Bioorganic & Medicinal Chemistry Letters 20 (2010) 6435–6438

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Apoptotic action of ursolic acid isolated from Corni fructus in RC-58T/h/SA#4 primary human prostate cancer cells

Seong-Hyuk Kwon [a], Hye-Young Park [a], Jae-Yong Kim [a], Il-Yun Jeong [b], Mi-Kyung Lee [a], Kwon-Il Seo [a,*]

[a] Department of Food and Nutrition, Sunchon National University, 413 Jungangro, Suncheon, Jeonnam 540-742, Republic of Korea
[b] Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Republic of Korea

ARTICLE INFO

Article history:
Received 7 June 2010
Revised 1 September 2010
Accepted 14 September 2010
Available online 17 September 2010

Keywords:
Corni fructus
Ursolic acid
Apoptosis
Primary human prostate cancer cells

ABSTRACT

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a major biological active component of Corni fructus that is known to induce apoptosis. However, the apoptotic mechanism of ursolic acid using primary malignant tumor (RC-58T/h/SA#4)-derived human prostate cells is not known. In the present study, ursolic acid significantly inhibited the growth of RC-58T/h/SA#4 cells in dose- and time-dependent manners. Ursolic acid induced cell death as evidenced by an increased proportion of cells in sub-G1 phase, the formation of apoptotic bodies, nuclear condensation, and DNA fragmentation. After ursolic acid treatment at concentrations above 40 μM, the activities of caspase-3, -8, and -9 were significantly increased compared that of control. Ursolic acid modulated the upregulation of Bax (pro-apoptotic) as well as the downregulation of Bcl-2 (anti-apoptotic). Ursolic acid also stimulated Bid cleavage, which indicates that the apoptotic action of caspase-8-mediated Bid cleavage leads to the activation of caspase-9. Thus, the apoptotic effect of ursolic acid was involved in extrinsic and intrinsic signaling pathways. In addition, ursolic acid increased the expression of the caspase-independent mitochondrial apoptosis factor (AIF) in RC-58T/h/SA#4 cells. The present results suggest that ursolic acid from Corni fructus activated apoptosis in RC-58T/h/SA#4 cells via both caspase-dependent and -independent pathways.

Prostate cancer is one of the most common malignancies and represents the second cause of cancer-related deaths in men worldwide.[1] In particular, prostate cancer has become more frequently diagnosed in Asian countries, potentially due to the Western dietary habits.[2] Epidemiologic studies suggest that dietary modifications could be an effective approach in reducing various cancers, including prostate cancer.[3] Recently, many types of medicinal plants were found to exhibit pharmacological effects, and many may have potential uses in cancer chemotherapy.[4] Therefore, many plants have been examined for the identification of new and effective anti-cancer compounds, as well as to elucidate the mechanisms of cancer prevention via apoptosis.

Corni fructus, the fruit of *Cornus officinalis* Sieb. Et Zucc. (family Cornaceae), has been traditionally used as a crude substance to treat tuberculosis, urination, allergy, asthma, hepatitis, lumbago, and chronic nephritis in Korea, Japan, and China.[5] Its major chemical constituents include ursolic acid, gallic acid, 5-hydroxymethylfurfural, morroniside, sweroside, and loganin.[6] These identified components were shown to have biological activities, including anti-inflammatory,[7] anti-oxidant,[8] anti-diabetic,[9] anti-microbial,[10] and anti-cancer activities.[11] Among them, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid compound, possesses a wide range of biological activities. Recently, many studies have been carried out to investigate the anti-tumor activity of ursolic acid, which is considered one of the most promising chemopreventive agents for cancer.[12] However, the mechanism of ursolic acid-induced apoptosis has never been investigated in RC-58T/h/SA#4 prostate cancer cells. In vitro human cell culture models are required to clarify the mechanism of prostate cancer progressing and for testing preventive and therapeutic agents. Many researchers have used human prostate cell lines (DU145, PC-3 and LNCaP) isolated from metastatic lesions.[13] However, these cells do not accurately reflect the genetic makeup or biological behavior of primary prostate tumors, whereas primary prostate tumor-derived cell lines retain the original phenotypes of primary cells and even express some prostate-specific markers.[14] RC-58T/hTERT cells would therefore constitute a novel human cancer culture model for the study of prostate cancer. Therefore, we have evaluated the apoptotic effect of ursolic acid from Corni fructus using primary tumor-derived human prostate cancer cells (RC-58T/h/SA#4).

