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# Initial Study on Naturally Occurring Products From Traditional Chinese Herbs and Vegetables for Chemoprevention

**Shu Zheng,* Hua Yang, Suzhan Zhang, Xianping Wang, Linlin Yu, Jieqing Lu, and Jun Li**

Cancer Institute, Zhejiang Medical University, Hangzhou, China

**Abstract** A number of naturally occurring products from vegetables and herbs exert chemopreventive properties against carcinogenesis. In this paper, two such compounds, isolated from garlic and from a traditional Chinese medicinal herb, are described for review. Elemene, isolated from the Chinese medicinal herb *Rhizoma zedoariae,* was shown to exhibit antitumor activity in human and murine tumor cells in vitro and in vivo. This novel antineoplastic agent has substantial clinical activity against various tumors. The in vitro effect of elemene on the growth of leukemia cells was evaluated by MTT assay. The $IC_{50}$ values of elemene for promyelocytic leukemia HL-60 cells and erythroleukemia K562 cells were 27.5 μg/mL and 81 μg/mL, respectively, while $IC_{50}$ for peripheral blood leukocytes (PBL) was 254.3 μg/mL. The inhibitory effect of elemene on proliferation of HL-60 cells was associated with cell cycle arrest from S to $G_2M$ phase transition and with induction of apoptosis. The apoptosis of tumor cells was confirmed by DNA ladder formation on gel electrophoresis and characteristic ultrastructural alterations. The results also demonstrated that inhibitory effects of allicin, a natural organosulfide from garlic, on proliferation of tumor cells were associated with the cell cycle blockage of $S/G_2M$ boundary phase and induction of apoptosis. These findings suggest that induction of apoptosis may contribute to the mechanisms of antitumor activity of elemene and allicin, which merit investigation as potential chemoprevention agents in humans. J. Cell. Biochem. Suppl. 27:106–112. © 1998 Wiley-Liss, Inc.

**Key words:** garlic; elemene; allicin; antitumor activity; carcinogenesis; leukemia

One of the most effective strategies in cancer control is chemoprevention [1], the prevention of cancer by ingestion of chemical agents that reduce the risk of carcinogenesis. Extrinsic factors, including nutrition, play a major role in the development of most human malignancies [2–4]. Foods contain not only numerous mutagens or carcinogens but also a variety of chemicals that can block carcinogenesis [5–7]. In principle, harmless agents should be considered first when selecting chemoprevention agents for high-risk or target populations. For this reason, we chose naturally occurring products and healthy foods to investigate as sources of potential chemoprevention agents. Chemoprevention agents inhibit or reverse cellular events associated with tumor initiation, promotion, and progression. The mechanism of chemoprotective activities might correlate and balance between phase I and phase II enzymes levels, and influence cellular macromolecules/transporters such as MDR glycoprotein, release of carcinogens, or DNA adducts and DNA repair [8–10].

With this view, we chose several related parameters, including $IC_{50}$, enzyme levels, cell cycle arrest, apoptosis, and others, as biomarkers for antitumor activity screening of several constituents recently isolated from edible vegetables and traditional Chinese medicinal herbs. Allicin (diallyl sulfide, DAS) a dietary compound, and beta-elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane), a natural compound isolated from a traditional Chinese medicinal herb (*Rhizoma zedoariae*), are described for review in this paper.

## MATERIALS AND METHODS

### Chemicals

Elemene Emulsion Injection (elemene) was originally provided by Dalian Jin Gang Pharmaceutical Co., Ltd. (Dalian, People's Republic of China [PRC]). 4,6-Diamidino-2-phenylindole (DAPI) and other laboratory reagents were pur-

*Correspondence to: Shu, Zheng, MD, Zhejiang Medical University, Hangzhou, 310031, China

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chased from Sigma (St. Louis, MO). Cisplatin (CDDP) was purchased from Jinzhou Pharmaceutical factory (Jinzhou, PRC). Injectio allitridi was purchased from Tian Feng Pharmaceutical factory (Shanghai, PRC).

### Cell Culture and Treatment

Human promyelocytic HL-60 cells were obtained from Shanghai Institute of Cell Biology, Academia Sinica. Human leukemia K562 cells were provided by Dr. Steinmann (Kiel University, Kiel, Germany). Human rectal carcinoma cell line HR-8348 was provided by Zhejiang Cancer Institute. These three cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in our lab. Human peripheral blood mononuclear cells (PBMCs) were isolated from the healthy donors using Ficoll-Hypaque gradient centrifugation. Cultures of 5 X $10^4$/mL were generally incubated with elemene, which was diluted in culture medium to obtain the desired concentration. Cells were incubated with various concentrations of elemene for 2 to 48 hours. The same aliquot of drug-free emulsion alone was added to the control cells.

