SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 27: 567-572, 2012

Genipin, a constituent of *Gardenia jasminoides* Ellis, induces apoptosis and inhibits invasion in MDA-MB-231 breast cancer cells

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Received August 18, 2011; Accepted October 4, 2011

DOI: 10.3892/or.2011.1508

Abstract. Genipin, a constituent of *Gardenia jasminoides* Ellis, is used in the treatment of hepatic disorders and inflammatory diseases in traditional medicine. Although mounting evidence suggests an anti-tumor activity of genipin in several cancer cell systems, the inhibitory effect of genipin on the growth of breast cancer cells has not been reported yet. The present study aimed to investigate the anti-proliferative activity of genipin in MDA-MB-231 human breast cancer cells. Herein, we showed that genipin efficiently induced apoptosis in MDA-MB-231 cells by the down-regulation of Bcl-2, up-regulation of Bax and proteolytic activation of caspase-3. Activation of JNK and p38 MAPK also increased by genipin. Importantly, genipin significantly inhibited invasive and migratory phenotypes of MDA-MB-231 cells. Taken together, this study demonstrates that genipin induces apoptosis and inhibits invasive/migratory abilities of highly invasive MDA-MB-231 human breast cancer cells, suggesting a potential application of genipin as a chemo-preventive agent that may prevent or alleviate metastatic breast cancer.

Introduction

Genipin, an aglycone derived from an iridoid glycoside called geniposide present in fruit of *Gardenia jasminoides* Ellis, has long been used as a traditional oriental medicine for the treatment of hepatic disorders and inflammatory diseases (1). Genipin exerts anti-inflammatory, anti-angiogenic, anti-proliferative and hepatoprotective activities in several cell lines including murine macrophage cells, human umbilical vein endothelial cells, human prostate cancer cells, and human hepatocarcinoma cells (2-5). Genipin has been shown to inhibit growth of human leukemia and prostate cancer cells (5,6). However, anti-proliferative activity of genipin in breast cancer cells is poorly understood. The present study aimed to investigate the inhibitory effect of genipin on the growth of MDA-MB-231 human breast cancer cells.

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Key words: genipin, cell invasion

Differentiated cells of multicellular organisms can induce death through the activation of a programmed cell suicide process known as apoptosis (7). Failure of cells to undergo apoptotic cell death may be involved in the pathogenesis of diseases including cancer (8). Induction of apoptosis is partly mediated by the balance of apoptosis-related gene products, such as Bcl-2, Bax, and caspases (9). The death-promoting protein Bax counteracts the anti-apoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2 (10). Caspase-3 is initially produced as a pro-caspase-3 (32 kDa) and then activated by cleavage events to generate a large and small subunit of 17 and 12 kDa, respectively (11).

In order to examine the chemopreventive potential of genipin on breast cancer *in vitro*, we investigated the effects of genipin on proliferation, apoptosis, invasion and migration of MDA-MB-231 human breast cancer cells. In the present study, we have demonstrated that genipin induces apoptosis in MDA-MB-231 human breast cancer cells, which is mediated by enhancing JNK and p38 kinase activity and decreasing the protein expression of Bcl-2 anti-apoptotic molecules.

Materials and methods

Reagents. Genipin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Sigma-Aldrich Inc., MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA).

Cell lines. MDA-MB-231 cells were cultured, as previously described (12). Cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin.

MTT assay. MDA-MB-231 cells ($3x10^4$ cells/well) cultured in a 96 well-plate were treated with various concentrations of genipin (0, 50, 100, 500, 1000 μM) for 24 h. Control cells were treated with dimethyl sulphoxide (DMSO) equal to the highest percentage of solvent used under the experimental conditions. Briefly, 25 mg/ml of 0.5% MTT (3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to the media and the cells were further incubated for 4 h. After the supernatant (100 μl) was replaced with the same volume of DMSO, absorbance was measured at 540 nm with an ELISA reader (EL 800, Bio-Tek Instruments Inc., Winooski, VT). The percentage