Ursolic acid from Corni fructus were isolated using a previous method.[15] Corni fructus were extracted with MeOH at 80 °C. After filtration and concentration, the resultant extracts were suspended with H_2O and then partitioned with *n*-hexane, $CHCl_3$, EtOAc, and butanol to give *n*-hexane-, $CHCl_3$-, EtOAc-, and butanol-extractable residues. Among these extracts, the EtOAc extracts showed the

* Corresponding author. Tel.: +82 61 750 3655; fax: +82 61 752 3657.
E-mail address: seoki@sunchon.ac.kr (K.-I. Seo).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.09.073

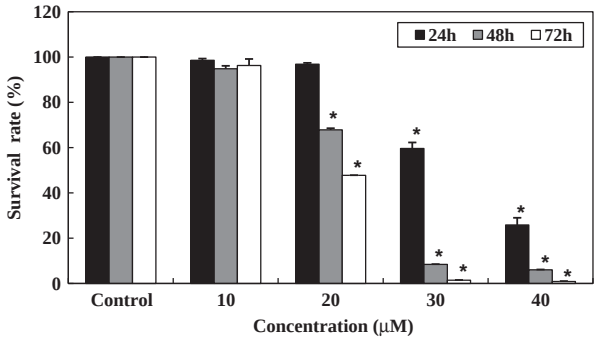

Figure 1. Cell growth inhibition effects in prostate cancer cells (RC58T/h/SA#4) treated with ursolic acid from Corni fructus for 24, 48, and 72 h by SRB assay.

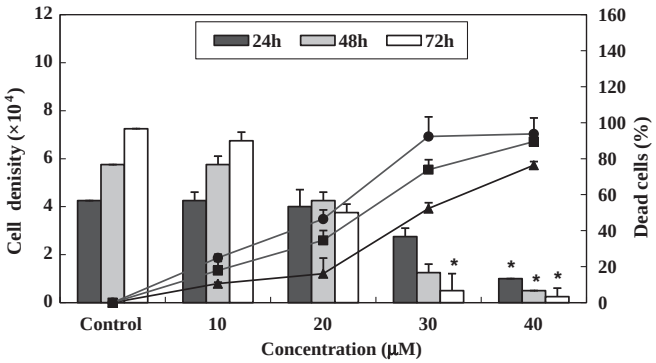

Figure 2. Cell growth inhibition effects in the prostate cancer cells (RC58T/h/SA#4) treated with ursolic acid from Corni fructus for 24, 48, and 72 h by trypan blue dye assay.

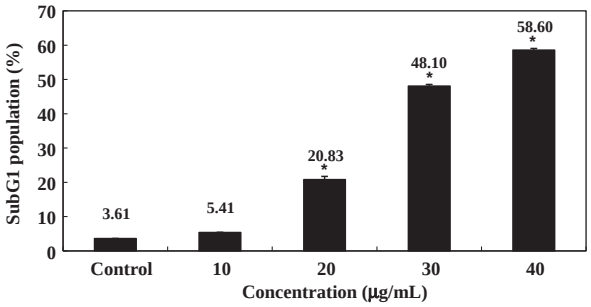

Figure 3. Sub-G1 population of RC58T/h/SA#4 cells treated with ursolic acid from Corni fructus for 24 h.

highest anti-cancer activity. Subsequently, bioassay-guided fractionation of the EtOAc-extractable residue, applying successive silica gel (or sephadex LH-20) column chromatography, led to the isolation of the compound. Based on the ^{1}H NMR, ^{13}C NMR and MS spectra, the isolated compound was easily identified as ursolic acid by comparison with literature data.

