RESEARCH COMMUNICATION

Growth Inhibitory Activities of Crude Extracts Obtained from Herbal Plants in the Ryukyu Islands on Several Human Colon Carcinoma Cell Lines

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Abstract

There is increasing interest in the use of herbs for the treatment of human diseases including cancer. Therefore, the purpose of this study was to determine whether crude extracts obtained from 44 herbal plants in the Ryukyu Islands might contain components capable of inhibiting the growth of a variety of human colon carcinoma cell lines. Leaves, roots and other parts of the plants were extracted with chloroform, and the crude extracts were dissolved in dimethylsulfoxide and used for the experiments. Extracts of *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* caused marked dose-dependent growth inhibition, with IC_{50} values in the range of 10-80 μg/ml. With the HCT116 cell line, the extracts of *Hemerocallis fulva* and *Ipomoea batatas* induced G1 cell cycle arrest after 48 h of treatment. In addition, we found that extracts of *Curcuma longa,* and *Nasturium officinale* induced apoptosis in these cells after 48 h of treatment. The present studies are the first systematic examination of the growth inhibitory effects of crude extracts obtained from herbal plants in the Ryukyu Islands. The findings provide evidence that several plants in the Ryukyu Islands contain components that may have anticancer activity.

Key Words: Growth inhibition - herbal plants - colon carcinoma cell line

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Introduction

There is increasing interest in the use of herbal products to maintain human health. Plants contain a wide variety of compounds that may have biological activities including anticancer effect (Boik, 2001). There are many kinds of herbal plants in the Ryukyu Islands, Okinawa, Japan. Some of them have been used for the treatment of patients who are affected with insomnia, severe infections, or enterocolitis in Okinawa from ancient times. Especially in women, Okinawa is known as one of longevity areas in Japan (Planning and information section, 2001), and lower incidence of cancer may contribute longer life span (Planning and information section, 2001). In a recent study, we found that the herbs *Peucedanum japonicum* and *Terminalia catuppa* that are widely distributed in Eastern Asian counties including the Ryukyu Islands inhibit the occurrence of aberrant crypt foci (ACF), known as putative preneoplastic lesions in rat colon carcinogenesis (Morioka et al., 2004; Morioka et al., 2005). Although the above-described epidemiological or experimental findings are noteworthy, so far there is only a limited number of studies that examined possible anticancer effects of plants in the Ryukyu Islands. Therefore, in the present study we used a spectrum of 5 human colon carcinoma cell lines to screen the extracts of 44 herbal plants in the Ryukyu Islands for their effects on cell proliferation, cell cycle progression and apoptosis in the hope of identifying valuable plant(s) that may have anticancer activity.

Materials and Methods

Plant Material

A total of 44 plants were obtained in a public market in mainland Okinawa (Table 1). Leaves, roots and other parts of these plants were extracted with chloroform or ethanol, filtered and evaporated under reduced pressure to remove chloroform or ethanol. The remaining crude extracts were dissolved in dimethlsulfoxide (DMSO)(Sigma Chemical Co., St. Louis, MO) and used for the following experiments.

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Cell Culture

Human colon cancer cell lines HCT116, SW480, CaCo2, HT29, and SW837 were generously provided by Dr. I. Bernard Weinstein (Columbia University Cancer Center, New York). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS)(ICN Biomedicals, Inc., Irvine, CA) in an incubator with humidified air with 5% CO_2 at 37˚C.

Cell Proliferation Assays

Cell proliferation was measured by using a MTT assay kit (Roche Diagnostics Corp., Indianapolis, IN) that colorimetrically measures a purple formazan compound produced by viable cells. Cells were plated onto 96-well plates ($2x10^3$ cells/well) in DMEM containing 10% FBS and cultured overnight to allow for cell attachment. Exponentially dividing cells were then treated with DMSO (<0.5%) or increasing concentrations (0.1-100 μg/ml) of each plant extract. After 48 h of treatment, cells were treated with 10 μl of MTT reagent for 4 h at 37˚C and then incubated with 100 μl of solubilization solution at 37˚C overnight. The quantity of formazan product was measured by using a spectrophotometric microtiter plate reader (Bio-Rad Laboratories, Hercules, CA) at 550 nm wavelength. Results were expressed as a percentage of growth, with 100% representing control cells treated with DMSO alone. All assays were performed in triplicate.