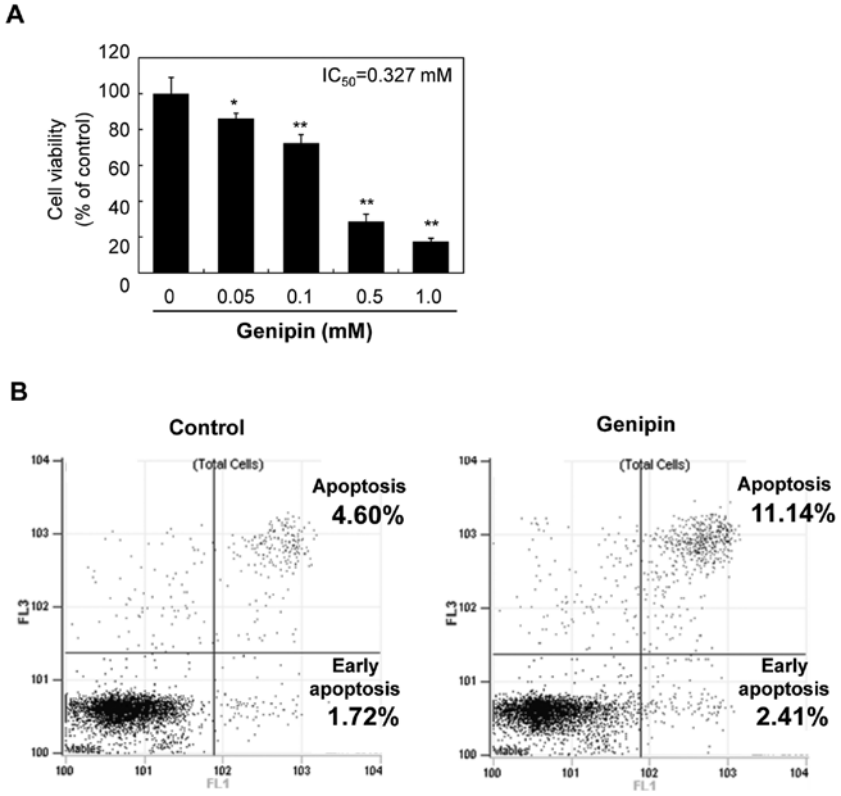

Figure 1. Genipin inhibits cell growth and induces apoptosis on MDA-MB-231 cells. (A) MDA-MB-231 cells ($3x10^4$) in a 24-well plate were treated with various concentrations of genipin for 24 h and counted. The results presented are ± standard error of the mean (SEM) of triplicates. (B) Cells were treated with 200 μM genipin for 24 h and incubated with annexin V-FITC and PI. The FITC and PI fluorescence was measured using a flow cytometer with FI-1 and FI-2 filters, respectively.

of surviving cells was defined as the relative absorbance of treated versus untreated cells.

Immunoblot analysis. Immunoblot analysis was performed as described previously (13). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-ERK1/2, anti-ERK1/2, anti-JNK, and phospho-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl2, anti-Caspase 3, anti-Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-actin antibody was purchased from Cell Signaling Technology.

In vitro invasion assays. *In vitro* invasion assay was performed using 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Cells were placed in the upper part of the Transwell plate, incubated for 17 h, fixed with methanol, and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting cells that migrated to the lower side of the filter under microscopy at x400. Thirteen fields were counted for each filter, and each sample was assayed in triplicate.

In vitro migration assay using Transwell. An *in vitro* migration assay was performed using a 24-well Transwell unit with polycarbonate filters, as previously described (13).

Flow cytometric analysis. For flow cytometry, MDA-MB-231 cells were grown in six-well plates and incubated for 24 h, and then treated with 200 μM genipin. After 24 h, cells were harvested and washed twice with PBS. Cells ($1x10^5$) were double stained with FITC-conjugated annexin V and propidium iodide for 15 min at room temperature in 1X binding, and then analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA).

Results

Genipin inhibits cell growth and induces apoptosis in MDA-MB-231 cells. In order to investigate the effects of genipin on the growth of MDA-MB-231 breast cancer cells were treated with various concentrations of genipin. As shown in Fig. 1A, treatment of cells with genipin for 24 h inhibited growth of MDA-MB-231 cells in a dose-dependent manner, with an IC_{50} value of 327 μM. To investigate whether the genipin-induced growth inhibition involves apoptosis, FACS analysis was conducted. Treatment with 200 μM genipin for 24 h resulted in a marked increase in annexin-positive apoptotic cells (11.14%) compared with the control cells (4.6%), demonstrating that genipin induced apoptosis of MDA-MB-231 cells (Fig. 1B). These results imply that the observed growth inhibitory effect of genipin may be due to the induction of apoptotic cell death.