### Cytotoxicity and MTT Assay

$IC_{50}$ value calculation for each cell line and PBMCs were determined by MTT assay as described [11]. For MTT assay, cells were seeded at 5 X $10^4$ cells/well and incubated overnight, then treated with equal volumes of medium containing final concentrations of elemene ranging from 0.01 to 320 µg/mL. All experiments were repeated at least twice, each in duplicate.

### Flow Cytometry Analysis

Cell sample preparation and DAPI staining for flow cytometry analysis were performed according to the method reported previously [12]. Cell cycle distribution was determined using a CA-II instrument (Partec, Germany). Resulting DNA distribution was then analyzed for the proportion of cells in apoptosis. Data was analyzed by Multicycle software (Phoenix Flow System, San Diego, CA).

### Electronic Microscope Examination

HL-60 cells were treated for 24 hours with different concentrations of elemene. Cell samples were conventionally fixed. Cells were examined and photographed using a Philips (Mahwah, NJ) EM410 transmission electron microscope (TEM).

### Agarose Gel Electrophoresis of Apoptotic DNA

Cells were treated with various concentrations of elemene, then harvested and washed with phosphate-buffered saline (PBS). Cell pellets (1 X $10^6$) were suspended in 1 mL of lysis solution containing 5 mmol/L Tris-HCl pH 8, 0.25% NP-40, 1 mmol/L EDTA, and 10 mg/mL RNase at 37°C for 1 hour. Twenty-five microliters of proteinase K (20 mg/mL) was added to the above suspension for 1 hour at 37°C. The supernatant of the cell lysat was analyzed by electrophoresis in a 1.5% agarose gel containing 0.2% µg/mL ethidium bromide (EB), and visualized under UV illumination, recorded by a IS-1000 Digital Image System. Dexamethasone-induced apoptotic DNA of rat thymus cells was used as a positive control in agarose gel electrophoresis [13,14].

## RESULTS

### Inhibitory Effect of Elemene on the Proliferation of Human Leukemia Cells

Proliferation of leukemia cells treated with elemene was evaluated by MTT assay. When leukemia cells in culture were treated with elemene for 72 hours, the growth of HL-60 leukemia cells and K562 leukemia cells was significantly inhibited. The $IC_{50}$ values of elemene for HL-60 and K562 cells were found to be 27.5 and 81.0 µg/mL, respectively, while treated with elemene for 72 hours. The IC50 for PBMCs from normal blood donors was 254.3 µg/mL.

### Effect of Elemene and Allicin on Cell Cycle Progression of Tumor Cells

When HL-60 cells were treated with 10 µg/mL of elemene, the proportion of cells at $G_2M$ phase was relatively decreased for 4-hour treatment. However, during 24- to 48-hour treatment, no significant effect of 10 µg/mL elemene on the cell cycle could be observed. When treated with 20 µg/mL elemene, the proportion of cells at $G_2M$ phase was markedly decreased and dependent on the time of drug exposure. In Figure 1, the distinct apoptotic features of sub-GI peak (Ap peak) are observed. The percentage of apoptotic cells for the treatment of 4, 24, and 48 hours were 41.5, 35.3, and 47.7%, respectively. When the dose of elemene increased to 40 µg/

