oils from naturally occurring plant dietary items such as onions, garlic and oranges have been reported to influence carcinogen-metabolizing enzymes.²⁾ Furthermore, essential oils have shown chemopreventive potential,³⁾ antitumor activity^{4–6)} and the ability to induce apoptosis in various cancer cell lines.⁷⁾

Apoptosis has been recognized to play an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells.⁸⁾ Genetic changes resulting in the loss of apoptosis or derangement of apoptosis-signaling pathways are likely to be critical components of carcinogenesis.^{9,10)} Additionally, induction of apoptosis of cancer cells is recognized as a valuable tool for cancer treatment.¹¹⁾ The execution process of cells by apoptosis is mediated by caspase-3, one of a family of cysteine proteases.^{12,13)} Reactive oxygen species (ROS) have been suggested to act as an upstream signal for caspase-3 activation.¹⁴⁾ Excessively produced ROS have been proposed as a common mechanism by which various agents induce apoptosis.¹⁵⁾ The apoptosis-inducing mechanisms of ROS include activation of proteases and nucleases, altered gene expression and changes in membrane permeability.^{16,17)}

Rutaceae *Zanthoxylum schinifolium* is an aromatic plant and its pericarp is widely used as a pungent condiment and seasoning in Korea and other East Asian countries. Thirty-three compounds were identified from extracted essential oils of the pericarp of *Zanthoxylum bungeanum* and *Zanthoxylum schinifolium*.¹⁸⁾ However, the antitumor effect of essential oils from the pericarp of *Zanthoxylum schinifolium* has not been studied. Only the root bark and stems of *Zanthoxylum schinifolium* showed antiplatelet aggregation, anti-HBV DNA replication and monoamine oxidase inhibitory activi-

ties.^{19–21)}

Thus, the purposes of this study were to investigate (i) whether the essential oils derived from volatile components in *Zanthoxylum schinifolium* pericarp induce apoptosis in HepG2 cells *in vitro* and regression of tumor size *in vivo*, and (ii) whether the activation of caspase-3 and production of ROS are involved in the mechanism of the volatile extract-induced apoptosis.

MATERIALS AND METHODS

Materials HepG2 human hepatoma cell line was purchased from American Type Culture Collection (Rockville, MA, U.S.A.). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Bis-(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). All other reagents whose suppliers are not indicated were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BAPTA/AM was prepared as a stock solution in dimethyl sulfoxide (DMSO), then diluted with aqueous medium to the final desired concentrations. The stock solutions of drugs were sterilized by filtration through 0.2 μ m disc filters (Gelman Sciences: Ann Arbor, MI, U.S.A.).

Extraction of Essential Oils *Zanthoxylum schinifolium* samples were purchased at Kyungdong (Seoul, Korea) market. A voucher specimen is preserved at the Department of Food Science and nutrition, The Catholic University of Korea, Puchon, Korea. The dried pericarp of *Zanthoxylum schinifolium* was powdered and extracted in 100 ml of dichloromethane and 100 ml of distilled water for 2 h using a

* To whom correspondence should be addressed. e-mail: jakim@yu.ac.kr

modified Likens and Nikerson type simultaneous distillation extraction apparatus at atmospheric pressure. Extracts were concentrated using a macro type Kuderna-Danish Concentrator (Cat NO. 6-4685, Supelco) to 3 ml. Concentrated volatile flavor component (VFC) extracts were re-concentrated to 1 ml under a gentle stream of nitrogen. The concentrates were identified by comparing retention times of unknowns with those of authentic compounds under identical conditions on gas chromatograph (Hewlett Packard 6890) and the mass spectra (Hewlett Packard model 5972).

Cell Culture HepG2 cells were grown at 37 °C in a humidified incubator with 5% CO₂/95% air in an Eagle's minimum essential medium supplemented with 10% FBS, 1 mM sodium pyruvate, 200 IU/ml penicillin and 200 µg/ml of streptomycin. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization with 0.25% trypsin-EDTA solution.

