

Proliferation effects of cinnamon extract on human HeLa and HL-60 tumor cell lines

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Abstract. – OBJECTIVE: To investigate the possible anti-cancer properties of cinnamon extract on two human tumor cell lines, HeLa cells and HL-60 cells.

MATERIALS AND METHODS: Two human tumor cell lines, HeLa cells and HL-60 cells, were exposed to increased concentrations of an extract prepared from cinnamon. The cell proliferation and cell cycle distribution were evaluated using MTT assay and flow cytometry, respectively. The possible action mechanism was also investigated by Western blot.

RESULTS: The results showed that cinnamon extract strongly inhibited tumor cell proliferation in a dose-dependent manner and exhibited dramatic increases in the percentage of cells in G2/M in parallel with exposure to increasing concentration of cinnamon extract. The Western blot results showed that cinnamon extract reduced the cyclin A, cyclin B1, ERK2, and p-ERK proteins expression.

CONCLUSIONS: Our study suggested that cinnamon extract inhibit the tumor cell survival by both down-regulated their target cell cycle regulation molecules and mitosis regulation molecules.

Key Words:

Cinnamon extract, Tumor cell lines, HeLa, HL-60, Proliferation, Cell cycle.

However, chemotherapy and radiotherapy have produced tremendous psychological and physical pain of patients during the treatment period and there are certain risks in the hematopoietic stem cells transplantation therapy^{4,5}. In contrast, because of relatively low adverse effects of naturally originated agents in common belief, these are the most desired. Cinnamon (*Cinnamomum cassia*) has been the focus of research and approved to be a compound that generally recognized as safe status by the Food and Drug Administration (FDA) recently. Cinnamon bark is the outer skin of an evergreen tall tree belonging to the family Lauraceae. Its extracts contain several active components including essential oils, mucus, tannin and carbohydrates^{6,7}, which have various biological activity such as anti-oxidant, anti-microbial, anti-inflammation, anti-diabetic and anti-tumor effect. Some of its active components such as essential oils, tannin and mucus, have been studied in detail. Previously, the anti-tumor properties of the aqueous cinnamon extract in a human cervical cancer cell line (SiHa) have been reported⁸⁻¹¹. Due to differences between the cell lines, these sorts of studies must be extended. In this study, we further identified the anti-tumor effect of cinnamon extracts on the growth of another two human cancer cell lines (HeLa, HL-60) and investigated the possible molecular mechanism.

Introduction

Cancers are the most life-threatening health problems in human being all the time and it is a worldwide problem to complete cure cancers patients^{1,2}. Since cancer is the second reason disease causing death in the world population, the discovery of novel anti-cancer drugs has been targeted³. Conventional cancer treatment medicine program has chemotherapy, radiotherapy and hematopoietic stem cell transplantation.

Materials and Methods

Cell Lines

HeLa and HL-60 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells maintained in Dulbecco's modified Eagle's medium (DMEM)

were supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT, USA) with 2 mM L-glutamine, 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells grown in 75 cm² flasks in a humidified atmosphere with 5% CO₂ at 37°C.

Preparation of Cinnamon Extract

Dried cinnamon bark was pulverized and extracted for six hours in absolute ethyl alcohol extractor and extracted twice. The extract solution was mixture well and dried in depressurization and heating condition. When the extract solution was a volume of 5 mL, the dry process was stopped and added into 30 mL of ultrapure water. The solution was mixed well and centrifugation at low speed at 20 min. The supernatant was filtered and concentrated with a rotary evaporator. The extract was then freeze dried resulting in a powder extract. The powder extract was dissolved with dimethyl sulfoxide (DMSO) at appropriate concentrations. The obtained solution was sterilized by passing through 0.22 µm filter and stored at -20°C freezer.