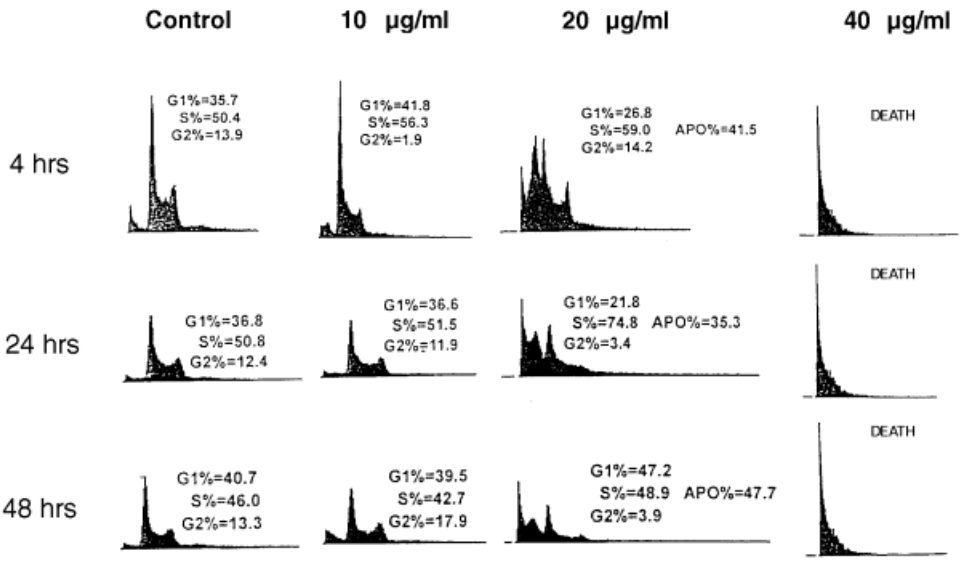


**Fig. 1.** Effect of elemene on cell cycle progression of tumor cells. Cell cycle phase arrest and subsequent apoptosis induced by varying concentrations of elemene in HL-60 cells. The cultures were treated for 4, 24, and 48 hours. At various times, cells were analyzed for apoptotic cell populations and for alterations in cell cycle stages by flow cytometry. Apoptotic cells have fractional DNA content and are represented by a "sub-G1" peak. Cells treated with 20 μg/mL of elemene beginning from 4 hours were arrested in S phase and also demonstrated significant apoptotic responses.

**TABLE I. Effect of DAS on Cell Cycle Progression of K562 Cells**

| Dose of DAS | 24 hours | | | 48 hours | | | 72 hours | | | 96 hours | | | 120 hours | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (μg/ml) | G1/0 | S | G2M | G1/0 | S | G2M | G1/0 | S | G2M | G1/0 | S | G2M | G1/0 | S | G2M |
| 0.0 | 30.5 | 60.0 | 9.4 | 35.3 | 47.6 | 17.1 | 38.3 | 57.8 | 4.0 | 44.9 | 47.9 | 7.2 | 44.2 | 48.0 | 7.8 |
| 0.1 | 33.9 | 55.6 | 10.5 | 35.0 | 47.9 | 17.1 | 42.1 | 50.6 | 7.3 | 46.6 | 39.3 | 14.1 | 42.7 | 46.0 | 11.4 |
| 1.0 | 30.5 | 41.2 | 28.3 | 32.3 | 46.4 | 21.3 | 32.2 | 55.7 | 12.1 | 41.8 | 43.3 | 14.9 | 38.8 | 43.7 | 17.5 |
| 5.0 | 20.8 | 36.2 | 43.0 | 22.3 | 55.0 | 22.7* | 40.3 | 37.8 | 21.9* | 36.9 | 28.9 | 34.3* | 23.8 | 54.0 | 22.2 * |
| 10.0 | 31.4 | 29.9 | 38.8 | 37.6 | 29.2 | 33.2 | 38.8 | 38.4 | 22.9* | 42.4 | 35.7 | 21.9* | ********** | | ********** * |

mL, total cells were undergoing death completely beginning from 4-hour treatment. Cell cycle analysis by flow cytometry revealed that elemene has great influence on the cell cycle progression of HL-60 cells and its effect is dependent on the dose and time of drug exposure. The inhibitory effect of elemene on proliferation of HL-60 cells was associated with cell cycle arrest from S to $G_2M$ phase transition and reduced the proportion of cells at the $G_2M$ phase, consequently inhibiting mitosis of tumor cells. On the other hand, the elemene-treated tumor cells were rapidly undergoing apoptosis.

Treating K562 cells and HR-8348 cells with DAS for 24 hours resulted in remarkable cell cycle blockage in $G_2M$ phase and a decline of S phase fraction in a dose-dependent manner (Tables I and II).

