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Crude Extract of Garlic Induced Caspase-3 Gene Expression Leading to Apoptosis in Human Colon Cancer Cells

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Abstract. *Garlic (Allium sativum) is a popular spice, a remedy for a variety of ailments and is also known for its medicinal uses as an antibiotic, antithrombotic and antineoplastic agent. Epidemiological and animal studies have shown that garlic consumption reduces the incidence of cancer e.g. in the stomach, colon, breast and cervix. The aim of this study was to investigate whether garlic extract has any influence on caspase-3 activity and gene expression and on the signal induction of apoptosis in vitro. As an assay system, the flow cytometry assay, Western blotting and cDNA microarray were applied in human colon cancer colo 205 cells. Our results indicated that garlic extract, when administered to the colo 205 cell cultures, reduced the percetange of viable cells, induced apoptosis, increased the levels of Bax, cytochrome c and caspase-3, but decreased the level of Bcl-2. The results also showed that raw extract of garlic decreased the mitochondrial membrane potential and increased the caspase-3 activity and gene expression. We conclude that crude extract of garlic can induce apoptosis in colo 205 cells through caspase -3 activity, by means of a mitochondrial-dependent mechanism.*

Garlic is identified as a class of natural *Allium* vegetables, and is also used as a food additive throughout the world. *Allium* vegetables, and/or their constituents, have been demonstrated to reduce the risk of cardiovascular diseases by lowering the serum cholesterol level (1), inhibiting platelet aggregation (2) and increasing fibrinolysis (3); decreasing cancer risk through inhibiting carcinogen activation and/or increasing detoxification of activated carcinogens (4, 5), and they also exhibit antitumour activities (6). It has also been reported that these vegetables have antibacterial (7), antifungal (8) and antiviral (9) effects, promote the immune system (10) and have anti-aging functions (11, 12).

Cell death can be divided into necrosis and apoptosis. Apoptosis is a tightly regulated process of programmed cell death, which is implicated in development and tissue homeostasis of multicellular organisms, to eliminate abundant and unwanted cells that occur in various physiological and pathological conditions (13-15). It is also well documented that abnormalities in cell death control can lead to a variety of diseases, including cancer. The pathways of apoptosis can be divided into mitochondrial or intrinsic pathways, that are initiated from internal events within the cell and death receptor, or extrinsic pathways, that occur at the cell surface, which result in the proteolytic activation of cysteine proteases (caspases) (16). Although garlic components had been demonstrated to induce apoptosis in human colon cancer cells (17, 18) and in leukemia HL-60 cells by caspase-3 activity (19), there have been no reports of crude garlic extract (CGE)-induced caspase-3 gene expression and apoptosis in human colon colo 205 cells. Therefore, the aim of this study was to examine whether or not CGE induces capspase-3 activity before leading to apoptosis in human colon colo 205 cells.

Materials and Methods

Chemicals and reagents. Trypan blue, ribonuclease-A, Tris-HCl, triton X-100 and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO) and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was bought from Boehringer Mannheim (Mannhein, Germany). CGE was prepared in our laboratory.

Human colon cancer cell line. The human colon adenocarcinoma cell line (colo 205) was obtained from the Food Industry

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Research and Development Institute (Hsinchu, Taiwan). The colon cancer cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBO and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin).

Effects of CGE on the viability of colo 205 cells. The colo 205 cells were plated in 12-well plates at a density of $2.5x10^5$ cells/well and grown for 24 h. CGE, at final concentrations 0, 0.5, 1 and 2 μg/mL, was then added. DMSO (solvent) was used as a control. For determining cell viability, the trypan blue exclusion test and flow cytometry were used, as described previously (20).

Flow cytometry analysis of DNA content for assaying apoptosis in colo 205 cells treated with various concentrations of CGE. Approximately $2.5x10^5$ cells/well of colo 205 in a 12-well plate with final concentrations (0, 0.5, 1 and 2 μg/ml) of CGE were incubated in an incubator for 48 h before harvesting the cells by centrifugation. The cells were fixed and assayed by flow cytometry, as described previously (20).