RC-58T/h/SA#4 cells were treated with various concentrations of ursolic acid. Cell viability was then assessed after 24, 48, and 72 h of incubation by both SRB assay and cell counting using trypan blue dye. This study show the anti-proliferative effect of ursolic acid on RC-58T/h/SA#4 cells in a time-dependent and dose-dependent manner ($\geqslant$30 μM) (Fig. 1). The number of viable cells decreased while the rate of cell death was increased in a dose-dependent manner in RC-58T/h/SA#4 cells treated with ursolic acid (Fig. 2).

To investigate the induction of the sub-G1 cell population, the DNA content of RC-58T/h/SA#4 cells treated with various concentrations (10, 20, 30 and 40 μM) of ursolic acid for 24 h was determined by flow cytometry as shown in Figure 3. We found that when ursolic acid ($\geqslant$20 μM) was added to the RC-58T/h/SA#4 cell culture, the sub-G1 cell population was significantly increased in a dose-dependent manner compared to the control.

To observe the morphologic characteristics of apoptosis, cells were stained with Hoechst 33258 after RC-58T/h/SA#4 cells were exposed to 30 and 40 μM of ursolic acid for 24 h. Control cells showed even distribution of the stain and round homogeneous nuclei. However, apoptosis in cells treated with ursolic acid was increased in a dose-dependent manner, accompanied by characteristic changes such as chromatin condensation and nuclear fragmentation (Fig. 4). For further assessment of apoptosis induced by ursolic acid, we assessed the integrity of DNA by agarose gel electrophoresis. As shown in Figure 5, incubation of RC-58T/h/SA#4 cells with 30 and 40 μM of ursolic acid elicited a characteristic DNA fragmentation "ladder" representing integer multiples of internucleosomal DNA in a dose-dependent manner.

To examine whether or not the apoptotic effects induced by ursolic acid activated caspase enzymes, the activities of initiator caspase (caspase-8, -9) and effector caspase (caspase-3) were investigated by fluorometric protease assay. Ursolic acid (40 μM) stimulated the activities of caspase-8, -9, and -3 in RC-58T/h/SA#4 cells by 1.8-, 1.1-, and 1.8-fold, respectively (Fig. 6). These results indicate that caspase-8, -9, and -3 were involved in apoptosis induced by ursolic acid.

As shown in Figure 7, although significantly elevated in a dose-dependent manner, the apoptotic effect of ursolic acid was attenuated in RC-58T/h/SA#4 cells treated with 10 μM Z-VAD-fmk, a general caspase inhibitor, compared to cells without Z-VAD-fmk. These results clearly indicate that ursolic acid-induced apoptosis was dependent on caspase activation.

Furthermore, we examined the cleavage of a well-known caspase-3 substrate, poly (ADP-ribose) polymerase, from its 116 kDa intact form into a 89 kDa fragment by Western blotting. Poly-(ADP-ribose) polymerase was processed into its predicted cleavage product of 89 kDa after ursolic acid treatment, but the processing did not occur in the untreated cells (Fig. 8).

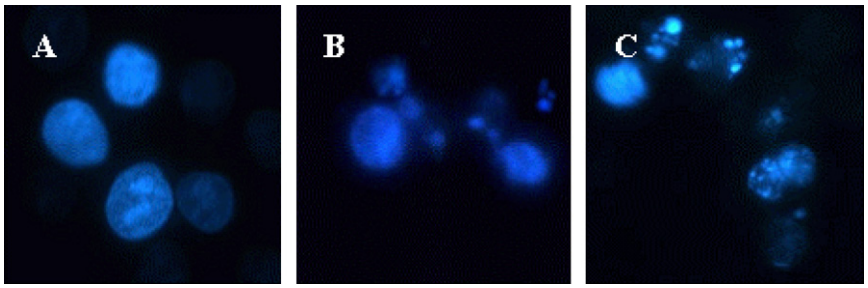

Figure 4. Nuclear fragmentation induced in the RC58T/h/SA#4 cells treated with ursolic acid from Corni fructus for 24 h.