Cell Viability Assay 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Staining Cell viability was assessed by the MTT staining method.²²⁾ Cells were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In the control experiments cells were grown in the same media containing drug-free vehicle. After incubation with the drug for 48 h, 100 µl of MTT (5 g MTT/l in H₂O) were added and cells were incubated for 4 h. Two hundred microliters of DMSO were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

Cell Death Assay (Trypan Blue Exclusion) Cells from 4- to 5-d-old cultures were seeded in equivalent amounts in 35 × 10 mm culture dishes at the density of 2×10^5 cells/dish. The volume of the medium in the dishes was 2 ml. The extracts of *Zanthoxylum schinifolium* were added to cultures 1 d after seeding in order to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 2 d. Cytotoxicity of the drug was assessed by counting the number of cells stained by trypan blue using a hemocytometer.

Flow Cytometric Analysis of Apoptosis For flow cytometry analysis, cells were collected and washed twice with a PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in the PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 µg/ml propidium iodide (PI), and 50 µg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACS Calibur (Becton Dickinson, U.S.A.). At least 20000 events were evaluated. All histograms were analyzed using Cell Quest (Becton Dickinson, U.S.A.) to determine the percentage of nuclei with hypodiploid content indicating apoptosis.²³⁾

The normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. Annexin-V, a Ca²⁺-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS) was employed to detect the early stage of apoptosis.²⁴⁾ It was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). Cells were washed with a cold PBS buffer, and resuspended in a binding buffer. A portion of cell suspension (500 µl) was exposed to Annexin-V-FITC. The cells were gently vortexed, incubated at room temperature for 20 min in

the dark, and then analyzed by FACS Calibur within 1 h of staining.

Intracellular ROS Measurement (Nitroblue Tetrazolium Reduction Assay) The intracellular superoxide generation was measured by the modified version of a previously described assay²⁵⁾ for the conversion of nitroblue tetrazolium (NBT) to formazan. NBT was added to the medium of cells to a final concentration of 1 mg/ml. After the drug treatment, microscopic examination verified the generation of insoluble formazan as dark purple granules. After removal of the medium, cells were lysed and formazan was dissolved with 2 M KOH and 1.4 volume of DMSO. The absorbance was read spectrophotometrically at 654 nm.

Caspase-3 Activity Assay Caspase-3 activity was measured by using an ApoAlert caspase colorimetric assay kit (BD Biosciences, CA, U.S.A.). Cell lysates were mixed with dithiothreitol (DTT) (10 mM)-rich reaction buffer containing 50 µM DEVD-pNA, a caspase-3 substrate, and incubated for 1 h at 37 °C. Enzyme-catalyzed release of pNA was monitored using a microplate reader at 405 nm.

Antitumor Assay in Xenografted Human Hepatocellular Carcinoma Cells Female BALB/c nude mice at 8 weeks of age were housed in stainless steel cages under a 12 h light/12 h dark cycle at a controlled temperature. The dorsal skin of the mice was inoculated with 2×10^7 cells of the Huh7 cells. Tumors developed in the skin of the control mice from 4 weeks after the injection of the Huh7 cells. Two hundred microliters of the extract (0.06 and 0.1%) was injected subcutaneously in the back of mice at the same day of cancer cell inoculation. The experiments were conducted in accordance with the University's guidelines for laboratory animal use and care.

Data Analysis All experiments were performed four times. Data were expressed as mean ± standard error of the mean (S.E.M.) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. *p* values less than 0.05 are considered statistically significant.

RESULTS

Induction of Apoptosis by Essential Oils from Pericarp of *Zanthoxylum schinifolium* in HepG2 Cells The volatile essential oils extracted from *Zanthoxylum schinifolium* contained sabinene (15.40%), citronellal (15.75%) and geranyl acetate (29.8%) as major components (>10%) as shown in Fig. 1. The minor volatile components in the extracts included β-myrcene, linalool, (–)-isopulegol, citronellyl acetate, 1,4-dimethyl pyrazole, α-terpinene, 3-methyl-6-(1-methylethyl)-2-cyclo-hexene-1-ol and trans-geraniol.