DNA fragmentation electrophoresis analysis. Colo 205 cells were plated in 10-cm dishes at a density of $5x10^6$ cells/well and grown for 24 h. They were then treated with 1 μg/ml CGE while only adding DMSO (solvent) for the control, and grown at 37˚C in humidified 5% CO_2 for 72 h for the DNA fragmentation electrophoresis assayed, as described previously (20).

Effects of CGE on the mitochondrial membrane potential of colo 205 cells. Cells were treated with or without CGE (final concentrations 0, 0.5, 1 and 2 μg/ml) for 24 h to detect the changes of mitochondrial membrane potential. The level of mitochondrial membrane potential of the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using $DiOC_6$ (4 mol/L). The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L) and incubated at 37˚C for 30 min, before analysis by flow cytometry (21).

Caspase-3 activity determination in colo 205 cells treated with or without CGE. Colo 205 cells were plated in 12-well plates at a density of $5x10^5$ cells/well and grown for 24 h. The various concentrations of CGE with the added DMSO (solvent) for the control were grown at 37˚C in humidified 5% CO_2 for 24 h. The cells were then harvested and lysed in lysis buffer to determine caspase-3 activity, as described previously (21).

Inhibition of CGE-induced apoptosis by the caspase inhibitor (z-VAD-fmk) in colo 205 cells. In order to examine whether or not caspase-3 activation was involved in the apoptosis triggered by CGE, the cells were pretreated with the cell-permeable broad-spectrum caspase inhibitor, z-VAD-fmk, 3 h prior to treatment with CGE. Apoptosis and caspase-3 activity were determined, as described above (21)

Western blotting to examine the effect of CGE on Bax, Bcl-2, cytochrome c and caspase-3 in colo 205 cells. The total protein was collected from colo 205 cells treated with or without various concentrations of CGE 24 h before the Bax, Bcl-2, cytochrome c and caspase-3 levels were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (20, 21)

Microarray hybridization to examine caspase-3 gene expression in colo 205 cells after treatment with CGE. The total RNA was extracted from colo 205 cells treated with or without 2 μg/mL CGE by using the Qiagen RNeasy Mini Kit at 24 h. cDNA microarrays were applied, as described previously (22).

Statistical analysis. The Student's *t*-test was used to analyze the statistical significance between the CGE-treated and control groups.

Results

Effects of CGE on the viability of colo 205 cells. The results demonstrated that the crude extract of garlic induced cytotoxicity on colo 205 cells, and that these effects were dose-dependent (Figure 1).

CGE induced apoptosis in colo 205 cells. The data from flow cytometry demonstrated that CGE induced apoptosis (sub-G1 occurrence), and that this effect was also dose-dependent (Figure 2). The DNA gel picture indicated that, in colo 205 cells treated with 2 μg/ml of CGE, DNA fragmentation was induced (Figure 3).

Effects of CGE on the mitochondrial membrane potential in colo 205 cells. The data from flow cytometry assays provided in Table I indicate that CGE decreased the mitochondrial membrane potential, and that this effect was also dose-dependent (Table I).

Caspase-3 activity determination in colo 205 cells treated with or without CGE. The results indicated that CGE increased the caspase-3 activity in colo 205 cells and that this effect was also dose-dependent (Figure 4A).

Inhibition of CGE-induced apoptosis by the caspase inhibitor (z-VAD-fmk) in colo 205 cells. The data demonstrated that CGE induced apoptosis, and that cells treated with caspase inhibitor showed a decrease in the percentage of apoptosis (Figure 4B). Apparently, caspase-3 is involved in CGE-induced apoptosis.

Western blotting to examinine the effect of CGE on Bax, Bcl-2, cytochrome c and caspase-3 expressions in colo 205 cells. The results indicate that CGE induced Bax, cytochrome c and caspase-3 expressions, but decreased Bcl-2 expression (Figure 5).

Microarray hybridization to examine the gene expression of caspase-3 in colo 205 cells after treatment with CGE. The results demonstrate that CGE induced caspase-3 gene expression (Figure 6).