### Apoptosis of HL-60 Cells Induced by Elemene

An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180 bp units, producing a characteristic ladder on agarose gel electrophoresis. To characterize elemene-induced apoptotic cell death in HL-60 cells, internucleosomal DNA fragmentation was analyzed after the cells were exposed to different concentrations of elemene from 2 to 24 hours. The distinct internucleosomal DNA fragmentation ladder could be observed in the elemene-treated HL-60 cells for 2 hours (Fig. 2A). Moreover, in comparison with 2-hour treatment, the dose of elemene for triggering DNA fragmentation was quite lower than that for 24-hour treatment (Fig. 2B). When morphological changes in the 24-hour elemene-

**TABLE II. Effect of DAS on Cell Cycle Progression of HR-8348 Cells**

| Dose of DAS (μg/ml) | 24 hours | | | 48 hours | | | 72 hours | | | 96 hours | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1/0 | S | G2M | G1/0 | S | G2M | G1/0 | S | G2M | G1/0 | S | G2M |
| 0.0 | 53.0 | 21.5 | 25.5 | 71.0 | 11.4 | 17.6 | 75.9 | 8.3 | 15.8 | 72.9 | 11.6 | 15.5 |
| 0.1 | 56.0 | 21.4 | 22.6 | 65.2 | 10.7 | 24.1 | 80.4 | 6.3 | 13.3 | 80.1 | 3.4 | 16.5 |
| 1.0 | 55.7 | 19.3 | 25.0 | 66.9 | 9.1 | 24.0 | 76.7 | 8.1 | 15.2 | 75.6 | 4.6 | 19.8 |
| 5.0 | 57.3 | 2.5 | 19.1 | 60.9 | 14.2 | 24.9 | 71.6 | 16.0 | 12.4 | 67.3 | 15.5 | 17.2 |
| 10.0 | 56.0 | 17.1 | 26.7 | 55.4 | 12.4 | 32.2 | 57.4 | 21.9 | 20.6 | 59.1 | 13.4 | 27.6 |

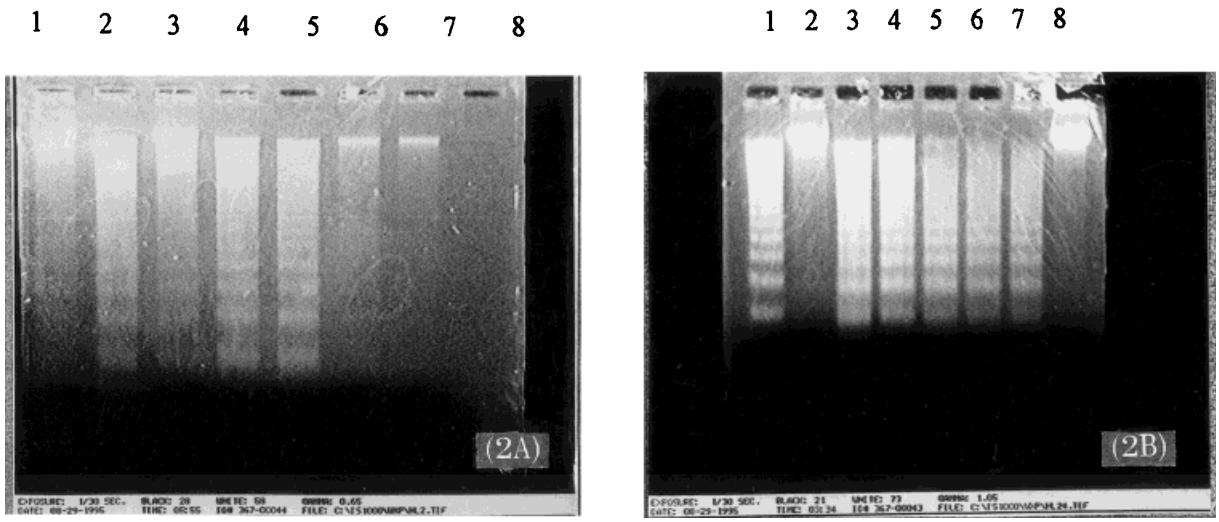


**Fig. 2.** Dose-response of elemenic-induced internucleosomal DNA fragmentation. **A:** HL-60 cells were treated with various concentrations of elemene for 2 hour control (**lane 1**), elemene 10 μg/mL (**lane 2**), 50 μg/mL (**lane 3**), 100 μg/mL (**lane 4**), 200 μg/mL (**lane 5**), 500 μg/mL (**lane 6**), and 1,000 μg/mL (**lane 7**). **B:** HL-60 cells treated with various concentrations of elemene for 24 hours. Dexamethasone-induced apoptotic DNA of rat thymus cells (**lane 1**), control (**lane 2**), elemene 10 μg/mL (**lane 3**), 50 μg/mL (**lane 4**), 100 μg/mL (**lane 5**), 200 μg/mL (**lane 6**), and 500 μg/mL (**lane 7**).

treated HL-60 cells were examined by TEM, distinct apoptotic features of condensed chromosomes and apoptotic bodies were observed (Fig. 3A–E). The characteristic ultrastructural alternations in the elemene-treated HL-60 cells were associated with the dose of drug exposure and were similar to those of the Cisplatin-treated HL-60 cells (Fig. 3F).

