RESEARCH COMMUNICATION

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

Tatsuya Kaneshiro¹, Masumi Suzui¹, Reika Takamatsu¹, Akira Murakami², Hajime Ohigashi², Tetsuya Fujino³, Naoki Yoshimi¹

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 IC₅₀ 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

Asian Pacific J Cancer Prev, 6, 353-358

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 catappa* 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 dimethylsulfoxide (DMSO)(Sigma Chemical Co., St. Louis, MO) and used for the following experiments.
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% CO₂ 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 (2x10⁴ 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 (1x10⁵/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 FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ). 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 (1x10⁵/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 (1x10⁵/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 adherent and floating cells were harvested and labeled with PI using a method described elsewhere.

Table 1. List of Plants Used for Screening

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

above. The percentage of cells that underwent apoptosis was
determined as the sub-G1 fraction in at least 1X10^6 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

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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Hemerocallis fulva</th>
<th>Ipomoea batatas</th>
<th>Curcuma longa</th>
<th>Nasturium officinale</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>50*</td>
<td>10</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td>SW480</td>
<td>50</td>
<td>30</td>
<td>&gt;100</td>
<td>20</td>
</tr>
<tr>
<td>CaCo2</td>
<td>25</td>
<td>80</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>HT29</td>
<td>30</td>
<td>20</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>SW837</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

* IC_{50} value (µg/ml)

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.

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 significant 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 these plant
extracts for 48 h induced 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 these plant
extracts for 48 h induced apoptosis in this cell line. After

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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DMSO (control)</th>
<th>Hemerocallis fulva</th>
<th>Ipomoea batatas</th>
<th>Curcuma longa</th>
<th>Nasturium officinale</th>
</tr>
</thead>
<tbody>
<tr>
<td>subG1</td>
<td>0.7</td>
<td>5.5</td>
<td>7.6</td>
<td>38.8</td>
<td>5.1</td>
</tr>
<tr>
<td>G1</td>
<td>52.9</td>
<td>60.1</td>
<td>55.1</td>
<td>36.2</td>
<td>62.2</td>
</tr>
<tr>
<td>S</td>
<td>24.2</td>
<td>16.5</td>
<td>13.6</td>
<td>12.4</td>
<td>16.6</td>
</tr>
<tr>
<td>G2/M</td>
<td>22</td>
<td>17.6</td>
<td>23.5</td>
<td>12.5</td>
<td>16.1</td>
</tr>
</tbody>
</table>

Asian Pacific Journal of Cancer Prevention, Vol 6, 2005 355
Table 4. Apoptotic Indices (%)

<table>
<thead>
<tr>
<th>DMSO (control)</th>
<th>Hemerocallis fulva 50 µg/ml</th>
<th>Ipomoea batatas 50 µg/ml</th>
<th>Curcuma longa 100 µg/ml</th>
<th>Nasturium officinale 10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>3.9±1.9</td>
<td>8.3±1.8°</td>
<td>34.4±8.0°</td>
<td>21.4±2.4°</td>
</tr>
</tbody>
</table>

°Significantly different from the control by Student’s t-test (P<0.001).
Inhibition of Colon Cancer Line Growth by Herbal Plants of the Ryukyu Islands

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). Nasturtium officinale is a Brassica rom which contains abundant isothiocyanate (Fahey et al., 2001; Gingras et al., 2004). It is demonstrated that isothiocyanate of Nasturtium 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 isothiocyanate derivative, phenyl isothiocyanate has been shown to enhance c-Jun N-terminal kinase (JNK) activity and this activity may lead to chemopreventive action of isothiocyanate (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.

Acknowledgements

This work was supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References