Genipin regulates apoptosis-related proteins. To evaluate the molecular mechanisms underlying the genipin-induced

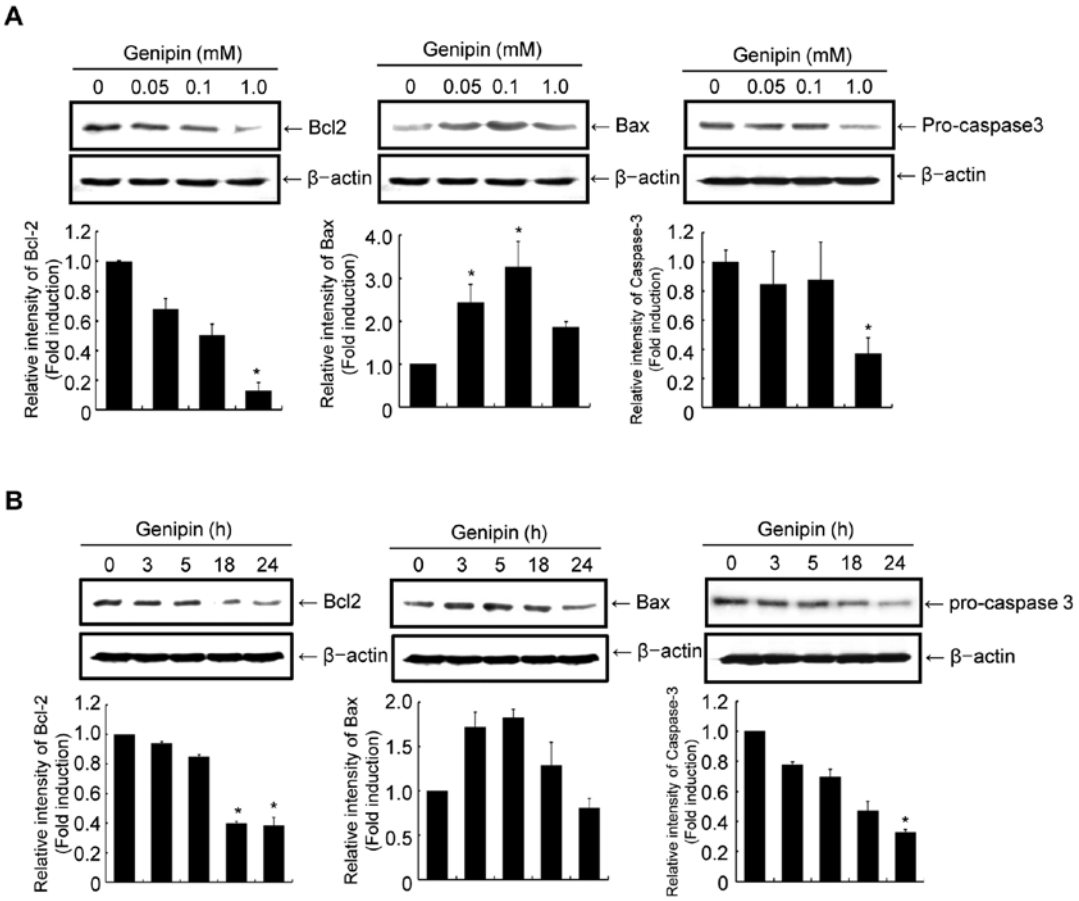

Figure 2. Genipin regulates expression of apoptosis-related protein in MDA-MB-231 cells. (A) Cells were treated with genipin at the indicated concentrations. The levels of Bcl-2, bax, and pro-caspase 3 were determined by immunoblot analyses using specific antibodies. (B) Cells were treated with 200 μM genipin at the indicated times.

apoptosis of MDA-MB-231 cells, we determined the protein levels of two key apoptosis-linked gene products, Bcl-2 and Bax. Cells were treated with various concentrations of genipin for 24 h. Expression of the anti-apoptotic oncoprotein Bcl-2 was decreased, whereas the death-promoting Bax expression was increased in MDA-MB-231 cells treated with genipin in a dose-dependent manner (Fig. 2A, left and center). These data demonstrate that genipin induced apoptosis by down-regulation of an anti-apoptotic protein Bcl-2 and up-regulation of a pro-apoptotic protein Bax in MDA-MB-231 cells.

