RESEARCH COMMUNICATION

Induction of Apoptosis in RKO Colon Cancer Cell Line by an Aqueous Extract of *Millingtonia hortensis*

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Abstract

Millingtonia hortensis **is an important medicinal plant in Southeast Asia, used for the treatment of asthma, sinusitis and as a cholagogue and tonic. The aim of this study was to compare the effects of aqueous and ethanol extracts of *Millingtonia hortensis* on the induction of apoptosis in an RKO human colon cancer cell line. Viability of RKO cells was assessed by MTT reduction assay. The aqueous extract, but not the ethanol extract of *M. hortensis* inhibited cell growth and proliferation in a dose- and time-dependent manner. Apoptotic cells were determined by flow cytometry and DNA fragmentation assay. Apoptotic cell numbers increased in a dose-dependent manner after treatment with aqueous extract. DNA ladders were clearly observed in RKO cells treated with 200, 300 and 400 μg/ml of the aqueous extract of *M. hortensis* for 48 h. These results indicate that the aqueous extract of *M. hortensis* inhibited cell proliferation in an RKO colon cancer cell line via the apoptosis pathway.**

Key Words: *Millingtonia hortensis* - DNA fragmentation - apoptosis - gel electrophoresis

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Introduction

Colorectal cancer is a common disease in developing countries (Rex, 2002; Dove-Edwin and Thomas, 2001; Peng et al., 2002; Zhang et al., 2002). It is increasing in Asian countries (Wargovich, 2001). During the past decade, the incidence of colorectal cancer has increased worldwide, with more than 500,000 cases being diagnosed per year (Chen et al., 2003). A number of risk factors have been suggested to increase the risk of colon cancer (Reddy et al., 1999). Colon carcinogenesis is linked with dietary habits (Emmons et al., 2005). Although the fact that many anticancer drugs have been developed, resistance to such drugs is ubiquitous. The research and development of new and safe drugs is necessary (Hosokawa et al., 2004). There is increasing evidence that increased consumption of fruits and vegetables reduces the risk of colon cancer (Ferguson et al., 2004; Frydoonfar et al., 2003; Ma et al., 2003; Olsson et al., 2004; Yang et al., 2000; Zhoa et al., 2004). Chemoprevention refers to the use of natural or synthetic compounds to prevent, reverse, or delay cancer development (Shih et al., 2004). Many edible and medicinal plants provide a potential resource for the research and development of chemopreventive agents for cancer (Chinwala et al., 2003).

Apoptosis is defined as programmed cell death. It plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells, or cells that have progressed to malignancy. Thus induction of apoptosis is a highly desirable mode as a chemotherapeutic for cancer control (Roy et al., 2002).

Millingtonia hortensis belongs to the Family Bignoniaceae. It is an important medicinal plant in Southeast Asia. In Thailand, the plant is called 'Peep' and used for the treatment of asthma and sinusitis (Takashi et al., 1995). At present, nothing is known concerning whether *M. hortensis* affects tumor cell growth. In this study, we compared the effects of aqueous and ethanol extracts from the bark of *M. hortensis* on an RKO colon cancer cell line.

Materials and Methods

Chemicals and Reagents

Minimum essential medium (MEM) was purchased from Gibco laboratories, Paisley, UK. Fetal bovine serum (FBS) and MTT [3-(4,5-di-methylthizol-2-yl)-2,5-diphenyl-tetrazolium bromide] were purchased from Sigma-Aldrich Chemical Company, Dorset, UK. An annexin V-FITC kit was purchased from Roche, Indianapolis, IN, USA. Ninety six-well flat bottom tissue culture plates were purchased from Nunc Inc, Hereford, UK.

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Plant Material

M. hortensis was identified by Dr. Chusie Trisonthi, Department of Biology, Faculty of Science, Chiang Mai University. Dried bark of *M. hortensis* was ground to a fine powder. One hundred grams of powder were extracted with 1,000 ml distilled water or with 80% ethanol by stirring for 4 h at room temperature. Then the filtrates from the ethanol extraction were directly filtered through Whatman filter paper number 1, while the filtrates from the water extraction were centrifuged at 3,500 rpm for 15 min. at 4˚C, before filtering. The filtrates were lyophilized to dryness. The residue was dissolved in culture medium, adjusted to 20 mg/ml final concentration and sterilized by passage through a Millipore filter membrane (0.22 μm).

Cell Culture

Human colon cancer cell line RKO was maintained and cultured in Minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37ºC in a humidified atmosphere containing 5% CO_2. Cells undergoing exponential growth were used throughout the experiments.

MTT Assay

Cytotoxicity was determined by the colorimetric MTT cleavage assay. Briefly, cells ($1x10^4$ per well) were plated in triplicate in 96-well tissue culture plates. Cells were treated with different concentrations of the extracts (200, 400 and 800 μg/ml) for 24, 48, 72 h. After incubation, culture media were discarded and new culture media containing 0.5 mg/ml of MTT were added. The plates were further incubated at 37ºC for 4 h. After the incubation, culture media were discarded and 0.1 ml of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazine crystals. The absorbance (OD) was measured at 540 nm using a microplate reader. Cell viability was expressed as a percentage of control culture.