Treatment of HepG2 human hepatoma cells with the volatile extract significantly reduced the cell viability examined by MTT assay. The decreased cell viability turned out to be due to cell death, which was determined by trypan blue exclusion assay (Fig. 2). In addition, the volatile extract induced apoptosis of the cells in a concentration- and time-dependent manner studied by flow cytometry by determining hypodiploid DNA content stained with PI and by using annexin-V binding assay to detect the loss of phospholipid asymmetry which occurred at the early stage of apoptosis, as depicted in Figs. 3A and B, respectively. These results clearly

Fig. 1. Total Ion Chromatogram of Volatile Compounds in the Pericarp of *Zanthoxylum schinifolium*

Fig. 2. The Volatile Extract from *Zanthoxylum schinifolium* Reduces Viability of HepG2 Human Hepatoblastoma Cells

Cells treated for 48 h with or without each concentration of the extract were analyzed for viability by MTT (A) and trypan blue exclusion (B) methods.

demonstrate that the essential oils induced apoptotic cell death in the HepG2 cells.

The Essential Oils from *Zanthoxylum schinifolium*-Induced Apoptosis Is Dependent on the Production of Reactive Oxygen Species But Not on Caspase Activation

Since it has been known that ROS are involved in apoptotic cell death induced by a variety of stimulators and that ROS act as an upstream signal for caspase-3 activation,²⁶⁾ which is the execution process of apoptosis,^{12,13)} we examined whether those signals are involved in the volatile extract-induced death of HepG2 cells. As shown in Fig. 4A, the volatile extract increased ROS level in a concentration-dependent manner assessed by measuring the reduction level of nitrobluetetrazolium 20 min after the treatment. However, caspase-3 activity did not change after treatment with different concentrations of the volatile extract for 48 h, as depicted in Fig. 4B. Pretreatment with Trolox, a well-known antioxidant, significantly blocked the volatile extract-induced apoptotic cell death (Figs. 5A, B), whereas the volatile extract-induced cell death was not prevented by either BAPTA, an intracellular Ca^{2+} chelator; PD98059, an inhibitor for extracellular signal-regulated protein kinase (ERK); wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor (Fig. 5A); or caspase inhibitors, Ac-DEVD-CHO and Z-VAD-fmk (Fig. 5B). These results suggest that apoptosis-inducing activity of the essential oils is mediated through ROS, but not

through other cellular signals including caspase-3.

In Vivo Antitumor Effect of the Volatile Essential Oils from *Zanthoxylum schinifolium* on Xenografted Huh-7 Human Hepatocellular Carcinoma Cells

The xenografted nude mice were used to investigate the cancer cell death-inducing effect of the volatile extract on Huh-7 human hepatocellular carcinoma cells *in vivo*. Huh-7 cells are the p53-mutated liver cancer cells, which is different from the HepG2 cells having wild type p53. In MTT assay we have also found that the extract decreased the viability of Huh-7 cells (data not shown here). In the skin of mouse that was not treated with the extract, tumors were developed after 4 weeks of being inoculated with Huh7 cells and from this time the incidence of the tumors increased progressively, as shown in Fig. 6B. On the other hand, the mice treated with 0.06% of the extract developed tumors at 6 weeks after the cancer cell inoculation. Furthermore, the treatment with 0.1% of the extract significantly inhibited tumorigenesis in xenografted nude mice.