Caspases are crucial mediators of apoptosis that depend on proteolytic activation of their pro-caspase forms to enzymatically active forms (14). The level of pro-caspase-3 was dose-dependently decreased by genipin treatment, suggesting that genipin induced the activation of caspase-3 in MDA-MB-231 cells (Fig. 2A, right).

We then performed a kinetic study to examine the effect of genipin on apoptosis-related proteins. As shown in Fig. 2B (left), treatment with 200 μM genipin caused a time-dependent decrease of Bcl-2 expression. In contrast, the expression level of Bax was increased 3 h after genipin treatment and decreased back to the basal level after 24 h. Pro-caspase-3 levels were decreased in a time-dependent manner, suggesting that genipin may enhance the proteolytic activation of caspase-3 (Fig. 2B, right). These results imply that genipin-induced apoptosis may be mediated by Bcl-2, Bax, and caspase-3 in MDA-MB-231 cells.

Genipin activates p38 MAPK and JNK in MDA-MB-231 cells. Activation of mitogen-activated protein kinases (MAPKs) are important intermediates in the signal-transduction pathway regulating cell proliferation and apoptosis (15-18). Two members of the MAPK superfamily, JNK and p38 MAPK, were shown to be activated in the apoptotic responses of cells (6,19,20). To examine the effect of genipin on the activation of these signaling molecules, MDA-MB-231 cells were treated with various concentrations of genipin. As shown in Fig. 3A, activation of p38 MAPK was increased by genipin treatment in a dose-dependent manner. Phosphorylated JNK was markedly increased by 50 μM genipin and decreased back to the basal level at 200 μM genipin. In contrast, activation of ERKs was not increased by the same treatment. A kinetic study showed that p38 MAPK and JNK were activated by genipin in a time-dependent manner, while phosphorylated ERKs were not affected (Fig. 3B).

Genipin inhibits invasion and migration of MDA-MB-231 cells. To investigate the effects of genipin on invasion and migration of MDA-MB-231 cells, we performed *in vitro* invasion and migration assays. As shown in Fig. 4, invasive and migratory abilities of MDA-MB-231 cells were significantly inhibited by genipin treatment in a dose-dependent manner. The results clearly demonstrate that genipin effectively inhibited the invasive and migratory phenotype of breast cancer cells.

