In Vitro Anticancer Activities of Leonurus heterophyllus Sweet (Chinese Motherwort Herb)

MAIMOOONA G. CHINWALA, M.S., 1 MIN GAO, Ph.D., 2 JIE DAI, Ph.D., 3 and JUN SHAO, Ph.D. 1

ABSTRACT

Objectives: To investigate the anticancer activities of Chinese motherwort herb (Leonurus heterophyllus Sweet; LHS).

Design: Dried LHS was extracted and reconstituted in phosphate-buffered saline. The in vitro antiproliferation activities of the extract were tested against seven human cancer cell lines. The DNA ladder assay and cell morphologic studies were performed to verify the drug's apoptotic activities. The possible pathway by which LHS induced apoptosis was also explored by examining mitochondrial depolarization, cytochrome c release, and caspase-3 activation.

Results: The LHS extract was effective in inhibiting the growth of all seven cancer cell lines tested. The IC₅₀ (50% inhibition concentrations, milligrams of raw material per milliliter) were in the range of 8.0–40.0 when the drug exposure time was 48 hours. The inhibitory action of the herbal extract was time- and dose-dependent. A significant decrease in activity was seen when the drug exposure time was shortened. Microscopic examination of the LN CaP and other cancer cell lines after treatment with LHS revealed morphologic changes that are typical of cells undergoing apoptosis. DNA fragmentation was obvious in the DNA latter assay and this confirmed the induction of apoptosis of the cancer cells by LHS. The mitochondria of the LHS-treated cells were found to undergo depolarization. Cytochrome c was released into the cytosol from the LHS-treated cells but not from the control cells. Cells treated with LHS showed cleavage of the full-length poly[ADP(ribose)] polymerase (PARP; 112 kd) to generate the 85-kd cleaved PARP fragment indicating the activation of caspase-3.

Conclusions: LHS was able to induce apoptosis of all the tumor cell lines tested. The antiproliferation effect was dose- and time-dependent. The mitochondrion was found to be involved in the apoptosis induced by the LHS extract.

INTRODUCTION

The low efficacy of current chemotherapy accompanied with severe adverse reactions has been driving an increasing number of patients toward alternative medicine. In the United States, half of all patients with cancer have tried complementary and alternative medicine (CAM) (McCann, 1997). Therefore, there is an urgent need to develop

1Biotechnology and Drug Delivery Laboratory, Department of Pharmacy and Administrative Sciences, College of Pharmacy and Allied Health Professions, St. John’s University, Jamaica, NY.
2Department of Radiation Oncology, Long Island Jewish Medical Center, Albert Einstein College of Medicine, New Hyde Park, NY.
3Department of Medicine, Weill Medical College of Cornell University, New York, NY.
new anticancer agents with minimum side-effects.

Traditional Chinese Medicine (TCM), which is an important part of CAM, has a 5000-year-old history (Comprehensive Dictionary of Traditional Chinese Medicine, 1979). Many edible and medicinal plants described in the classic Chinese Materia Medica provide a potential resource for the research and development of chemopreventive agents of cancer (Han, 1999). Preliminary studies conducted on Chinese leek (Allium tuberosum Rottler), a common vegetable, have shown that the oral administration of Chinese leek extract was effective against B16-F10 melanoma lung metastases in C57BL mice (Shao et al., 2001). The efficacy of a prescription called Dang Gui Lu Hui Wan, which consists of 11 Chinese herbs, has been demonstrated for the treatment of chronic myelocytic leukemia (Han, 1999). In a review on anticancer Chinese herbal medicines, many herbal drugs recommended for use in various cancers such as hepatoma, esophageal cancer, stomach cancer, cervical cancer, breast cancer, lung cancer, et cetera, have been described (Yeh, 1973). This suggests that TCM is a good resource to explore for newer anticancer agents having relatively low toxicity.