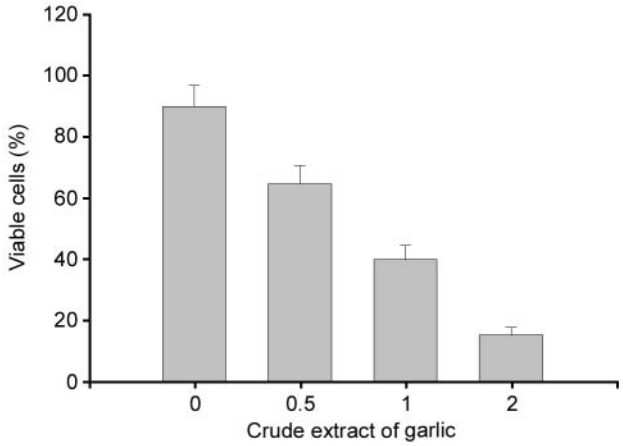

Figure 1. *Percentage of the viable colo 205 cells after CGE treatment. Colo 205 cells (1x10^{5}cells/ well; 12 well plates) were cultured in RPMI 1640 medium + 10% FBS with CGE for 24 h. Then the cells were collected by centrifugation and the viable cells were determined by flow cytometry, as described in Materials and Methods. The data represent the mean±S.D. of three experiments. *p<0.05*

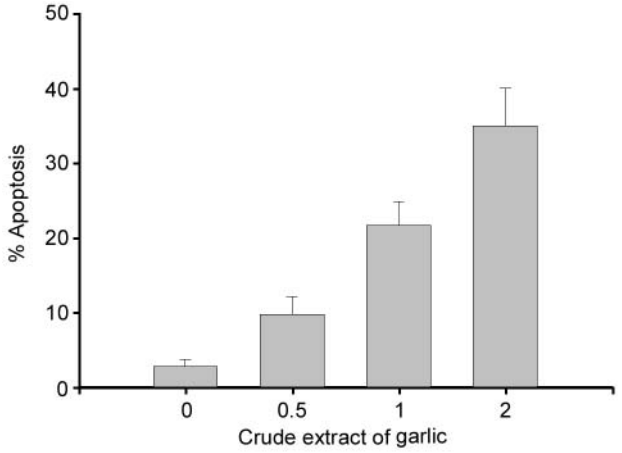

Figure 2. *Effects of CGE on colo 205 cells apoptosis (sub-G1 group). Colo 205 cells were cultured with 0, 0.5, 1 and 2 μg/mL CGE for 48 h, and the cells were harvested and analyzed for the sub-G1 group. The percentage of colo 205 cells in apoptosis was evaluated by flow cytometry as described in Materials and Methods. The data represents the mean±S.D. of three experiments. *p<0.05*

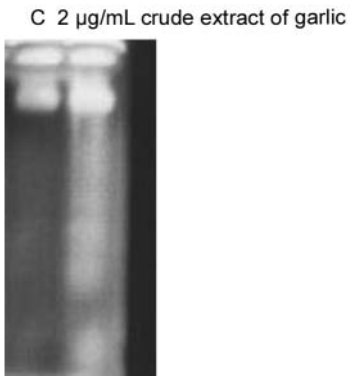

Figure 3. *CGE induced DNA laddering fragmentation in colo 205 cells. Colo 205 cells were incubated with or without CGE for 48 h, then DNA degradation was analyzed by gel electrophoresis, as described in Materials and Methods.*

Table I. *Flow cytometric analysis of mitochondrial membrane potential in human colon cancer cells (colo 205) with or without various concentrations of CGE treatment for 24 h.*

Crude extract of garlic (CGE) (μg/mL)	Percentage of cells stained by $DiOC_6$
0 (control)	96.2±13.9
0.5	78.4±10.2
1	51.6±8.2*
2	24.1±4.1*

Values are mean±S.D. n=3. The colo 205 cells (5x10^5 cells/ml) were treated with various concentrations of CGE. The zero concentration was defined as the control. The percentage of cells that were stained by the $DiOL_6$ dye, and the stained cells were determined by flow cytometry, as described in the Materials and Methods section.
*differs between CGE and control. $p<0.05$.