## DISCUSSION

The main active component of elemene injection, a newly approved anticancer drug in China, is β-elemene, which has been effective for treatment of cervical carcinoma and cancer of lung, liver, brain, and other tissues. Few side effects of elemene injection were reported in clinical trials [15, 16]. No overt signs of elemene-induced toxicity of the function of liver and kidney and no suppression of bone marrow were observed.

Pharmacological study [17] indicated that β-elemene was active against a variety of tumor cell lines in in vitro tests. It was found that elemene possesses direct cell-killing activity via inhibition of the synthesis of DNA, RNA, and protein in tumor cells, but the mechanism by which elemene leads to cell death is not entirely clear. We are currently investigating the possibility that human tumor cells may undergo apoptosis after treatment with β-elemene. Our results [18] indicate that β-elemene causes inhibition of the growth of tumor cells and an apparent block in cell cycle progression from S to $G_2M$ phase, subsequently resulting in apoptosis in tumor cells. Our data suggest that the induction of apoptosis contributes to the mechanisms for antitumor activity of β-elemene.

Garlic compounds have been shown to inhibit growth of animal tumors and to modulate the activity of diverse chemical carcinogens [19,20].

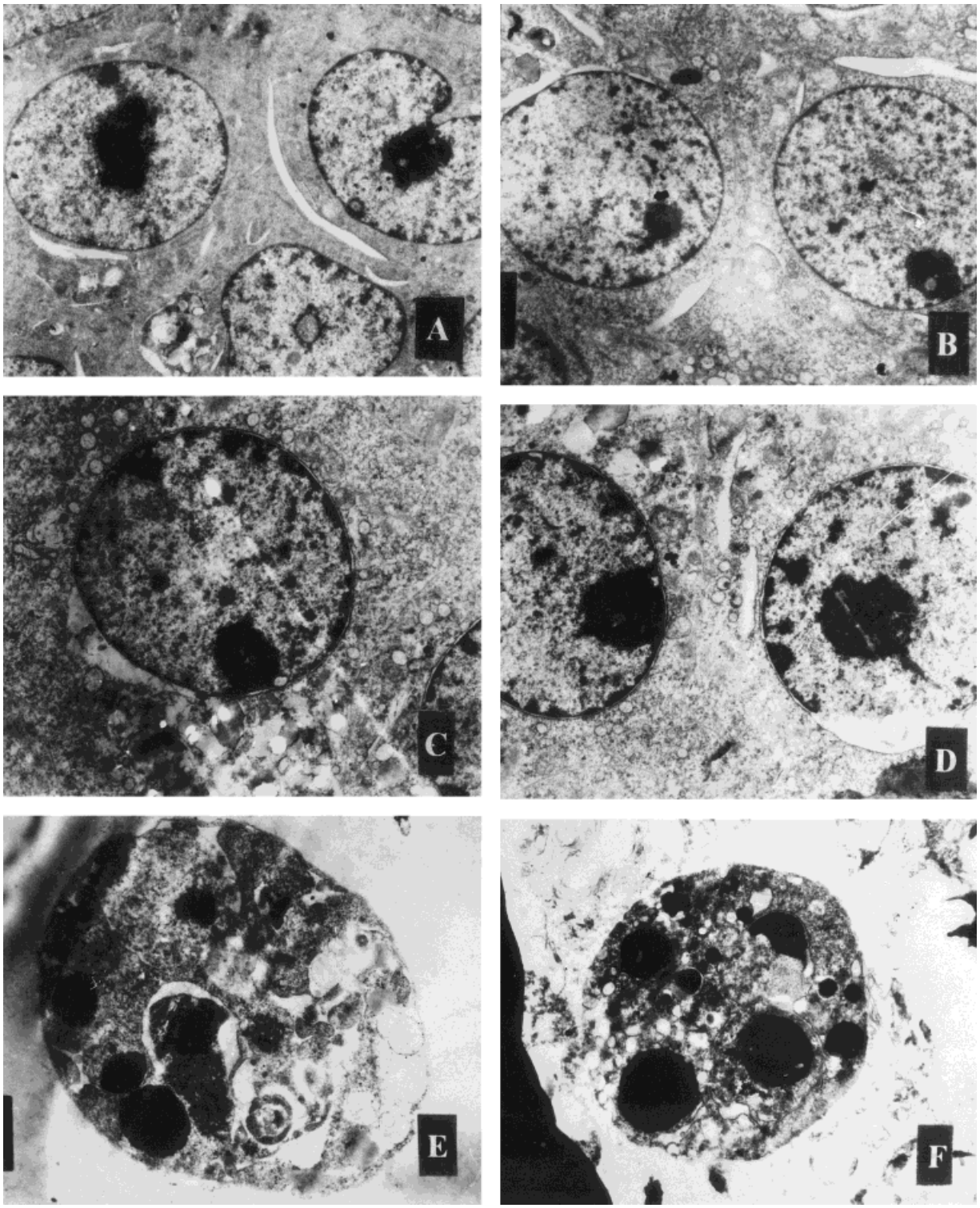


**Fig. 3.** Analysis of morphological changes of HL-60 cells induced by elemene. Control of HL-60 leukemia cells (**A**); cells treated with 10 μg/mL of elemene, structure of nucleus still in good condition (**B**); cells treated with 20 μg/mL of elemene, chromatin anchored to inner side of nucleus membrane, numbers of endoplasmic reticulum (ER) increased, mitochondrion in good condition (**C**); cells treated with 20 μg/mL of elemene, local lesion of nucleus membrane demonstrated (**D**); cells treated with 30 μg/mL of elemene, chromosome condensation, rudimental nucleus membrane, and vacuolization of degenerative cells (**E**); cells treated with 10 μg/mL of CDDP, karyopyknosis, and apoptotic body surrounded by nucleus membrane (**F**).

Garlic may exert an anti-carcinogen effect by modulating detoxification enzymes such as s-transferase (GST) [21,22]. The mechanism of garlic in tumor growth inhibition remains unclear. We have investigated the effect of diallyl sulfide (DAS) on the proliferation of tumor cells. The results demonstrated that inhibitory effects of DAS on proliferation of tumor cells were associated with the cell cycle blockage of $S/G_2M$ boundary phase and induction of apoptosis (data not shown). Induction of apoptosis by garlic could contribute to cancer chemoprevention.