Cell Cycle Assays

Exponentially growing cells were treated with the indicated concentrations of each plant extract and were harvested by trypsinization at the indicated time. Cells ($1x10^5$/ml) were seeded in 10-cm diameter culture plates, and cultured to yield 50-60% confluence. Cells were then treated with either DMSO or 10-100 μg/ml each plant extract. After 48 h of treatment, both adherent cells were harvested, washed twice with PBS, fixed with 70% ethanol, centrifuged, resuspended in 400 μl of 2 mg/ml RNase (Sigma), stained 400 μl of 0.1 mg/ml propidium iodide (PI, Sigma) and filtered through 60 μm nylon filters (Ikemoto Scientific Technology Co., Ltd., Tokyo). Flow cytometric analysis was performed on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ) as described elsewhere (Suzui et al., 2002). The distribution of cells in the G1, S, and G2-M phases of the cell cycle were calculated using CELL Quest computer program (Becton Dickinson).

Apoptosis Assays

Cells ($1x10^5$ /ml) were plated in 10-cm diameter culture dishes and then treated with either DMSO or the indicated concentrations of each plant extract in DMEM containing 10% FBS for 48 h. DNA was extracted by a method described earlier (Mori et al., 2001). DNA fragmentation was visualized by agarose gel electrophoresis followed by ethidium bromide staining. To examine changes in nuclear morphology, cells were collected and fixed with 1% glutaraldehyde and stained with 1 mM Hoechist 33258 stain solution (Sigma). Nuclear condensation of cells was observed by a fluorescence microscope. We also performed flow cytometry analysis to assess apoptosis. Cells ($1x10^5$ / ml) were plated in 10-cm diameter culture dishes and then treated with either DMSO (control) or the indicated concentrations of each plant extract in DMEM/10% FBS. After 48 h of treatment, both adhesive and floating cells were harvested and labeled with PI using a method described

Table 1. List of Plants Used for Screening

No.	Name (part of the plants used for the experiment)	No.	Name (part of the plants used for the experiment)
1	*Ixeris dentata Nakai* (leaf)	23	*Citrus depressa Hayata* (leaf)
2	*Daucus carota L. var. satira DC.* (root)	24	*Limonium wrightii Hance O.K.* (leaf)
3	*Hemerocallis fulva L. var. sempervirens M. Hotta* (root)	25	*Limonium wrightii Hance O.K.* (stalk)
4	*Ipomoea batatas L.* (root)	26	*Alangium platanifolium Harms var. trilobum Ohwi* (leaf)
5	*Momordica charantia L.* (fruit)	27	*Alangium platanifolium Harms var. trilobum Ohwi* (stalk)
6	*Saccharum officinarum L.* (stalk)	28	*Ficus pumila L.* (leaf)
7	*Curcuma longa L.* (root)	29	*Terminalia catappa L.* (leaf)
8	*Capsicum annuum var. angulosum* (fruit)	30	*Polygonum chinense L.* (leaf)
9	*Solanum tuberosum L.* (root)	31	*Polygonum chinense L.* (root)
10	*Cymbopogon citratus A. DC. Stapf* (leaf, stalk)	32	*Ricinus communis L.* (leaf)
11	*Cassia obtusifolia L.* (leaf)	33	*Rubus parvifolius L.* (leaf)
12	*Carthamus tinctorius L.* (flower)	34	*Carica papaya L.* (leaf)
13	*Camelia sinensis O. Kuntze* (leaf)	35	*Bupleurum falcatum L.* (leaf)
14	*Hibisucus rosa-sinesis L.* (flower)	36	*Crossostephium chinese Mak.* (leaf)
15	*Brassica chinensis L.* (leaf)	37	*Ternstroemia japonica Thunb* (stalk)
16	*Dioscorea japonica Thunberg* (root)	38	*Camellia japonica L.* (leaf)
17	*Digenea simplex C. Ag* (whole)	39	*Camellia japonica L.* (stalk)
18	*Brassica compestris L. var. pervilidis* (leaf)	40	*Pinus luchuensis Mayr* (leaf)
19	*Nasturium officinale R. Br.* (leaf)	41	*Artemisia campestris L.* (leaf)
20	*Zizania latifolia Stapf* (seed)	42	*Hibiscus rosa-sinensisL.* (leaf)
21	*Alpinia speciosa K. Schum* (leaf)	43	*Saccharum officinarum L.* (sugarcane bagasse)
22	*Youngia japonica DC.* (leaf)	44	*Saccharum officinarum L.* (bagasse fly ash)