Cellular Morphology by Microscope

The cells grown in DMEM complemented with 10% fetal bovine serum in 75 cm² flasks in a humidified atmosphere with 5% CO₂ at 37°C incubator until confluency and propagated after trypsinization. Direct microscopy observation was used for finding any changes in morphological form of cells, detachment, shrinkage and colony forming of cells at 0, 24, 48 and 72 h after treatment of cinnamon extract by inverted microscope (Nikon, Tokyo, Japan).

MTT Assay

Cultured cells were grown in 96-well plates. Apoptosis was measured using flow cytometry. Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded (1×10⁴ cells/well in 100 µL) and cultured for 24 h. Then, the medium was removed and the media containing cinnamon extract at different concentrations were added. MTT was performed at 24, 48, 72, 96, 120 and 144 h. The absorbance at 450 nm was measured after incubation with 20 µL of MTT for 4 h. The curve of cell proliferation was then drawn and the proliferation efficiency was examined. The experiments were repeated three times independently.

Cell Cycle Analysis

The effect on cell division by cinnamon extract was determined by assessing cellular DNA content using propidium iodide (PI) staining. Briefly, cells were treated with 1 mg/mL or without of cinnamon extract and incubated at 48 h in the incubator and then each sample was harvested and fixed in 70% ethanol overnight. After fixation, cells were centrifuged and washed with PBS and treated with 0.5 µg/mL of DNase-free RNase (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature and stained with 100 µg/mL of PI in 0.1 M sodium citrate buffer (pH 7.4) for 30 min at 4°C. The cell cycle distribution was determined by flow cytometric analysis (FACS).

Western Blotting

Proteins were extracted from whole cell lysates and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The anti-cyclin B1, anti-cyclin A, anti-ERK2 and anti-p-ERK (1:1000; Abcam, Cambridge, USA) were used. The membranes were then incubated with secondary anti-bodies (1:5000; Abcam, Cambridge, MA, USA). Next, the membrane was treated with chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as per the manufacturer's instructions and analyzed by Image J software.

Statistical Analysis

Data were expressed as mean ± SD. A two-tailed student's *t*-test was employed where *p* < 0.05 was considered to be statistically significant. Each assay was repeated three times.

Results

The Cinnamon Extract Influences the Cellular Morphology

The normal cells showed a flat polygonal shape with good permeability. The cell morphology was complete, clear nucleolus and well adherence (Figure 1A). After cinnamon extract treatment, the HeLa cellular morphology and the growth pattern have changed and apparently time-effect relationship. After treating 30, 120 and 180 min, the volume of cells has been atrophying, however, the adherence and growth have less influenced (Figures 1B, 1C, 1D). After incubation with cin-