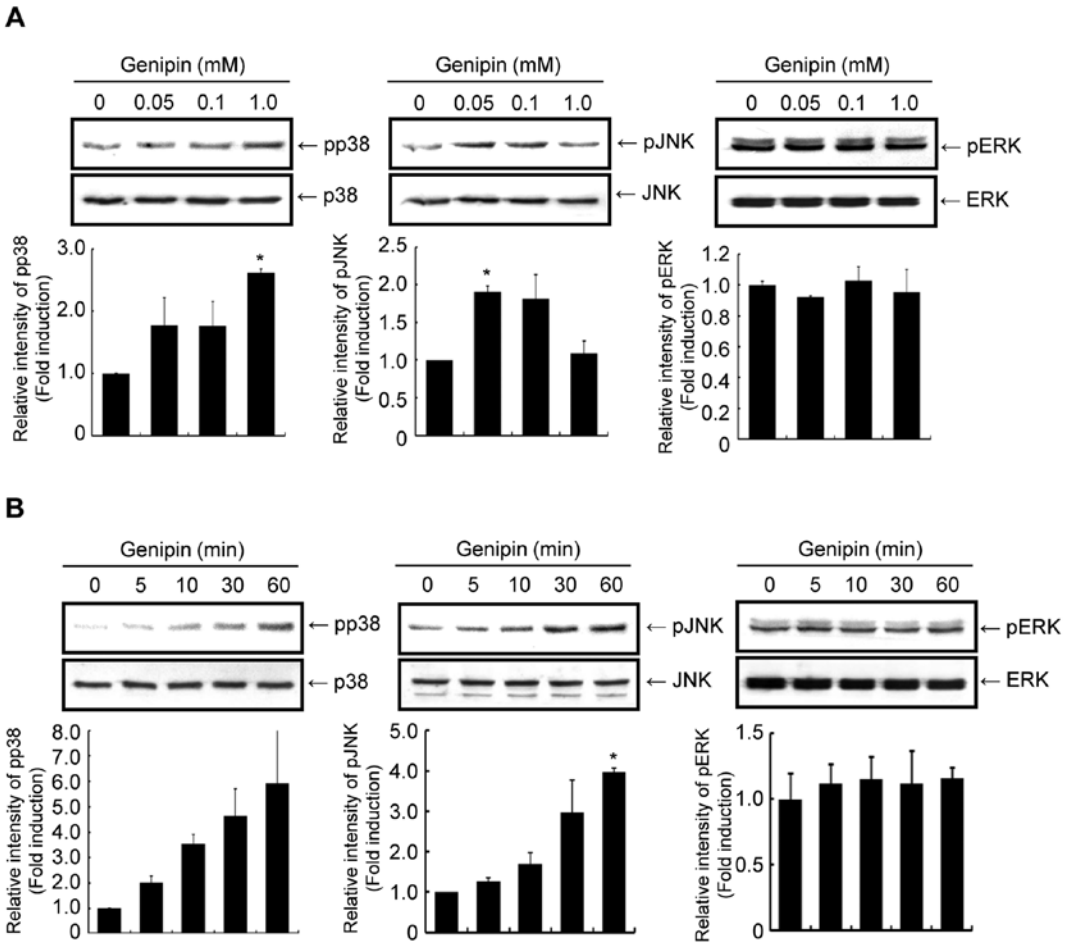

Figure 3. Genipin activates p38 and JNK in MDA-MB-231 cells. (A) Cells were treated with genipin at the indicated concentration. The levels of activated ERK1/2, p38 MAPK, and JNK were determined by immunoblot analyses using phospho-specific antibodies pERK1/2, pp38, and pJNK, respectively. (B) Cells were treated with 200 μM genipin at the indicated times.

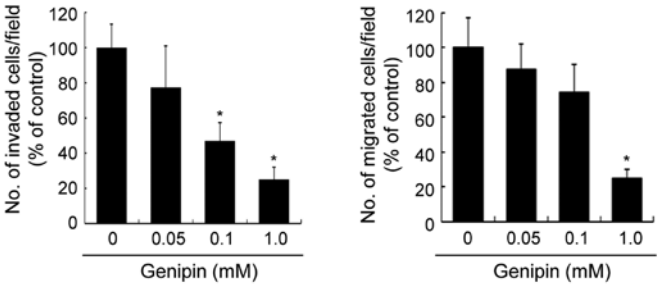

Figure 4. Genipin inhibits invasion and migration of MDA-MB-231 cells. *In vitro* invasion and Transwell migration assays were conducted on MDA-MB-231 cells treated with the indicated concentrations of genipin for 17 h. The results represent the means + SE of triplicates. *Statistically different from control at p<0.01.

Discussion

Cancer chemoprevention using natural products that inhibit the development of invasive cancer has emerged as a powerful strategy against cancer (21,22). Bioactive compounds and phytochemicals from plants have been shown to suppress early and late stages of carcinogenesis and can consequently inhibit proliferation of malignant cancer cells (23-25). In the present study, we showed that genipin efficiently inhibited the growth of MDA-MB-231 human breast cancer cells. Given that *G. jasminoides* has been widely used as an oriental medicine for many years (26), any potential adverse effect for anti-cancer agents would not be significant. Our findings suggest a potential role of genipin in regulation of proliferation, invasion and migration of highly invasive MDA-MB-231 human breast cancer cells.

Since apoptosis is arguably the most potent natural defense against cancer (7), efforts have been made to develop strategies that trigger apoptosis in malignant cancer cells. Apoptotic cell death plays an important role in breast cancer. The level of Bcl-2 expression correlates with breast cancer both *in vitro* and *in vivo* (27,28). Our study demonstrates that genipin induces apoptosis in human breast cancer cells with a prominent decrease of Bcl-2, preventing apoptosis by blocking the release of cytochrome C from mitochondria (29). Bax, which directly induces cytochrome C release from mitochondria and consequently triggers caspase activation (30), was increased by genipin treatment. Consistent with these reports, our results suggest that genipin may proteolytically activate pro-caspase-3. These results suggest that genipin-induced apoptosis of breast cancer cells is mediated by Bcl-2 and Bax.