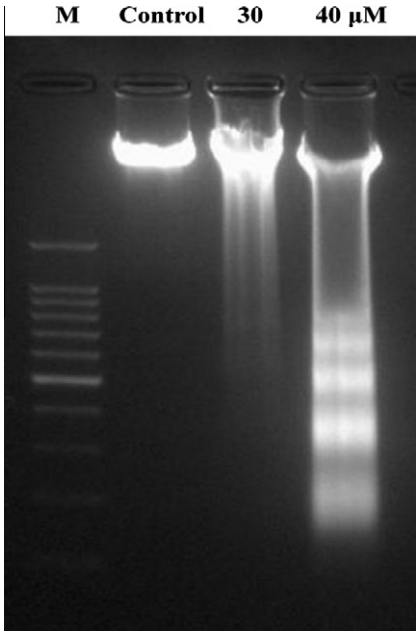

Figure 5. DNA fragmentation by treatment of ursolic acid in RC58T/h/SA#4 cells for 24 h.

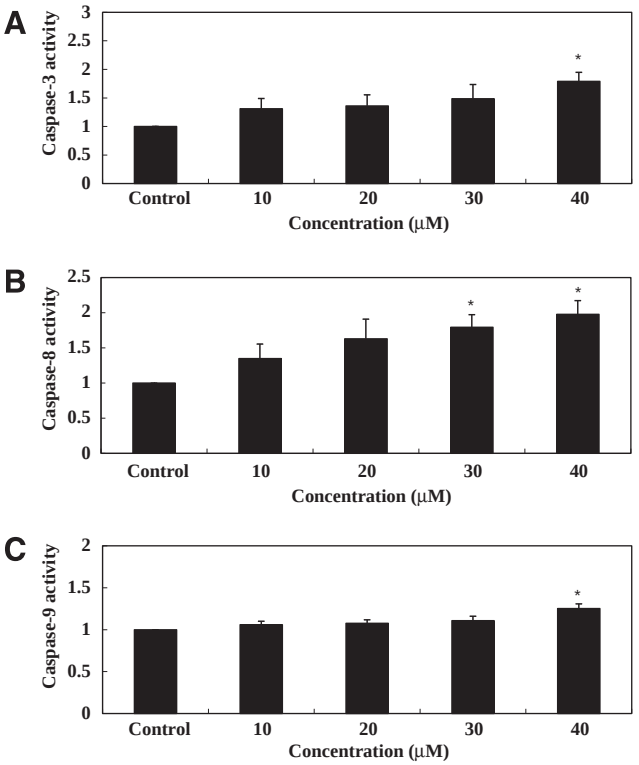

Figure 6. Caspase activities in the RC58T/h/SA#4 cells treated with ursolic acid from Corni fructus.

To determine whether or not apoptotic induction of ursolic acid was involved in the mitochondrial pathway, we examined the levels of Bcl-2 proteins such as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) by Western blotting. As shown in Figure 9A, ursolic acid dose-dependently increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic protein Bcl-2 in RC-58T/h/SA#4 cells.

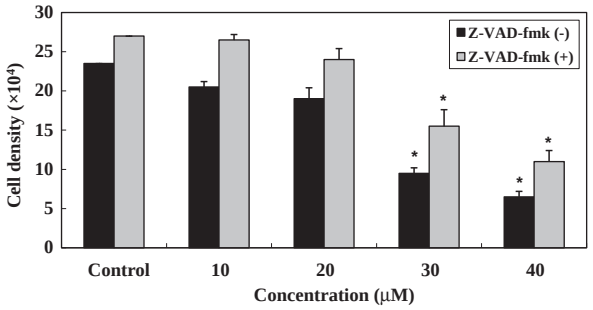

Figure 7. Effect of a caspase inhibitor (Z-VAD-fmk) on cell death induced by ursolic acid from Corni fructus.

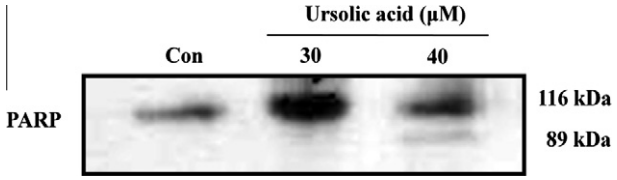

Figure 8. Apoptotic PARP cleaved fragmentation in the RC58T/h/SA#4 cells treated with ursolic acid from Corni fructus for 24 h by Western blot.