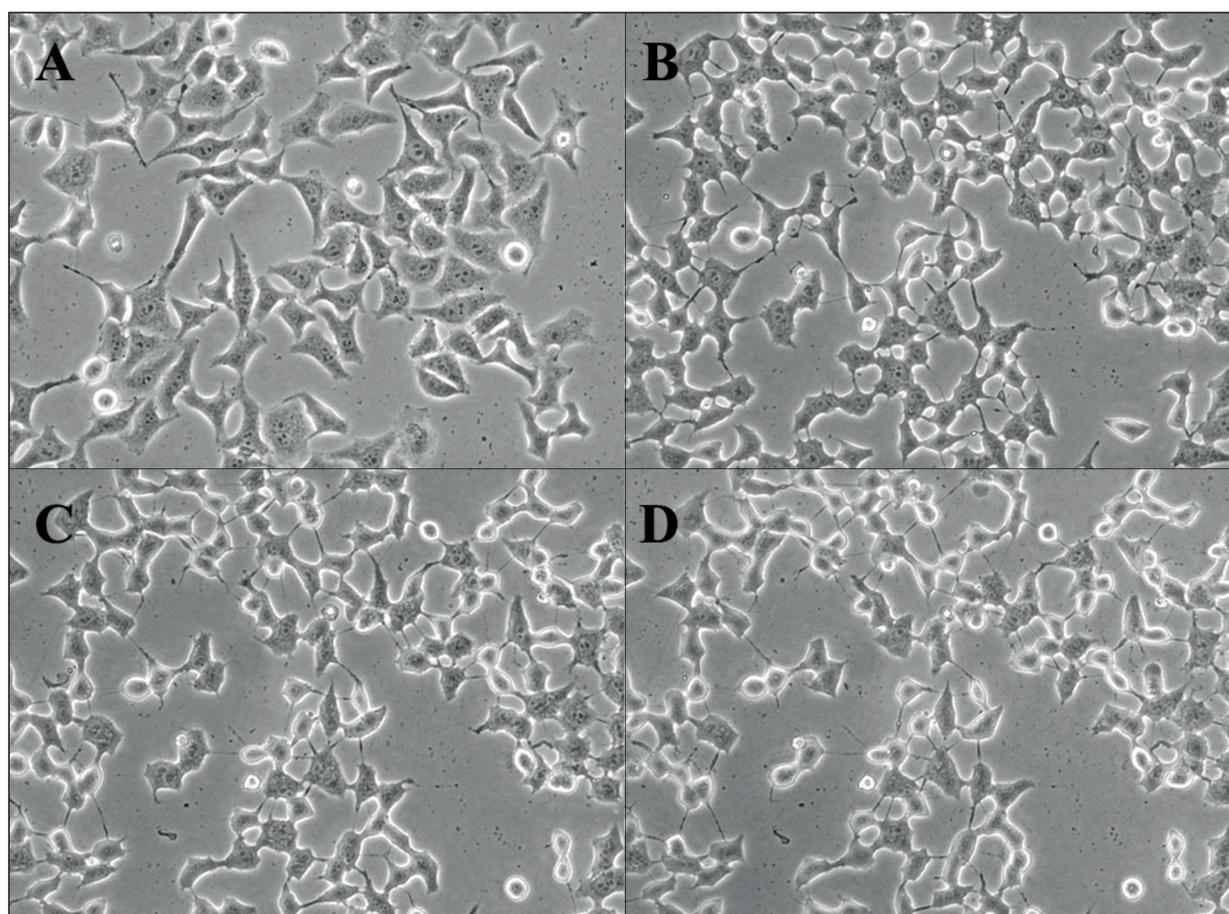


Figure 1. The cellular morphology of HeLa cell with or without cinnamon extract treatment. *A*: the normal HeLa cell morphology. *B*, *C* and *D* were the HeLa cellular morphology with cinnamon extract treatment after 30, 120 and 180 min, respectively.

namon extract 48 h, the form of some cells was long fusiform or circle, the adherence was poor, and some cells have begun to appear apoptosis (Figure 2). The HL-60 cell morphology, which influenced by cinnamon extract were similar to the HeLa cell.

Decreasing Cell Proliferation After Cinnamon Extract Treatment

To confirm the effects of cinnamon treatment on HeLa and HL-60 cell survival, proliferation and cell viability were measured. In the presence of cinnamon extract, the rate of tumor cell growth was significantly inhibited. Figure 3 illustrated the dose-dependent decreases in percentage of cell number compared to the non-treated control for each of the two cell lines as the amount of cinnamon extract increased from 0.1 to 1.0 mg/mL.

Cell Cycle Arrest After Cinnamon Extract Treatment

We first tested whether cinnamon extract induced cell cycle arrest in HeLa and HL-60 cell lines. The results showed that a decreasing pattern in the percentage of cells in G0/G1 was observed for the two cell lines as the amount of cinnamon extract was increased from 0 to 0.8 mg/mL. Percentages in this phase for the two cell lines ranged from 42-45% for untreated controls to 12-18% for the highest cinnamon extract concentration tested. Treatment with cinnamon extract had a more variable effect on the S phase distribution patterns among the two cell lines. All two-cell lines exhibited dramatic increases in the percentage of cells in G2/M in parallel with exposure to increasing concentration of cinnamon extract (Figures 4 and 5).