Several natural products have been screened in our initial investigation to find novel potential chemoprotectors. Some data are presented as follows for review.

1. The extract of *Brassica pekinensis* cabbage has an inhibitory effect on KB cells. The $IC_{50}$ of sample No. 1628 and 1603 was 61.2 and 192.4 µg/mL, respectively.
2. **Ling Zhi** (*Ganoderm lucidum*, Fr. Karst) extracts had weak cytotoxicity but strongly inhibited of P-glycoprotein or chemosensitizer of chemotherapy. As compared with verapamil on multidrug resistant (MDR) cells (K562/VCR), the IC50 of Doxorubicin (DOX) was 0.55 µg/mL with 100 µg/mL Ling Zhi extract, and 0.29 µg/mL with 3 µg/mL verapamil, while IC50 of DOX in control was 30 µg/mL.
3. **Kanglaite,** an injection preparation from traditional Chinese food, was cytotoxic to tumor cells with $IC_{50}$ 22.6, 38.5, and 136.4 µl/mL on KB, K562, and K562/VCR cell lines, respectively. The cytoxocity of **kanglaite** to K562/VCR cells was 23% at 40 µl/mL and 1% at 4 µl/mL, while cytotoxicity of DOX at 10 µg/mL was 22.9 and 81.6% when combined with 4 µl **kanglaite** and 10 µl/mL DOX.
4. **Shenmai,** an injection prepared from traditional Chinese herbs, exhibited lower cytotoxicity and MDR reversal, which increased the intracellular accumulation of DOX and vincristine in resistant tumor cell lines.

In conclusion, the results of our initial study demonstrate that dietary constituents and natural products from traditional Chinese herbs have potential inhibitory effects on carcinogenesis and tumor progression. Although the exact mechanisms involved in their protective effects against carcinogenesis are not clearly understood at present, our results should provide a stimulus to design further studies on chemoprevention agents. It was recently reported that antitumor activity had been found in a variety of Chinese cabbages. This suggests that more chemoprevention agents could be found from naturally occurring substances present in diet and herbs, which are plentiful sources for cancer prevention.

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