Discussion

Recently, there has been increased inerest in the chemoprevention of gastrointestinal cancer. So far, many minor dietary components have been found to inhibit various stages of carcinogenesis (23). The anticarcinogenic effects of *Allium* vegetables, that derive from epidemiological data as well as from laboratory animal studies, have been reported. A case-control study involving 564 patients with stomach cancer and 1131 controls documented an inverse correlation between the dietary intake of *Allium* vegetables and cancer risk (24). Consumption of garlic was inversely associated with colon cancer risk (25). The use of garlic in health products and herbal remedies in Nigeria was also reported (26).

A more recent population-based, case-control study conducted in Shanghai, China, examined the association between the risk of prostate cancer and the intake of *Allium* vegetables (garlic, onions, scallions, chives and leeks). This study, involving 238 cases with histologically-confirmed prostate cancer and 471 control subjects, concluded that men

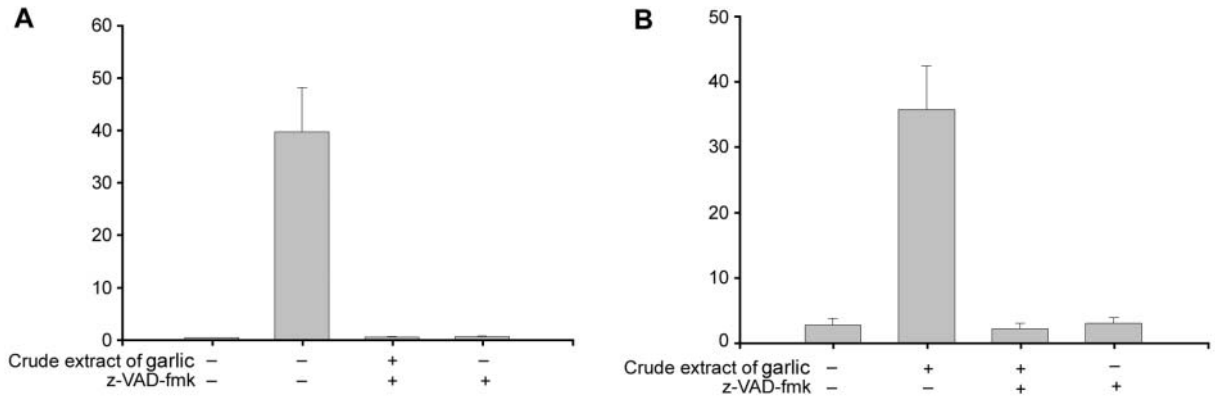

Figure 4. *Flow cytometric analysis of the effects of CGE on colo 205 caspase-3 activity and apoptosis. The colo 205 cells were incubated with 2 μg/mL CGE and/or with or without z-VAD-fmk treatment for caspase-3 activity (panel A) and apoptosis determination (panel B), as described in Materials and Methods. The data represent the mean±S.D. of three experiments. *p<0.05*