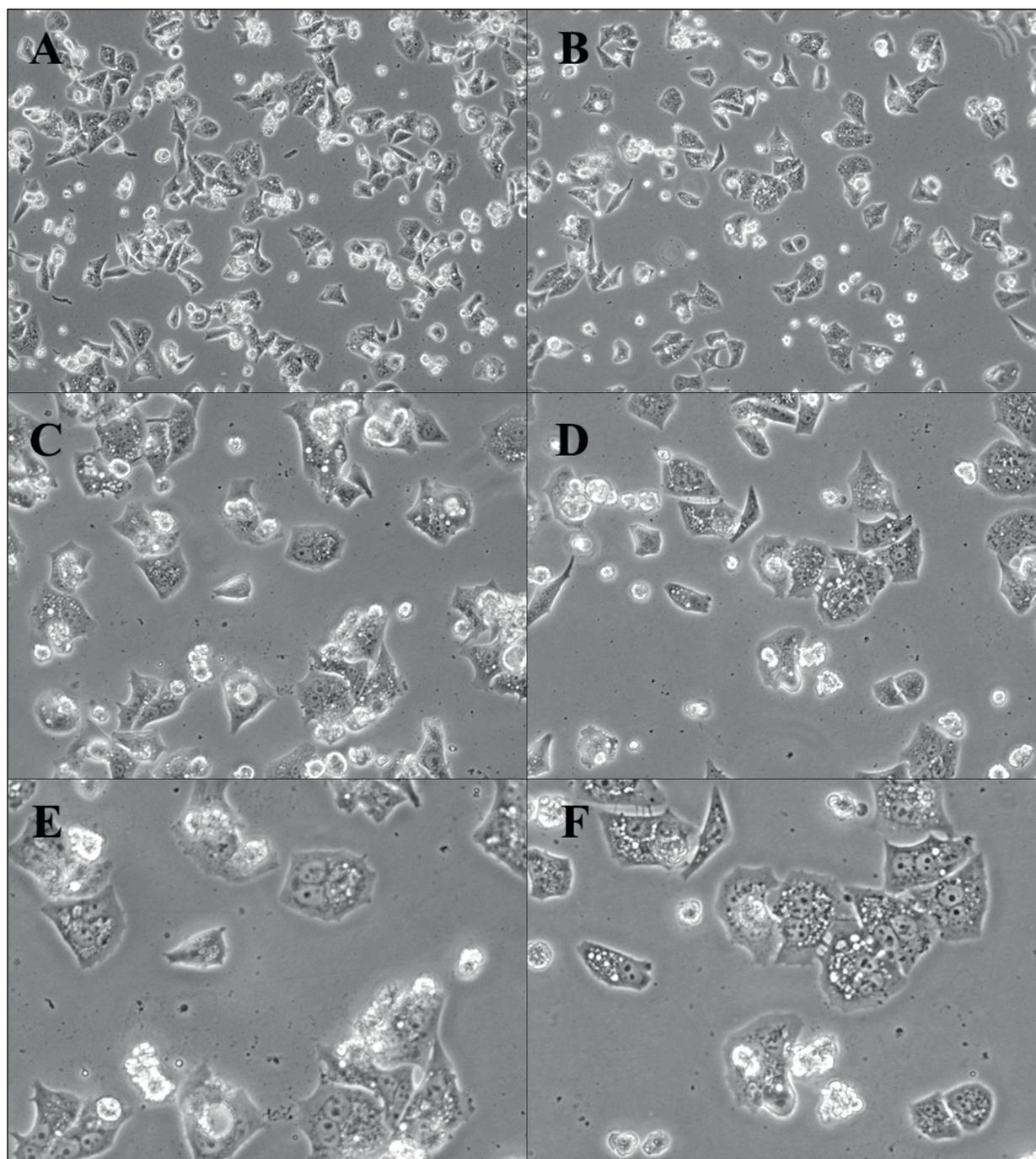


Figure 2. The cellular morphology of HeLa cell after incubation 48 h with or without cinnamon extract treatment. *A, C, E* were the cellular morphology of HeLa cell after incubation 48 h without cinnamon extract at $\times 10$, $\times 40$ and $\times 100$ microscope, respectively. *B, D, F* were the cellular morphology of HeLa cell after incubation 48 h with cinnamon extract at $\times 10$, $\times 40$ and $\times 100$ microscope, respectively.