No. 1-21: chloretorm extracts, No. 22-24: ethanol extracts

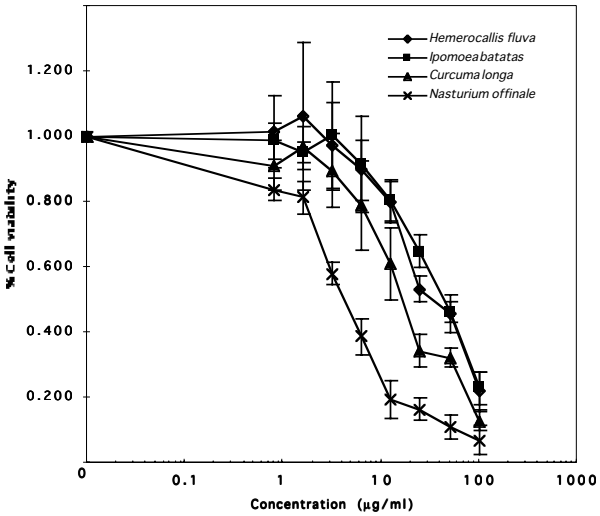

Figure 1. Representative Results of a Dose-response Curve of HCT116 cells.

above. The percentage of cells that underwent apoptosis was determined as the sub-G1 fraction in at least $1X10^4$ cells using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ) with the CELL Quest computer program (Becton Dickinson) (Suzui et al., 2002).

Statistical Analysis

Statistical differences between cells treated with DMSO (control) and those treated with each plant extract were evaluated by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Several plant extracts in the Ryukyu Islands cause a dose-dependent growth inhibition in several human colon carcinoma cell lines

To examine the growth inhibitory activity of each extract derived from 44 plants in the Ryukyu Islands in a variety of human colon carcinoma cell lines, we generated dose-response curves using MTT assays. In the HCT116, SW480, CaCo2, HT29, and SW837 cell lines, extracts from *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* caused a significant growth inhibition in a dose-dependent fashion, with IC_{50} values in the range of 10-80 μg/ml (Figure 1 and Table 2). However, the IC_{50} values of extracts from *Hemerocallis fulva* and *Ipomoea batatas* were more than 100 μg/ml when CaCo2 cells were treated with these 2 plant extracts (Table 2). The HCT116 cells treated with extracts from *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* displayed lower growing rate than that of cells treated with DMSO alone during these cells were grown for 24-72 h (data not shown). However, the extracts derived from the other 40 plants did not cause a growth-inhibitory effect in any of these cell lines (data not shown). Additional studies were then done with the HCT116 cells because this cell line was more sensitive than the other 4 human colon carcinoma cell lines.

Table 2. Growth Inhibitory Effect of Plant Extracts on 5 Human Colon Carcinoma Cell Lines

	HCT116	SW480	CaCo2	HT29	SW837
Hemerocallis fulva	50*	10	>100	100	100
Ipomoea batatas	50	30	>100	20	20
Curcuma longa	25	80	60	75	30
Naturium officinale	10	25	50	30	30

* IC_{50} value (μg/ml)

Extracts of Hemerocallis fulva and Ipomoea batatas induce G1 cell cycle arrest in the HCT116 human colon carcinoma cell line

In view of the above-described inhibitory effects of several plant extracts on cell growth, we examined whether HCT116 cells treated with these plant extracts for 48 h arrest cells in a special phase of the cell cycle by using flow cytometry analysis. When HCT116 cells were treated with 20 μg/ml of extracts from *Hemerocallis fulva* or *Ipomoea batatas*, these extracts caused a significiant increase of cells in G1 by about 7-9% and this was associated with a concomitant decrease of cells in the S and G2-M phase of the cell cycle (Table 3).

Several plant extracts in the Ryukyu Islands induce apoptosis

Because we found that several plant extracts cause a marked growth inhibition on the HCT116 cells, we were further interested in determining whether these plant extracts also induce apoptosis in this cell line. After treating cells with extracts of the following 4 plants, *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale*, at concentrations of 50-100 μg/ml for 48 h, we extracted DNA and examined their apoptosis-inducing activity by observing DNA fragmentation in agarose gel electrophoresis. DNA isolated from the HCT116 cells treated with the extracts of *Curcuma longa* and *Nasturium officinale* displayed evident DNA fragmentation when compared with DNA derived from cells treated with DMSO alone (Figure 2). We then examined whether extracts of *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale*

Table 3. Cell Cycle Distribution of the HCT116 Cell Line after Treatment of Each Plant Extract (%)