DISCUSSION

The risk of human cancer is strongly influenced by choice in life style and diet is also reported to be an important factor for determining the mortality of cancer.^{27,28)} Recently, it has been greatly reviewed that dietary phytochemicals possess a

Fig. 3. The Volatile Extract Induces Apoptosis in a Concentration- and Time-Dependent Manner in HepG2 Human Hepatoma Cells

In the experiments of (A) cells were treated for 48 h with or without each concentration of the extract. The number of apoptotic cells was measured by flow cytometry as described in the text. The region to the left of the G_0/G_1 peak was defined as cells undergoing apoptosis-associated DNA degradation. In the experiments of (B), the cells were incubated in the absence (black-filled) or in the presence (gray-filled) of the extracts for each designated time. Cells were stained with Annexin-V-FITC and analyzed by flow cytometry. Note that in the presence of the extracts there is a shift in Annexin-V-FITC fluorescence. This is due to the binding of annexin-V to membrane phospholipids of cells undergoing apoptosis. Results are representative of the four experiments. In bar graphs the data represent the mean values of four replications with bars indicating S.E.M. * $p < 0.05$ compared to control.

Fig. 4. The Volatile Extract Enhances the Production of Reactive Oxygen Species with No Changes of Caspase-3 Activity in HepG2 Human Hepatoma Cells

The data (A) show the level of ROS measured by NBT reduction assay. The cells were treated with different concentrations of the extract for 20 min. In the experiments of (B) changes in caspase-3 activity was measured by colorimetric assay as described in the text. The data represent the mean values of four replications with bars indicating S.E.M. * $p < 0.05$ compared to control.

Fig. 5. Trolox, an Antioxidant, Markedly Reduces the Extract-Induced Apoptotic Death of HepG2 Cells.

The cell viability (A) was measured by MTT assay and the measurement of apoptotic cells (B) was the same as in Fig. 3. The cells were pretreated for 4 h with BAPTA (1 μ M) and 30 min with Trolox (200 μ M), PD98059 (100 μ M), Wortmannin (100 nM) or caspase inhibitors, Z-VAD-fmk (10 μ M) and DEVD-CHO (5 μ M) before the extract (1 μ l/ml) application. In bar graphs the data represent the mean values of four replications with bars indicating S.E.M. * p <0.05 compared to the control. # p <0.05 compared to the group treated with the extracts alone.

Fig. 6. The Volatile Extract Suppresses the Growth of Huh7 Human Hepatocarcinoma Cells in Nude Mice

The mice (B, C, D) were injected with 23×10^7 cells of the Huh7 cells. The mice were treated with extracts dissolved in PBS in a concentration of 0.06 (C) and 0.1 % (D) subcutaneously at the same day of the cancer cell inoculation. Representative mice from three independent experiments were photographed 4 weeks after the injection of Huh7 cell line. The arrows point out the tumor masses.

cancer chemoprevention activity.²⁹⁾ Some essential oils from various plants including onions, garlic and ginger have also been reported to have the anticarcinogenic properties.^{30–32)}

The pericarp of *Zanthoxylum schinifolium* is used as one of the sources for a pungent condiment and seasoning in China, Korea, Japan and Taiwan. Previously, essential oils from the pericarp of *Zanthoxylum bungeanum* and *Zanthoxylum schinifolium* were identified as thirty-three compounds.¹⁸⁾ Our present study identified more than thirty-three compounds and found that geranyl acetate was the most abundant among the constituents in the essential oils from pericarp of *Zanthoxylum schinifolium*. Geranyl acetate (3,7-dimethyl-2,6-octadiene-1-ol acetate) is a natural constituent of more than 60 essential oils, including Ceylon citronella, palmarosa, lemon grass, petit grain, neroli bigarade, geranium, coriander, carrot, and saffras. Geraniol, generated from geranyl acetate by geranyl acetate esterase, has been reported to possess *in vitro* and *in vivo* antitumor activity.²⁶⁾