Since ursolic acid-induced apoptosis involves the initiation of death receptor and mitochondrial signaling, it is possible that ursolic acid activated the mitochondrial apoptotic pathway through caspase-8-mediated Bid cleavage, resulting in caspase-9 activation. Accordingly, we confirmed the status of Bid during ursolic acid-induced apoptosis. Figure 9B shows that the full size Bid (22 kDa) protein was cleaved to yield a 15 kDa fragment following treatment with ursolic acid, an event that closely matched the appearance of caspase-8 activation.

AIF is a mitochondrial protein that can be translocated into nuclei during caspase-independent apoptosis, whereupon it mediates nuclear condensation and DNA fragmentation.[17] We determined whether or not AIF pays a role in ursolic acid-induced apoptosis by analyzing changes in the levels of AIF by Western blotting (Fig. 10A), as well as AIF translocation into the nucleus by immunostaining (Fig. 10B). The results suggest that AIF translocation into the nucleus is required for ursolic acid-induced apoptosis in RC-58T/h/SA#4 cells.

The induction of apoptosis is known to be an efficient strategy for cancer therapy.[18] Recently, bioactive components isolated from a variety of medicinal plants have been observed to trigger apoptosis. The present study demonstrated for the first time that ursolic acid exhibited an anti-proliferative effect on primary malignant tumor (RC-58T/h/SA#4)-derived human prostate cells. This effect of ursolic acid was due to apoptosis induction. Apoptosis is characterized by a series of morphological and biochemical alterations to the cell such as cell shrinkage, chromatin condensation, plasma membrane blebbing, DNA fragmentation, and sub-G1 DNA accumulation.[19] We observed that ursolic acid induced such characteristic morphological changes and increased the accumulation of RC-58T/h/SA#4 cells in sub-G1 phase.

The caspase family plays an important role in driving apoptosis as a part of both the extrinsic and intrinsic apoptotic pathway.[20] Caspase-8 is known to propagate the apoptotic signal either by direct cleavage and activation of downstream caspases or by the cleavage of Bid.[21] Death receptor-triggering activates the mitochondrial pathway via the caspase-8-mediated cleavage of Bid.[16] Our data shows that the activation of both caspase-8 and -9 significantly increased ursolic acid-induce apoptosis in RC-58T/h/SA#4 cells. We also observed that ursolic acid enhanced Bid cleavage.

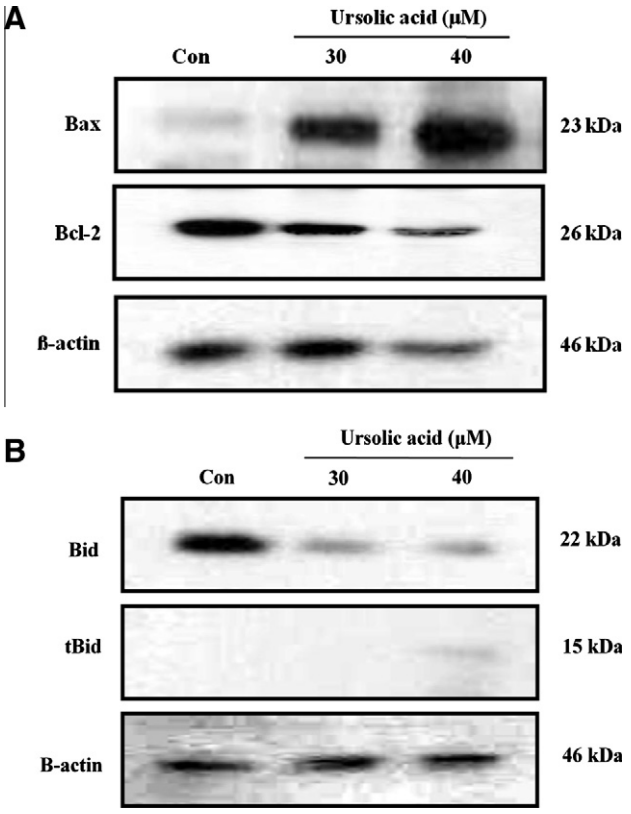

Figure 9. Ursolic acid from Corni fructus induced apoptosis via the mitochondrial pathway.