Cinnamon Extract Inhibits the Regulate Proteins Expression

Cyclins (cyclin A and B1) and extracellular signal regulated kinase (ERK) play a central role in the cell cycle and mitosis progression and

may also affect response to cinnamon extract. Hence, we determined the cell cycle arrest activity of cinnamon extract is linked with changes in the levels of cyclin A, cyclin B1, ERK2 and p-EKR proteins. Interestingly, cinnamon extract

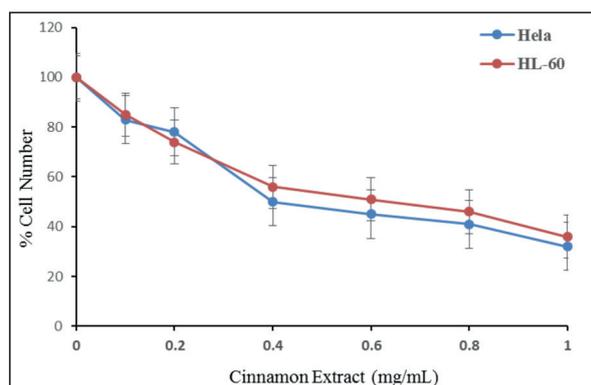


Figure 3. Dose-dependent growth inhibition induced by cinnamon extract after 48 h in the HeLa and HL-60 cell lines. The results were showed by the percent decrease in cell number normalized to untreated control (100%). Data was showed as means \pm SD, n=3.

decreased the amount of cyclin A, cyclin B1, ERK2 and p-EKR proteins in total cell lysates in a time-dependent manner (Figure 6). These results suggest that cinnamon extract inhibit the tumor cell survival by both down-regulated their target cell cycle regulation molecules and mitosis regulation molecules.

Discussion

Cancer is the largest cause of mortality in the world at present. The use of natural agents with chemoprevention effects is one the promising strategies to combat cancer¹². After decade's studies, the natural plant used for anti-cancer therapy has improved remarkably. The herbal medicine could be used for adjuvant chemotherapy, which have ideal efficacy for colorectal cancer, pancreatic cancer, breast cancer, cervical cancer, stomach cancer and liver cancer¹³⁻¹⁶. The anticancer herbal medicine has the advantages of obvious efficacy, small side effects, specific to cancer cells and decrease the suffering of cancer patients without surgical operation¹². Researchers from United States have isolated the active component from *Ganoderma lucidum*, which could promote CAD enzyme activation so that destroy the DNA double helix and restrain division in the cancer cells¹⁷. Further studies suggested that the extract of *Ganoderma lucidum* contained about 20 kinds of triterpenoids and about 10 of these triterpenoids had anticancer activity¹⁸. Since cinnamon is used as a spice for food preparation and folklore medicine as well as in modern pharmacy,