In the present study, we report for the first time that volatile essential oils from pericarp of *Zanthoxylum schinifolium* possess an antitumor activity on HepG2 human hepatoma cells by inducing apoptosis. The effect of the volatile extract on the viability of Chang human hepatocytes was less potent than that in HepG2 cells (data not shown here), suggesting that the effect of the extract is more selective to cancer cells than to normal hepatocytes. The apoptosis-inducing action of the extract seems to be mediated through ROS production since the extract generated ROS,

and the antioxidant Trolox significantly inhibited apoptosis induced by the extract. ROS has been reported as a key signal molecule in apoptotic cell death induced by various agents including tamoxifen,³³⁾ capsaicin^{34,35)} and garlic.^{36,37)}

Even though many cellular studies have shown that intracellular Ca^{2+} plays a role in cancer cell death through interplay with ROS production,^{38,39)} the volatile extract-induced apoptosis of HepG2 cells did not seem to be related to changes in intracellular Ca^{2+} level. The previous studies have shown that the antiproliferative effect of some of phytochemicals is mediated through actions in cell-signaling kinases such as mitogen-activated protein kinases (MAPK), protein kinase C and PI3K. On the contrary, PD98059 and wortmannin, the inhibitors of ERK and PI3K, respectively did not block the apoptotic cell death induced by the volatile extract from *Zanthoxylum schinifolium*, indicating that MAPK or PI3K seems to be not involved in the apoptosis. More importantly, the extract-induced apoptosis was blocked by an antioxidant. Overall, the results suggest a pivotal role for ROS exists in the apoptosis induction by the essential oils extracted from *Zanthoxylum schinifolium*. The apoptosis-inducing action of the essential oils in HepG2 cells was further supported by the *in vivo* anti-tumor effect on Huh-7 cells xenografted into nude mice. The results represent that the HepG2 cells and Huh-7 cells, hepatocellular carcinoma (HCC) cells, are susceptible to the extract regardless of their p53 status.

In conclusion, our results suggest a plausible utilization of the essential oils from *Zanthoxylum schinifolium* pericarp as an anti-tumor agent in HCC therapy.

Acknowledgements This work was supported by a grant (02-PJ2-PG3-20802-0001) from the Ministry of Health and Welfare, R.O.K.