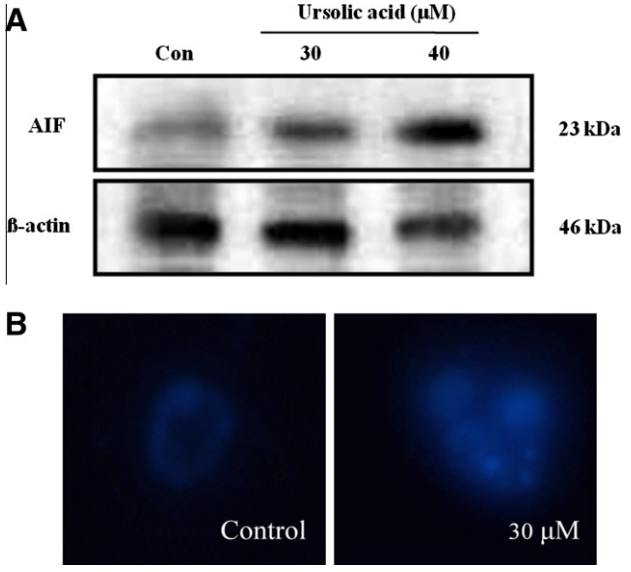

Figure 10. Induction of apoptosis through caspase-independent pathway in the RC58T/h/SA#4 cells treated with ursolic acid from Corni fructus.

Thus, the induction of apoptosis ursolic acid might have been mediated through the death receptor pathway.

The intrinsic apoptosis pathway is dependent on Bcl-2 family members that regulate cytochrome c as well as on the release apoptotic protease activating factor-1 (Apaf-1), which leads to caspase-9 activation. The active forms of caspase-9 can activate downstream effector caspase-3.[22] After caspase-3 activation, a specific substrate such as poly (ADP) ribose polymerase (PARP) is cleaved, which is an important step in apoptosis.[23] We found that ursolic acid increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic Bcl-2. This subsequently activated caspase-9, which in turn activated caspase-3 in RC-58T/h/SA#4 cells. Further, when performing a PARP 116 kDa band became faint while the 89 kDa band became dark in cells after 24 h of ursolic acid treatment. These data indicate that ursolic acid-induced apoptosis is caspase-dependent and involves the activation of a mitochondrial pathway.

On the other hand, ursolic acid elevated AIF protein expression and AIF translocation into the nucleus. AIF is a mitochondrial apoptosis-inducing factor implicated in apoptosis as well as a mitochondrial flavoprotein that translocates to the nucleus in response to apoptotic stimuli. In the nucleus, AIF induces partial DNA fragmentation and chromatin condensation. AIF appears to promote nuclear apoptosis independent of caspase activation, although it likely acts in a cooperative manner with other factors.

In conclusion, these results suggest that ursolic acid from Corni fructus could inhibit RC-58T/h/SA#4 prostate cancer cell growth by apoptotic induction, which was associated with the activation of caspase-dependent and -independent mitochondrial pathways. These findings will be useful for evaluating the potency of ursolic acid as an anti-tumor agent.

Acknowledgment

The authors wish to thank Dr. Rhim Johng S of Center for Prostate Disease Research (CPDR) for providing the RC-58T/h/SA#4 primary human prostate cancer cells.