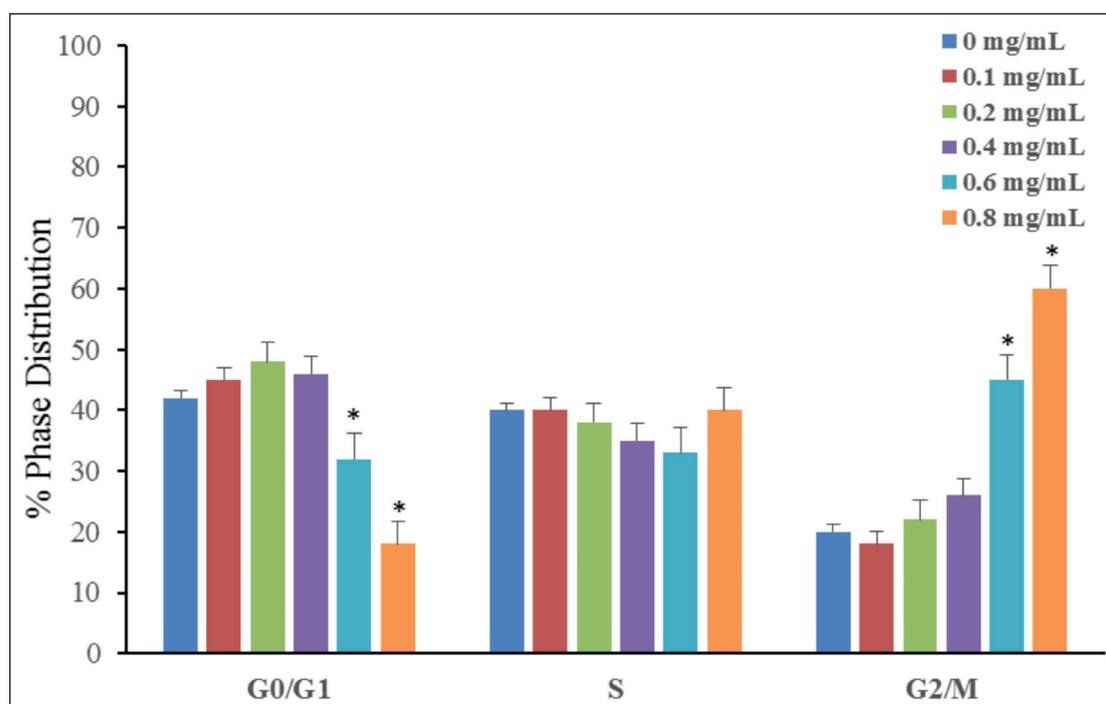


Figure 4. Effect of increasing concentration of cinnamon extract on the percentage of DNA distributed in the three phases of the cell cycle in HeLa cells. It is a significant difference from the non-treated control value within the phase after cinnamon extract treatment with 0.6 and 0.8 mg/mL, * $p < 0.05$.