Apoptosis Assay

Apoptosis was assessed by detecting the expression of phosphatidyl serine on the membrane of apoptotic cells. The apoptosis assay was performed as described (Bunpo et al., 2005). Briefly, after treatment with *M. hortensis* extract, cells were harvested and washed twice with cold PBS. Then cells were resuspended in 100 μl of a reaction mixture containing annexin V-FITC and PI according to the manufacturer's instructions. In all experiments, fluorescence was determined from the combined collection of floating and attached cells using a flow cytometer.

DNA Fragmentation Assay

DNA fragmentation assay was performed as described (Mihara et al., 2003), with slight modifications. Briefly, RKO cells ($1x10^6$ cells) were treated with various concentrations of *M. hortensis* extract for 48 h. After incubation, cells were collected and washed twice with ice cold PBS. The pellets were lysed with 100 ml lysis buffer for 10 min at 4˚C. Cell lysates were harvested by centrifugation at 15,000 rpm for 5 min. Supernatants were collected and incubated for 1 h at 37˚C with 10μl of RNase A (10 mg/ml). Then 5μl of proteinase K (10 mg/ml) was added and further incubated at 50˚C for 30 min. DNA was precipitated with 1 ml of isopropanol and 20μl of 5 M sodium chloride at -20˚C overnight. The DNA pellet was collected by centrifugation, washed with ice-cold 70% ethanol once and dried. The DNA pellet then was dissolved in TE buffer. The DNA solution was electrophoresed on 2% agarose gel containing 0.5μg/ml of ethidium bromide. DNA fragments were visualized under UV light.

Statistical Analysis

Significant differences between mean values were assessed via the Mann-Whitney U test. A p value of <0.05 was considered significant.

Results

Reduction of RKO cell viability by the aqueous extract

To examine the effects of *M. hortensis* on RKO colon cancer cell growth, we incubated RKO cells in the presence

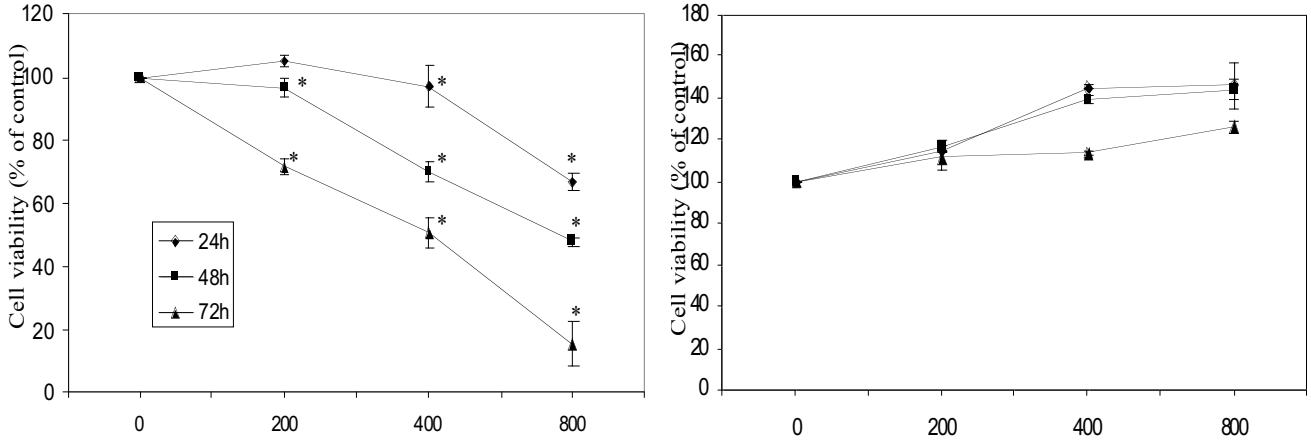

Figure 1. Effects of *M.hortensis* Treatment on the Viability of RKO Cells. Cells were exposed to various concentrations of (A) aqueous extract (B) ethanol extract of *M. hortensis* for 24, 48 and 72 h. The percentage cell viability was determined by the MTT dye reduction assay. Values are the means ±SD of triplicate analyses (n = 3, $p<0.05$).