References and notes

1. Forti, G.; Selli, C. *Int. J. Androl.* **1996**, *19*, 1.
2. Quinn, M.; Babb, P. *BJU Int.* **2002**, *90*, 162.
3. Kim, S. Y.; Park, K. W.; Kim, J. W.; Shon, M. Y.; Lee, S. T.; Kim, K. H.; Rhim, J. S.; Yamada, K.; Seo, K. I. *Int. J. Oncol.* **2008**, *32*, 869.
4. Bonaccorsi, I.; Altieri, F.; Sciamanna, I.; Oricchio, E.; Grillo, C.; Contartese, G.; Galati, E. M. *Cancer Lett.* **2008**, *263*, 130.
5. Choi, W. Y.; Chun, H. J.; Lee, J. H.; Beak, S. H. *Korean J. Pharmacogn.* **2003**, *34*, 70.
6. Du, W.; Cai, H.; Wang, M.; Ding, X.; Yang, H.; Cai, B. *J. Pharm. Biomed. Anal.* **2008**, *48*, 194.
7. Kroes, B. H.; van den Berg, A. J.; Quarles van Ufford, H. C.; van Dijk, H.; Labadie, R. P. *Planta Med.* **1992**, *58*, 499.
8. Xu, H.; Shen, J.; Liu, L.; Shi, Y.; Li, L.; Wei, M.; Can, J. *Physiol. Pharmacol.* **2006**, *4*, 1267.
9. Jang, S. M.; Yee, S. T.; Choi, J.; Choi, M. S.; Do, G. M.; Jeson, S. M.; Yeo, J.; Kim, M. J.; Seo, K. I.; Lee, M. K. *Int. Immunopharmacol.* **2009**, *9*, 113.
10. Wu, V. C. H.; Qiu, X.; Hsieh, Y. H. P. *Food Microbiol.* **2008**, *25*, 190.
11. Lee, K. H.; Lin, Y. M.; Wu, T. S.; Zhang, D. C.; Yamagishi, T.; Hayashi, T.; Hall, I. H.; Chang, J. J.; Wu, R. Y.; Yang, T. H. *Planta Med.* **1988**, *54*, 308.
12. Kassi, E.; Papoutsi, Z.; Aligiannis, M.; Moutsatsou, P. *J. Cancer Res. Clin. Oncol.* **2007**, *133*, 493.
13. Yasunaga, Y.; Nakamura, K.; Ko, D.; Srivastava, S.; Moul, J. W.; Sesterhenn, I. A.; McLeod, D. G.; Rhim, J. S. *Oncogene* **2001**, *20*, 8036.
14. Gu, Y.; Li, H.; Miki, J.; Kim, K. H.; Furusato, B.; Sesterhen, I. A.; Chu, W. S.; McLeod, D. G.; Srivastava, S.; Ewing, C. M.; Isaacs, W. B.; Rhim, J. S. *Exp. Cell Res.* **2006**, *312*, 831.
15. Kim, B. H.; Park, K. W.; Kim, J. Y.; Jeong, I. Y.; Yang, G. H.; Cho, Y. S.; Yee, S. T.; Seo, K. I. *Korean J. Food Sci. Technol.* **2004**, *36*, 1001.
16. Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. *Cell* **1998**, *94*, 491.
17. Kim, S. Y.; Park, K. W.; Kim, J. Y.; Shon, M. Y.; Yee, S. T.; Kim, K. H.; Rhim, J. S.; Yamada, K.; Seo, K. I. *Int. J. Oncol.* **2008**, *32*, 869.
18. Wu, S. J.; Ng, L. T.; Lin, D. L.; Huang, S. N.; Wang, S. S.; Lin, C. C. *Cancer Lett.* **2004**, *215*, 199.
19. Huh, J. E.; Kang, K. S.; Ahn, K. S.; Kim, D. H.; Saiki, I.; Kim, S. H. *Life Sci.* **2003**, *73*, 2249.
20. Yun, J. M.; Kweon, M. H.; Kwon, H.; Mukhtar, H. *Carcinogensis* **2006**, *27*, 145.
21. Kruidering, M.; Evan, G. I. *IUBMB Life* **2000**, *50*, 85.
22. Verma, M.; Singh, S. K.; Bhushan, S.; Sharma, V. K.; Datt, P.; Kapahi, B. K.; Saxena, A. K. *Chem. Biol. Interact.* **2008**, *171*, 45.
23. Kim, J. B.; Koo, H. N.; Jeong, H. J.; Lyu, Y. S.; Park, S. G.; Won, J. H.; Kim, Y. K.; Hong, S. H.; Kim, H. M. *J. Pharmacol. Sci.* **2005**, *97*, 138.