Signaling mediated by MAPKs is involved in the regulation of cell apoptosis (15). Among the MAPK pathways, JNK and

p38 MAPK pathways are generally activated by stress agents, and implicated as key regulators of stress-induced apoptosis (6,31). Apoptosis induced by chemotherapeutic drugs was mediated by the p38 MAPK-caspase signaling pathway in human pancreatic cancer cells (32). Consistent with these reports, our results demonstrated that genipin activated p38 MAPK and JNK, but not ERKs, in MDA-MB-231 cells, suggesting the involvement of p38 MAPK and JNK in genipin-induced apoptosis in human breast cancer cells. The effect of genipin on the activation of MAPK family members varies in different cell systems. Genipin was shown to induce apoptosis through the activation of JNK, but not p38 MAPK, in hepatocarcinoma and prostate cancer cells (3,5). Genipin also induced the activation of JNK in rat hepatoma and HeLa cells (3,33). Further investigation on the association of signaling pathways with apoptotic cell death induced by genipin needs to be performed.

Breast cancer is considered the most commonly diagnosed type of cancer and the second most common cause of cancer-related death among women (34). Metastasis, a characteristic of highly malignant cancers with poor clinical outcome, is one of the major causes of mortality in breast cancer patients. Therefore, anti-tumor agents that may inhibit invasion and migration of breast cancer cells have been extensively pursued in many laboratories, including ours (35,36). Although many studies have reported the anti-tumor activity of genipin in various cancer cells, there is a limited amount of information on the anti-invasive activity of genipin. The present study clearly demonstrated that genipin inhibits invasion and migration of the highly invasive MDA-MB-231 human breast cancer cell line in a dose-dependent manner.

Taken together, the present study showed that genipin inhibits cell growth and the invasive/migratory phenotypes of human breast cancer cells. Given that metastatic breast cancer is one of the most lethal malignancies in women, our novel findings suggest a potential application of genipin as a chemopreventive agent for breast cancer patients.

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) grants funded by the Ministry of Education, Science and Technology (MEST) (No. ROA-2008-000-20070-0, and No. 20110001205).