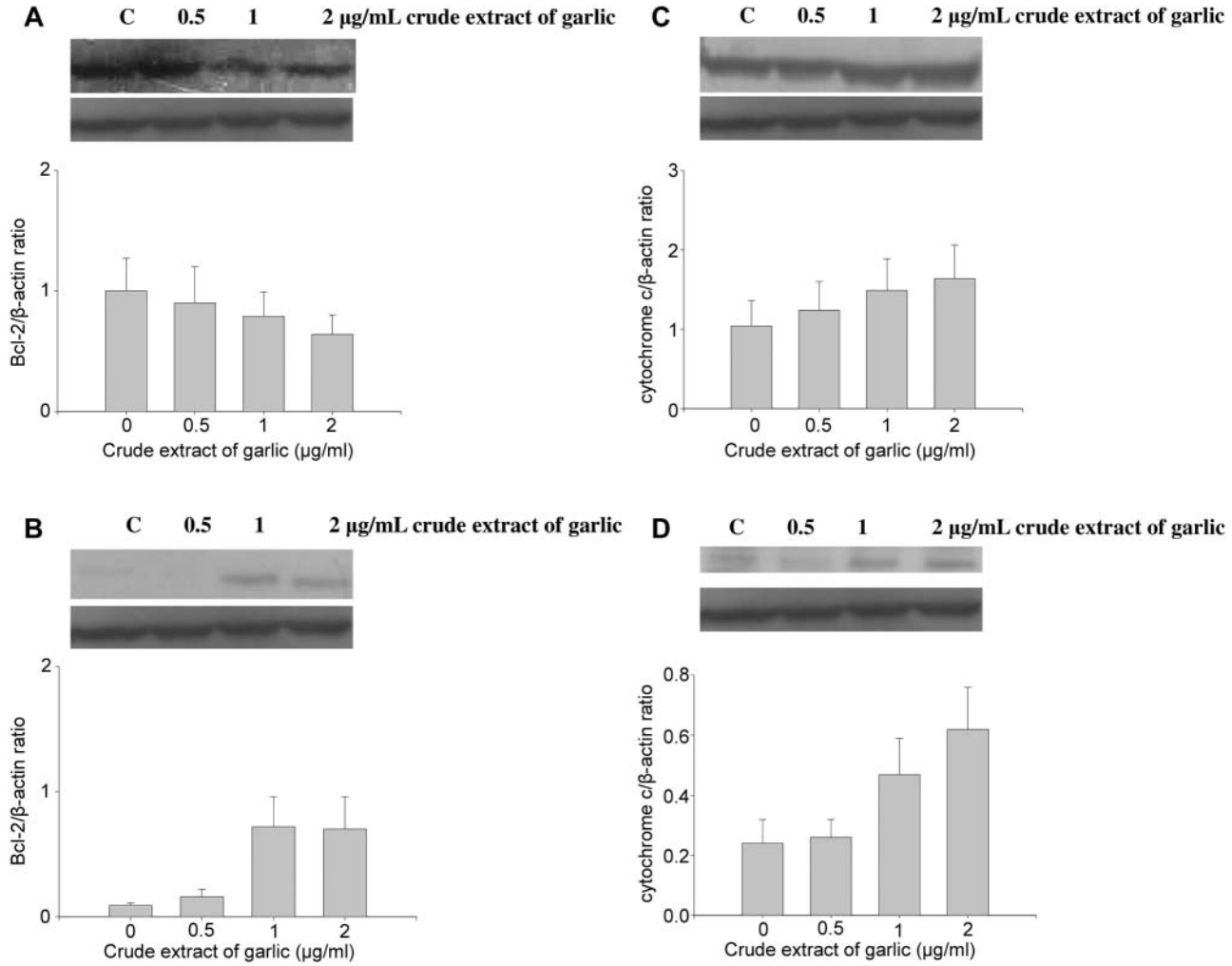

Figure 5. *Representative Western blot showing changes in the levels of Bcl-2, Bax, , cytochrome c and caspase-3 in colo 205 cells after exposure to CGE. The colo 205 cells (5x10^6/ml) were treated with 0, 0.5, 1 or 2 μg/mL CGE for 24 h then the cytosolic fraction and total protein were prepared and determined, as described in Materials and Methods. Evaluation of the levels of Bcl-2, Bax, cytochrome c and caspase-3 expressions were estimated by Western blotting as described in Materials and Methods.*