Fraction	DMSO	*Hemerocallis fulva*			*Ipomoea batatas*			*Curcuma longa*		*Nasturium officinale*	
	(control)	20 μg/ml	50 μg/ml	100 μg/ml	20 μg/ml	50 μg/ml	100 μg/ml	25 μg/ml	100 μg/ml	10 μg/ml	50 μg/ml
subG1	0.7	5.5	7.6	38.8	5.1	7.9	18	31.7	94.5	36.2	97.7
G1	52.9	60.1	55.1	36.2	62.2	57.1	55	27.5		30.7	
S	24.2	16.5	13.6	12.4	16.6	18.1	12.3	18.8	5.4	18.2	2.3
G2/M	22	17.6	23.5	12.5	16.1	16.7	14.6	21.8		14.9	

A: DMSO (control)
B: 100 μg/ml *Curcuma longa* extract
C: 50 μg/ml *Nasturium officinale* extract

Figure 2. DNA Fragmentation Assay. Note fragmented DNAs in lanes B and C.

induce sub-G1 fraction of these cells. We found that at concentrations between 10-100 μg/ml the extracts of *Curcuma longa*, and *Nasturium officinale* caused a marked sub-G1 fraction (31-97%) of cells in a dose-dependent manner (Table 3). Moreover, the extract (100 μg/ml) of *Hemerocallis fulva* and *Ipomoea batatas* caused a marked sub-G1 fraction (18-38%) of these cells. We further examined whether these extracts alter the nuclear morphology of cells treated with the indicated concentrations of the plant extracts using a Hoechist 33258 stain solution. After treating HCT116 cells with the extracts of *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* displayed evidence of apoptosis representing an increase of nuclear condensation (Figure 3, right panel). While these cells treated with 0.5% DMSO did not show morphological changes in the nuclei (Figure 3, left panel). We then calculated apoptotic index (apoptotic cells/total cells counted) of each plant extract (Table 4). Higher concentrations (approximately IC_{90} value) of the extracts of *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* caused a significant increase in apoptotic index. Lower concentrations (approximately IC_{50} value) of these plant extracts also caused a significant increase in the index except the extract (50 μg/ml) from *Ipomoea batatas* (Table 4).

Discussion

In this study, we screened the extracts obtained from 44 herbal plants in the Ryukyu Islands for the growth-inhibitory effects in a variety of human colon carcinoma cell lines (HCT116, SW480, CaCo2, HT29, SW837). Of these 44

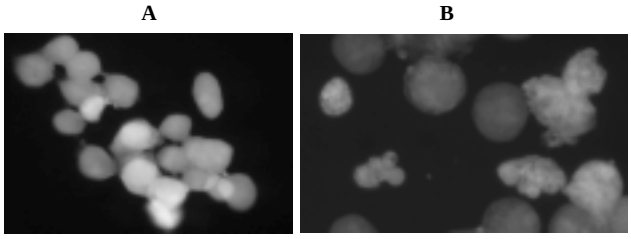

Figure 3. Morphological Changes in the Nuclei of HCT116 Cells. Morphological changes were determined by fluorescence microscopy. After treatment of HCT116 with indicated concentrations of the plant extracts for 48 h, cells were stained with a Hoechst33258 solution. Representative results are shown in cells treated with 0.5% DMSO (A), or 100 μg/ml *Curcuma longa* extract (B).

plants, extracts of *Hemerocallis fulva*, *Ipomoea batatas*, *Curcuma longa*, and *Nasturium officinale* caused a dose-dependent growth inhibition in these colon carcinoma cells. The IC_{50} values of these 4 plant extracts were about 50 μg/ml, 50 μg/ml, 25 μg/ml, and 10 μg/ml, respectively, when HCT116 cells were treated with the extracts in DMEM plus 10% FBS for 48 h. We have further examined the mechanisms of action of these extracts in the HCT116 cell line. We found that extracts of *Hemerocallis fulva* and *Ipomoea batatas* induce G1 cell cycle arrest up to about 10% at a lower concentration (20 μg/ml) in these cells. This increase in G1 was associated with a concomitant decrease of cells in the S and G2-M phases of the cell cycle. These findings suggest that the extracts of Hemerocallis fulva and Ipomoea batatas contain more than one component(s) that induce G1 cell cycle arrest. Because we found that several plant extracts in the Ryukyu Islands arrest cells in G1 in the HCT116 cells, we further determined whether these extracts also induce apoptosis in this cell line. We found that extracts of *Curcuma longa* and *Nasturium officinale* caused a marked and dose dependent sub-G1 fraction of cells. We also found that these extracts caused fragmented DNAs in agarose gel electrophoresis and nuclear condensation of cells in a Hoechist33258 staining assay, suggesting that these extracts can induce apoptosis. The present results clearly indicate that several plant extracts in the Ryukyu Islands have the activity to induce G1 cell cycle arrest and apoptosis in HCT116 human colon carcinoma cells. Taken together, the growth inhibitory activity of these extracts may be associated with the inhibition of cancer cell growth and the induction of apoptosis.