REFERENCES AND NOTES

- 1) The authors are equally contributed to this work.
- 2) Banerjee S., Sharma R., Kale R. K., Rao A. R., *Nutrition Cancer Internat. J.*, **21**, 263—269 (1994).
- 3) Salim E. I., Fukushima S., *Nutrition Cancer Internat. J.*, **45**, 195—202 (2003).
- 4) Shoff S. M., Grummer M., Yatvin M. B., Elson C. E., *Cancer Res.*, **51**, 37—42 (1991).
- 5) Yu S. G., Hildebrandt L. A., Elson C. E., *J. Nutr.*, **11**, 2763—2767 (1995).
- 6) Carnesecchi S., Schneider Y., Ceraline J., Duranton B., Gosse F., Seiler N., Raul F., *J. Pharmacol. Exp. Ther.*, **298**, 197—200 (2001).
- 7) Buhagiar J. A., Podesta M. T., Wilson A. P., Micallef M. J., Ali S., *Anticancer Res.*, **19**, 5435—5443 (1999).
- 8) Nayfield S. G., Karp J. E., Ford L. G., Dorr F. A., Kramer B. S., *J. Natl. Cancer Inst.*, **83**, 1450—1459 (1991).
- 9) Kastan M. B., Canman C. E., Leonard C. J., *Cancer and Metastasis Review*, **14**, 3—15 (1995).
- 10) Schulte-Hermann R., Bursch W., Low-Baselli A., Wagner A., Grasl-Kraupp B., *Cell Biol. Toxicol.*, **13**, 339—348 (1997).
- 11) Kornblau S. M., *Leukemia*, **12**, S41—S46 (1998).
- 12) Polverino A. J., Patterson S. D., *J. Biol. Chem.*, **272**, 7013—7021 (1997).
- 13) Thornberry N. A., *Chemistry and Biology*, **5**, R97—R103 (1998).
- 14) Jacobson M. D., Raff M. C., *Nature (London)*, **374**, 814—816 (1995).
- 15) Kamata H., Hirata H., *Cell Signalling*, **11**, 1—14 (1999).
- 16) Yu B. P., *Physiological Reviews*, **74**, 139—162 (1994).
- 17) Schiaffonati L., Tiberio L., *Liver*, **17**, 183—191 (1997).
- 18) Liu S., Wei L., *Zhongguo Zhong Yao Za Zhi*, **16**, 359—360, 383 (1991).
- 19) Chen I. S., Lin Y. C., Tsai I. L., Teng C. M., Ko F. N., Ishikawa T., Ishii H., *Phytochemistry*, **39**, 1091—1097 (1995).
- 20) Tsai I. L., Lin W. Y., Teng C. M., Ishikawa T., Doong S. L., Huang M. W., Chen Y. C., Chen I. S., *Planta Medica*, 618—623 (2000).
- 21) Jo Y. S., Huong D. T., Bae K., Lee M. K., Kim Y. H., *Planta Medica*, **68**, 84—85 (2002).
- 22) van de Loosdrecht A. A., Nennie E., Ossenkoppele G. J., Beelen R. H., Langenhuijsen M. M., *J. Immunol. Methods*, **141**, 15—22 (1991).
- 23) Hockenbery D., Nunez G., Millman C., Schreiber R. D., Korsmeyer S. J., *Nature (London)*, **348**, 334—346 (1990).
- 24) Vermes I., Haanen C., Steffens-Nakken H., Reutelingsperger C., *J. Immunol. Methods*, **184**, 39—51 (1995).
- 25) Rook G. A., Steele J., Umar S., Dockrell H. M., *J. Immunol. Methods*, **82**, 161—167 (1985).
- 26) Ueda S., Masutani H., Nakamura H., Tanaka T., Ueno M., Yodoi J., *Antioxidants and Redox Signal*, **4**, 405—414 (2002).
- 27) Doll R., Peto R., *J. Natl. Cancer Inst.*, **66**, 1191—1308 (1981).
- 28) Key T. J., Schatzkin A., Willett W. C., Allen N. E., Spencer E. A., Travis R. C., *Public Health Nutrition*, **7**, 187—200 (2004).
- 29) Surh Y. J., *Nature Reviews Cancer*, **3**, 768—780 (2003).
- 30) Belman S., *Carcinogenesis*, **4**, 1063—1065 (1983).
- 31) Sadhana A. S., Rao A. R., Kucheria K., Bijani V., *Cancer Lett.*, **40**, 193—197 (1988).
- 32) Wattenberg L. W., Coccia J. B., *Carcinogenesis*, **12**, 115—117 (1991).
- 33) Lee Y. S., Kang Y. S., Lee S. H., Kim J. A., *Cell Death and Differentiation*, **7**, 925—932 (2000).
- 34) Ito K., Nakazato T., Yamato K., Miyakawa Y., Yamada T., Hozumi N., Segawa K., Ikeda Y., Kizaki M., *Cancer Res.*, **64**, 1071—1078 (2004).
- 35) Lee Y. S., Kang Y. S., Lee J. S., Nicolova S., Kim J. A., *Free Radical Research*, **38**, 405—412 (2004).
- 36) Kwon K. B., Yoo S. J., Ryu D. G., Yang J. Y., Rho H. W., Kim J. S., Park J. W., Kim H. R., Park B. H., *Biochem. Pharmacol.*, **63**, 41—47 (2002).
- 37) Filomeni G., Aquilano K., Rotilio G., Ciriolo M. R., *Cancer Res.*, **63**, 5940—5949 (2003).
- 38) Bae G. U., Seo D. W., Kwon H. K., Lee H. Y., Hong S., Lee Z. W., Ha K. S., Lee H. W., Han J. W., *J. Biol. Chem.*, **274**, 32596—32602 (1999).
- 39) Supinski G., Nethery D., Stofan D., DiMarco A., *J. Appl. Physiol.*, **87**, 2177—2185 (1999).