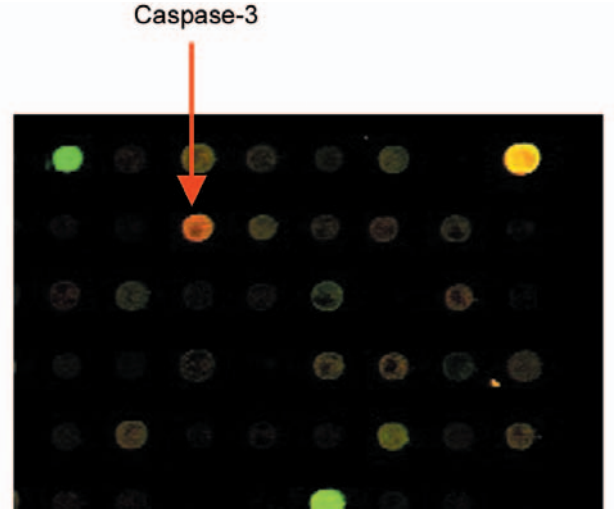

Figure 6. *Up-regulation of caspase-3 gene in colo 205 cells treated with 2 μg/mL CGE and assayed by cDNA microarray. Colo 205 cancer cells ($5x10^6$ cells/mL) in 6-well plates treated with or without 2 μg/mL CGE for 24 h. Red color spot represents up-regulation and green color spot down-regulation. Circle marks the caspase-3 gene up-regulation.*

with a high intake of total *Allium* vegetables (>10 g/day) had a statistically significantly lower risk of prostate cancer than those with low intake (27). Results from investigators based on antiproliferation studies on cancerous cell-based models suggest that the tumor-suppressive effect of garlic corresponds with cell cycle inhibition and apoptosis induction (19, 28, 29). However, the exact mechanism of garlic-induced apoptosis in human colon cancers still remains unclear. Therefore, it was worth investigating the effects of CGE on human colon cancer cells to find the mechanism and signal pathway, and how they lead to apoptosis in colo 205 cells.

Our results demonstrated that CGE was cytotoxic against colon cancer cells. The data also showed that cell death after treatment with CGE was the result of apoptosis, and that increased Bax levels and decreased Bcl-2 levels led to a decrease in the mitochondrial membrane potential, which caused caspase-3 levels to increase, finally leading to DNA fragmentation and apoptosis. The homeostasis of the mitochondrial membrane potential is supervised mainly by Bcl-2 family membranes (30). The ratio of Bax/Bcl-2 is associated with the potential of the mitochondrial membrane. We found that CGE caused apoptosis in colo 205 cells through increased caspase-3 activity, and this death pathway was caspase-3-dependent since the caspase inhibitor z-VAD-fmk caused the decrease in caspase-3 activity, leading to a decrease in the percentage of apoptosis in colo 205 cells. It was reported that the link between mitochondrial changes and caspase activation could involve two levels (30, 31). We examined the effect of CGE on the production of radical oxygen species (ROS) in colo 205 cells, and the data did show that the CGE increased ROS production (data not shown). It was also reported that ROS increased the permeability of the mitochondrial membrane (32). Further investigations on the chemopreventive properties of CGE are justified.

Acknowledgements

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References

1 Steiner M and Lin RS: Changes in platelet function and susceptibility of lipoproteins to oxidation associated with administration of aged garlic extract. J Cardiovasc Pharmacol *31*: 904-908, 1998.
2 Agarwal KC: Therapeutic actions of garlic constituents. Med Res Rev *16*: 111-124, 1996.
3 Rahman K: Historical perspective on garlic and cardiovascular disease. J Nutr *131*: 977-979, 2001.
4 Yang CS, Chhabra SK and Hong JY *et al*: Mechanisms of inhibition of chemical toxicity and carcinogenesis by diallyl sulfide (DAS) and related compounds from garlic. J Nutr *131*: 1041-1045, 2001.
5 Milner JA: Mechanisms by which garlic and allyl sulfur compounds suppress carcinogen bioactivation. Garlic and carcinogenesis. Adv Exp Med Biol *492*: 69-81, 2001.
6 Block E: The chemistry of garlic and onions. Sci Am *252*: 114-119, 1985.
7 Cellini L, Campli E D and Masulli M *et al*: Inhibition of *Helicobacter pylori* by garlic extract (*Allium sativum*). FEMS Immunol Med Microbiol *13*: 273-277, 1996.
8 Avato P, Tursil E and Vitali C *et al*: Allylsulfide constituents of garlic volatile oil as antimicrobial agents. Phytomedicine *7*: 239-243, 2000.
9 Guo NL, Lu DP and Woods GL *et al*: Demonstration of the anti-viral activity of garlic extract against human cytomegalovirus *in vitro*. Chin Med J (Engl.) *106*: 93-96, 1993.
10 Lau BH, Yamasaki T and Gridley DS: Garlic compounds modulate macrophage and T-lymphocyte functions. Mol Biother *3*: 103-107, 1991.
11 Moriguchi T, Saito H and Nishiyama N: Aged garlic extract prolongs longevity and improves spatial memory deficit in senescence-accelerated mouse. Biol Pharm Bull *19*: 305-307, 1996.
12. Nishiyama N, Moriguchi T and Saito H: Beneficial effects of aged garlic extract on learning and memory impairment in the senescence-accelerated mouse. Exp Gerontol *32*: 149-160, 1997.
13 Green DR: Apoptotic pathways: paper wraps stone blunts scissors. Cell *102*: 1-4, 2000.
14 Reed JC: Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med *7*: 314-319, 2001.
15 Ferri KF and Kroemer G: Organelle-specific initiation of cell death pathways. Nat Cell Biol *3*: 255-263, 2001.
16 Budihardjo I, Oliver H and Lutter M *et al*: Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol *15*: 269-290, 1999.