References

1. Peng CH, Tseng TH, Liu JY, Hsieh YH, Huang CN, Hsu SP and Wang CJ: Penta-acetyl geniposide-induced C6 glioma cell apoptosis was associated with the activation of protein kinase C-delta. Chem Biol Interact 147: 287-296, 2004.
2. Koo HJ, Song YS, Kim HJ, Lee YH, Hong SM, Kim SJ, Kim BC, Jin C, Lim CJ and Park EH: Antiinflammatory effects of genipin, an active principle of gardenia. Eur J Pharmacol 495: 201-208, 2004.
3. Kim BC, Kim HG, Lee SA, Lim S, Park EH, Kim SJ and Lim CJ: Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway. Biochem Pharmacol 70: 1398-1407, 2005.
4. Wang GF, Wu SY, Rao JJ, Lü L, Xu W, Pang JX, Liu ZQ, Wu SG and Zhang JJ: Genipin inhibits endothelial exocytosis via nitric oxide in cultured human umbilical vein endothelial cells. Acta Pharmacol Sin 30: 589-596, 2009.
5. Hong HY and Kim BC: Mixed lineage kinase 3 connects reactive oxygen species to c-Jun NH2-terminal kinase-induced mitochondrial apoptosis in genipin-treated PC3 human prostate cancer cells. Biochem Biophys Res Commun 362: 307-312, 2007.
6. Feng Q, Cao HL, Xu W, Li XR, Ren YQ and Du LF: Apoptosis induced by genipin in human leukemia K562 cells: involvement of c-Jun N-terminal kinase in G_2/M arrest. Acta Pharmacol Sin 32: 519-527, 2011.
7. Raff MC: Social controls on cell survival and cell death. Nature 356: 397-400, 1992.
8. Bursch W, Oberhammer F and Schulte-Hermann R: Cell death by apoptosis and its protective role against disease. Trends Pharmacol Sci 13: 245-251, 1992.
9. Chiarugi V, Magnelli L, Cinelli M and Basi G: Apoptosis and the cell cycle. Cell Mol Biol Res 40: 603-612, 1994.
10. Kobayashi T, Ruan S, Clodi K, Kliche KO, Shiku H, Andreeff M and Zhang W: Overexpression of Bax gene sensitizes K562 erythroleukemia cells to apoptosis induced by selective chemotherapeutic agents. Oncogene 16: 1587-1591, 1998.
11. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM: Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801-809, 1995.
12. Koh MS and Moon A: Activation of H-Ras and Rac1 correlates with epidermal growth factor-induced invasion in Hs578T and MDA-MB-231 breast carcinoma cells. Biochem Biophys Res Commun 406: 25-29, 2011.
13. Kim MS, Lee EJ, Kim HR and Moon A: p38 kinase is a key signaling molecule for H-Ras-induced cell motility and invasive phenotype in human breast epithelial cells. Cancer Res 63: 5454-5461, 2003.
14. Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland MS, Kwon JT, Hwang SK, Jin H, Churchwell MI, Cho MH, Doerge DR, Helferich WG and Hergenrother PJ: Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nat Chem Biol 2: 543-550, 2006.
15. Schaeffer H and Weber M: Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 19: 2435-2444, 1999.
16. Lin C, Zimmer SG, Lu Z, Holland RE Jr, Dong Q and Chambers TM: The involvement of a stress-activated pathway in equine influenza virus-mediated apoptosis. Virology 287: 202-213, 2001.
17. Lin MT, Juan CY, Chang KJ, Chen WJ and Kuo ML: IL-6 inhibits apoptosis and retains oxidative DNA lesions in human gastric cancer AGS cells through up-regulation of anti-apoptotic gene mcl-1. Carcinogenesis 22: 1947-1953, 2001.
18. Tominaga K, Higuchi K, Sasaki E, Suto R, Watanabe T, Fujiwara Y, Oshitani N, Matsumoto T, Kim S, Iwao H and Arakawa T: Correlation of MAP kinases with COX-2 induction differs between MKN45 and HT29 cells. Aliment Pharmacol Ther 1: 143-150, 2004.
19. Chuang SM, Wang IC and Yang JL: Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. Carcinogenesis 21: 1423-1432, 2000.
20. Kutuk O, Pedrech A, Harrison P and Basaga H: Pramanicin induces apoptosis in Jurkat leukemia cells: a role for JNK, p38 and caspase activation. Apoptosis 10: 597-609, 2005.
21. Ferguson LR: Antimutagens as cancer chemopreventive agents in the diet. Mutat Res 307: 395-410, 1994.
22. Stavric B: Antimutagens and anticarcinogens in foods. Food Chem Toxicol 32: 79-90, 1994.
23. Karikas GA: Anticancer and chemopreventing natural products: some biochemical and therapeutic aspects. J BUON 15: 627-638, 2010.
24. Wu J, Omene C, Karkoszka J, Bosland M, Eckard J, Klein CB and Frenkel K: Caffeic acid phenethyl ester (CAPE), derived from a honeybee product propolis, exhibits a diversity of anti-tumor effects in pre-clinical models of human breast cancer. Cancer Lett 308: 43-53, 2011.
25. Chien SY, Wu YC, Chung JG, Yang JS, Lu HF, Tsou MF, Wood WG, Kuo SJ and Chen DR: Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. Hum Exp Toxicol 28: 493-503, 2009.
26. Tseng TH, Chu CY, Huang JM, Shiow SJ and Wang CJ: Crocetin protects against damage in rat primary hepatocytes. Cancer Lett 97: 61-67, 1995.

27. Formby B and Wiley TS: Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. Ann Clin Lab Sci 28: 360-369, 1998.
28. Buchholz TA, Davis DW, McConkey DJ, Symmans WF, Valero V, Jhingran A, Tucker SL, Pusztai L, Cristofanilli M, Esteva FJ, Hortobagyi GN and Sahin AA: Chemotherapy-induced apoptosis and Bcl-2 levels correlate with breast cancer response to chemotherapy. Cancer J 9: 33-41, 2003.
29. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129-1132, 1997.
30. Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D and Reed JC: Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 95: 4997-5002, 1998.
31. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ and Davis RJ: Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270: 7420-7426, 1995.
32. Habiro A, Tanno S, Koizumi K, Izawa T, Nakano Y, Osanai M, Mizukami Y, Okumura T and Kohgo Y: Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells. Biochem Biophys Res Commun 316: 71-77, 2004.
33. Cao H, Feng Q, Xu W, Li X, Kang Z, Ren Y and Du L: Genipin induced apoptosis associated with activation of the c-Jun NH2-terminal kinase and p53 protein in HeLa cells. Biol Pharm Bull 33: 1343-1348, 2010.
34. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer Statistics. CA Cancer J Clin 57: 43-66, 2007.
35. Kang HJ, Soh Y, Kim MS, Lee EJ, Surh YJ, Kim HR, Kim SH and Moon A: Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells. Int J Cancer 103: 475-482, 2003.
36. Koh MS, Hwang JS and Moon A: Lycopene inhibits proliferation, invasion and migration of human breast cancer cells. Biomol Ther 18: 92-98, 2010.