In the present study, we found that HCT116 and SW480 cells are more sensitive to growth inhibition by the extracts of *Hemerocallis fulva* and *Ipomoea batatas* because CaCo2

Table 4. Apoptotic Indices (%)

	DMSO	*Hemerocallis fulva*		*Ipomoea batatas*		*Curcuma longa*		*Nasturium officinale*	
	(control)	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml	10 μg/ml	50 μg/ml
Mean±SD	3.9 ± 1.9	8.3 ± 1.8*	34.4 ± 8.0*	4.0 ± 1.3	13.2 ± 0.9*	21.4 ± 2.4*	42.2 ± 2.9*	15.9 ± 2.9*	29.8 ± 2.3*

* Significantly different from the control by Student's *t*-test ($P<0.001$).

cells require much more higher concentration (>100 μg/ml) of the extract for growth inhibition than HCT116 or SW480 cells (Table 2). In a previous study, it is demonstrated that highly proliferating cells are critical to growth inhibition by the extract of Bilberry (*Vaccinium myrtillus*) (Katsube et al., 2003). Because the growing rate of the HCT116 and SW480 cells is as about 5-10 times as much higher than that of the CaCo2 cells (Maoret et al., 1999), these findings may explain why susceptibility to growth inhibition differs depending on cell lines.

Hemerocallis fulva is widely distributed in Eastern Asian countries (Cichewicz et al., 2004). Its root has been in use for hundreds of years by people in the Ryukyu Islands for the purpose of treatment of insomnia, jaundice, and anemia (Uezu, 1997). Anthraquinone derivatives obtained from *Hemerocallis* have been recently shown to have growth inhibitory effect in human breast, brain, lung, and colon carcinoma cell lines (Cichewicz et al., 2004). In addition, the *Hemerocallis* species contain several compounds that have antioxidant activity (Cichewicz and Nair, 2002). *Ipomoea batatas* contains anthocyanin compounds (Konczak-Islam et al., 2003). Anthocyanins are commonly included in colored fruits and vegetables (Hou, 2003). Anthocyanins obtained from these fruits inhibit tumor cell proliferation in a variety of human carcinoma cell lines (Hou, 2003; Katsube et al., 2003). Also, purified anthocyanin inhibits both the occurrence of ACF in the rat colon induced by dimethylhydrazine (DMH) and rat colon carcinogenesis induced by DMH plus 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Hagiwara et al., 2001). Curcumin is considered as one of active components in the herb *Curcuma longa*. This compound has been reported to have a growth inhibitory effect and to induce apoptosis in a wide variety of human carcinoma cell lines (Bhaumik et al., 1999; Kawamori et al., 1999; Ushida et al., 2000; Araujo and Leon, 2001). Antioxidant and anti-inflammatory activities of curcumin have also been demonstrated (Ruby et al., 1995; Banerjee et al., 2003; Kim et al., 2003). *Nasturium officinale* is a *Brassica rom* which contains abundant isothiocyanete (Fahey et al., 2001; Gingras et al., 2004). It is demonstrated that isothiocyanete of *Nasturium officinale* caused a marked inhibition on growth in human leukemia, myeloma, colon carcinoma, breast carcinoma, and hepatoma cell lines (Zhang et al., 2003). In addition, an isothiocyanete derivative, phenyl isothiocyanete has been shown to enhance c-Jun N-terminal kinase (JNK) activity and this activity may lead to chemopreventive action of isothiocyanete (Yu et al., 1996; Chen et al., 1998).

The present studies provide evidence that several plant extracts in the Ryukyu Islands inhibit the growth and induce G1 cell cycle arrest and apoptosis in cell cultures of human colon carcinoma cells, strongly indicating that these plant extracts contain a component(s) that may have anticancer activity. It is of interest whether these plant extracts are useful in the chemoprevention and/or therapy of human colon cancer. Thus, important consideration is whether or not, following oral administration, sufficient blood and tissue levels can be achieved and these extracts exert significant adverse side effects. To address these issues, further studies are in progress to identify the active component of these extracts and the precise molecular mechanism by which these extracts inhibit the growth of human colon carcinoma cells.

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