17 Sundaram SG and Milner JA: Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis *17*: 669-673, 1996.
18 Park EK, Kwon KB and Park KI *et al*: Role of Ca^{2+} in diallyl disulfide-induced apoptotic cell death of HCT-15 cells. Exp Mol Med *34*: 250-257, 2002.
19 Kwon KB, Yoo SJ and Ryu DG *et al*: Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. Biochem Pharmacol *63*: 41-47, 2002.
20 Tan TW, Tsai HY and Chen YF *et al*: Induction of apoptosis in human promyelocytic leukemia HL-60 cells by *Ampelopsis cantoniensis* crude extract. In Vivo *18*: 457-462, 2004.
21 Lu H F, Sue CC and Yu CS *et al*: Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. Food Chem Toxicol *42*: 1543-1552, 2004.
22 Yang CC, Yang JH and Lu HF *et al*: N-acetyltransferase activity is involved in paclitaxel-induced N-acetylation of 2-aminofluorene in human bladder cancer cells (T24). Anticancer Res *24*: 1501-1506, 2004.
23 Wattenburg LW: Inhibition of carcinogenesis by minor dietary constituents. Cancer Res *52*: 2024-2029, 1992.
24 You WC, Blot WJ and Chang YS *et al*: *Allium* vegetables and reduced risk of stomach cancer. J Natl Cancer Inst *81*: 162-164, 1989.
25 Steinmetz KA, Kushi LH and Bostick RM *et al*: Vegetables, fruit, and colon cancer in the Iowa Women's Health Study. Am J Epidemiol *139*: 1-15, 1994.
26 Iwalokun BA, Ogunledun A and Ogbolu DO *et al*: *In vitro* antimicrobial properties of aqueous garlic extract against multidrug-resistant bacteria and *Candida* species from Nigeria. J Med Food *7*: 327-333, 2004.
27 Hsing AW, Chokkalingam AP and Gao YT *et al*: *Allium* vegetables and risk of prostate cancer: a population-based study. J Natl Cancer Inst *94*: 1648-1651, 2002.
28 Nakagawa H, Tsuta K and Kiuchi K *et al*: Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines. Carcinogenesis *22*: 891-897, 2001.
29 Hong YS, Ham YA and Choi JH *et al*: Effects of allyl sulfur compounds and garlic extract on the expression of Bcl-2, Bax, and p53 in non small cell lung cancer cell lines. Exp Mol Med *32*: 127-134, 2000.
30 Totth A, Sebestyen A and Barna G *et al*: TGFβ1 induces caspase-dependent but death-receptor independent apoptosis in lymphoid cells. Anticancer Res *21*: 1207-1212, 2001.
31 Scaffidi C, Kirchoff S and Krammer PH *et al*: Apoptosis signaling in lymphocytes. Curr Opin Immunol *11*: 277-285, 1999.
32 Ribeiro A, Bronk SFand Roberts PJ *et al*: The transforming growth factor β-inducible transcription factor, TIEG1, mediates apoptosis through oxidative stress. Hepatology *30*: 1490-1497, 1999.

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