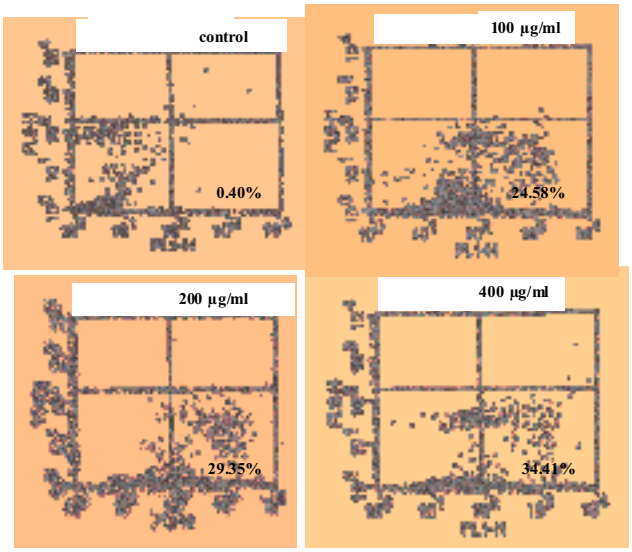

Figure 2. Apoptosis Induction of RKO cells by the Aqueous Extract of *M. hortensis*. Cells were incubated with various concentrations of aqueous extract for 24h, harvested by trypsinization, stained with annexin V-FITC, and analyzed by flow cytometry. The percentages are proportions of apoptotic cells in the whole population.

or absence of various concentrations of extracts. The rate of cell survival was significantly decreased when treated with different concentrations of aqueous extract. The decrease in cell viability was found to be time- and dose-dependent (Figure 1A). However, the ethanol extract did not affect the rate of cell survival (Figure 1B).

Induction of RKO cell apoptosis by the aqueous extract

To evaluate the early stages of apoptotis, we analyzed phosphatidyl serine on the outer leaflet of apoptotic cell membranes using Annexin V-FITC staining followed by flow cytometry. As shown in Figure 2, apoptotic cell numbers increased after treatment with the aqueous extract for 24h. DNA fragmentation was similarly observed after 48 h at a concentration of 200 µg/ml (Figure 3).

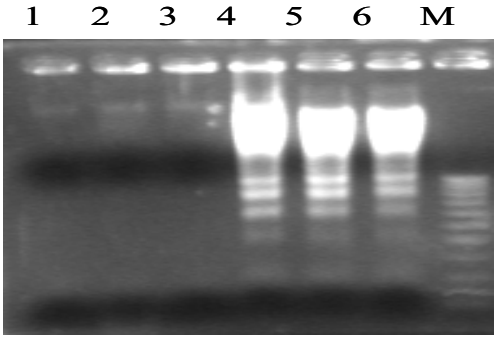

Figure 3. Electrophoretic Separation of Fragmented DNA of RKO Cells. Treatment was with aqueous extract of *M. hortensis* for 48h. DNA was extracted and electrophoresed on 2% agarose gel containing ethidium bromide. DNA fragments were visualized under UV light. Lane 1 = control, Lane 2 = 50 µg/ml of extract, Lane 3 = 100 µg/ml of extract, Lane 4 = 200 µg/ml of extract, Lane 5 = 300 µg/ml of extract, Lane 6 = 400 µg/ml of extract, M = molecular weight marker.

Discussion

Cellular proliferation and apoptosis in normal tissues maintain a balance between cell proliferation and cell death. Unlimited growth and proliferation in the absence of apoptosis are major characteristics of malignant tumor cells. Inhibiting cancer cell proliferation via apoptosis is a novel method for cancer therapy (Yuan et al., 2004).

In the Asian and Pacific area, a wide variety of plants have been traditionally used as foods and medicines. A large number of candidate compounds, which may be practically useful for human cancer prevention, have been identified (Nishino et al., 2000). *M. hortensis* is a widely distributed herb in Southeast Asia, and traditionally has been an important medicinal plant (Takeshi et al., 1996).

In this study, we focused on the effects of *M. hortensis* extracts on colon cancer activity. To the best of our knowledge, this study is the first to show that the aqueous extract of *M. hortensis* induces apoptosis of RKO cells.

Although the aqueous extract was able to inhibit cell proliferation and induce cell death in RKO colon cancer cells, the ethanol extract was not. We suggest that water soluble compounds found in *M. hortensis* have the ability to induce cancer cell death. DNA fragmentation is a hallmark of cells undergoing apoptosis. Typically, the DNA of apoptotic cells is cleaved into a population of fragments, each of which is composed of multimers of 180-200bp in length. Accumulation of extra-nuclear fragmented DNA, which typically appears as a ladder pattern of DNA fragmentation, is due to internucleosomal cleavage associated with apoptosis. DNA fragmentation was found in the cells treated with *M. hortensis* extract. The induction of apoptosis is known to be an efficient strategy for cancer therapy. Recently, extracts prepared from a variety of plants have been found to possess the ability to trigger the apoptotic process (Pezzuto, 1997; Yoon et al., 1999).

In summary, the aqueous extract from bark of *M. hortensis* has the ability to inhibit colon cancer cell growth and proliferation. Cell death by this extract was mediated via the apoptosis pathway. These results suggest the possibility that components of *M. hortensis* might have a therapeutic potential for treating human colon cancer. The molecular mechanisms underlying these effects are under investigation.

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