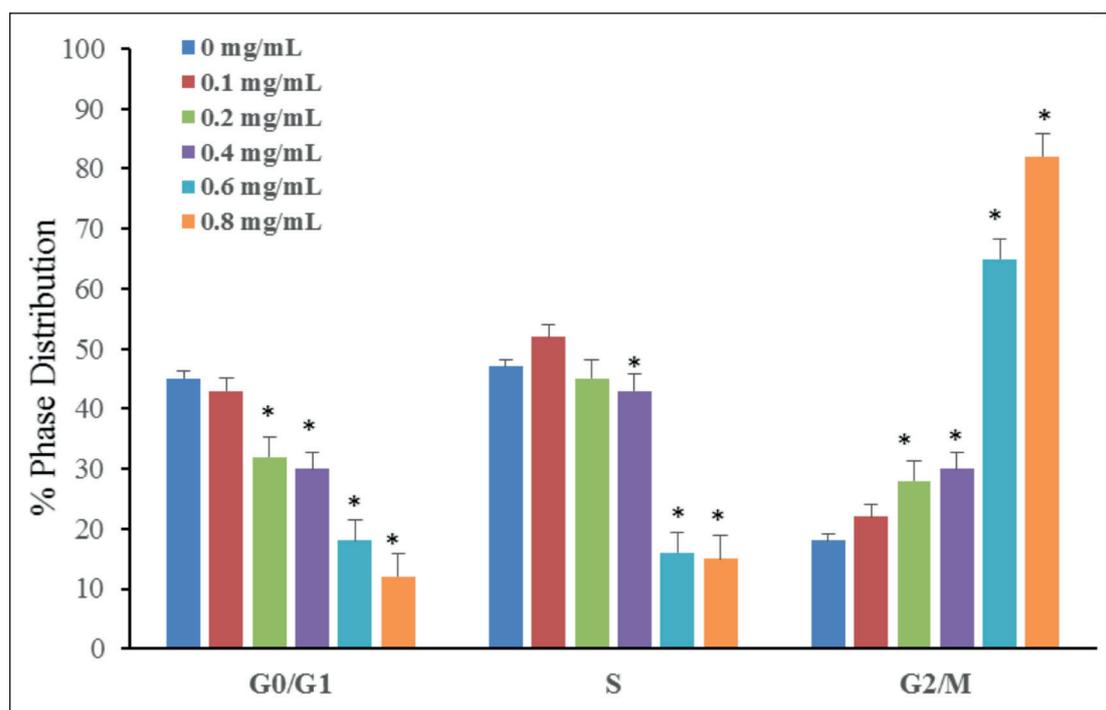


Figure 5. Effect of increasing concentration of cinnamon extract on the percentage of DNA distributed in the three phases of the cell cycle in HL-60 cells. It is a significant difference from the non-treated control value within the phase after cinnamon extract treatment with from 0.2 to 0.8 mg/mL, $*p < 0.05$.

it has been reputed to be useful in the cure of numerous diseases. Several recent studies have found the cinnamon extract contains anticancer activity. Cinnamon extract was shown to inhibit hematologic cancer cell proliferation *in vitro*, melanoma tumor growth in mice and VEGF expression in melanoma^{19, 20}. Much attention has been paid to the influence of cinnamon on insulin action, which may provide benefits for diabetic patients. More recently, a water-based cinnamon extract was found to inhibit Tau aggregation and filament formation associated with Alzheimer's disease²¹. In the present study, we have demonstrated the alcohol-soluble fraction reduced cellular growth in a dose-dependent manner in both HeLa and HL-60 cell lines. Sensitivity of growth to the extract was similar among the cell lines. Analysis of DNA distribution across cell cycle phases demonstrated a differential effect on the regulation of progression by the cinnamon extract compounds. The HL-60 cells displayed increasingly greater percentages of cells at G2/M with increasing cinnamon extract compared to HeLa cell lines. Cyclin A and B1 are considered potential candidate biomarkers in cancer²². Cyclin B1 has been shown to be the rate-limiting acti-

vator of mitotic G1 to S phase transition²³. While over expression of cyclin A which regulates the S-G2-M phase transition of the cell cycle correlates closely with clinic-pathologic parameters and prognosis in patients with cancers²⁴. The canonical RAS-RAF-MEK-ERK mitogen-activated protein kinase signaling pathway coordinates cell growth, differentiation, survival and migration in healthy tissues²⁵. In this work, we further demonstrated the anti-tumor effects of cinnamon extract are also linked with the inhibition proliferation in a cancer specific manner. The results showed that cinnamon extract decreased the amount of cyclin A, cyclin B1, ERK2 and p-EKR proteins in total cell lysates in a time-dependent manner, which suggested that cinnamon extract inhibit the tumor cell survival by both down-regulated their target cell cycle regulation molecules and mitosis regulation molecules. Although cinnamon extracts inhibited proliferation in various cancer cell lines such as HeLa and HL-60, *in vivo* animal studies are necessary to test whether cinnamon extracts have also anti-tumor effects in other types of cancers. In summary, anti-tumor effects of cinnamon extract appear to be mediated by multiple mechanisms.

Conclusions

Cinnamon extract potently inhibited various tumor cell growths *in vitro* studies. Anti-cancer effect of cinnamon extract is mediated by both down-regulated their target cell cycle regulation molecules and mitosis regulation molecules. Hence, cinnamon extract could lead to development of potent anti-tumor agent or complementary and alternative medicines for the treatment of diverse cancers.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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