Leonurus heterophyllus Sweet is also called Chinese motherwort herb. According to ancient Chinese medical texts, LHS has functions such as promoting circulation, clearing clots, treating breast pain and swelling, and detoxification (Comprehensive Dictionary of Traditional Chinese Medicine, 1979). LHS might have been used for breast cancer treatment in ancient China. A single report is available studying the effect of fractions of Leonurus sibiricus L for its antineoplastic effect in which the authors emphasize the importance of the synergistic action of several components of the herb in order to obtain maximal benefit (Nagasawa et al., 1992). Accordingly, we thought it valuable to further test the anticancer activity of LHS without separation of its components.

We conducted an in vitro antiproliferation study of LHS against seven human cancer cell lines and found that LHS inhibited growth of all tested cancer cell lines. We have also investigated the apoptotic effect of LHS on LN CaP human prostate cancer cell line. Furthermore, we demonstrated that the mechanism of LHS-induced apoptosis in LN CaP cells involved cytochrome c release from mitochondria and subsequent activation of caspase signaling.

**MATERIALS AND METHODS**

**Materials**

Herb extract. The dried aerial part of Leonurus heterophyllus Sweet (Chinese motherwort herb) was used for the preparation of the extract.

Cancer cell lines. Seven human cancer cell lines were used. The descriptions and sources of these cell lines are given in Table 1.

Reagents, antibodies, and laboratory supplies. All cell culture reagents and chemicals were purchased from Hyclone Laboratories (Logan, UT). Cell culture plates and flasks were purchased from Costar Corporation (Cambridge, MA). MTT microtiter; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was purchased from Alfa Aesar (Ward Hill, MA). Anticytochrome c mouse monoclonal immunoglobulin G (IgG) was purchased from Pharmingen (San Diego, CA). Anti-Cox4 mouse monoclonal IgG was purchased from Molecular Probes (Eugene, OR). Anti-α-actin goat polyclonal IgG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Anti-poly [ADP (ribose)] polymerase (PARP) (Ab-2) mouse monoclonal IgG was purchased from Oncogene Research Products (Boston, MA). The secondary antibodies used in this study for Western blotting were purchased from Santa Cruz Biotechnology, Inc.

Methods

Dried Chinese motherwort herb (Leonurus heterophyllus Sweet) was extracted and subsequent dilutions prepared in phosphate-buffered saline (PBS) as follows. Twenty (20) grams of the dried herb (aerial part) was extracted with 250 mL of distilled water for a period of 1 hour at room temperature. It was then heated at 70°C for 3 minutes and then
further kept at room temperature for a period of 1 hour. The extract was then filtered and collected. This extraction procedure was repeated once. The filtrates obtained from both the extraction steps were combined and centrifuged at 4000 rpm for 10 minutes. The supernatant of the extract was then collected, filtered, and freeze-dried. A stock solution of 0.250 g raw material per milliliter was prepared from the freeze-dried powder of the herbal extract using equal volumes of distilled water and PBS. This stock solution was sterilized by filtering it through a 0.2-μm membrane. This solution had a pH in the range of 7.1–7.4. A series of the diluted solutions were made in PBS from the 0.250 g/mL stock solution, and stored at 4°C.

**Tumor cell culture.** The cells were cultured at 37°C, 5% CO₂ with relatively high humidity. The culture media for all the cell lines are listed in Table 1. Cells were harvested either by using cell dissociation solution or trypsin-ethylenediaminetetraacetic acid (EDTA) followed by the addition of cold fresh culture media. Only those cells with viability greater than 95% were used for further experiments.

In vitro **antiproliferation assay.** The live cancer cells were first seeded into each well on a 96-well plate (4 × 10³ cells per 200 μL per well for the A-549 and the TsuPr1 cell lines, 5 × 10³ cells per 200 μL per well for the C-33A, MCF7, DU 145, and the LN CaP cell lines and 6 × 10³ cells per 200 μL per well for the MDA-MB-453 cell line. The cells were cultured for 15–20 hours before the addition of the LHS extract of different concentrations (50 μL per well, 6 wells per concentration). The final LHS concentration in the culture media was in the range of 0.048–50 mg/mL. To the control wells, 50 μL of PBS instead of LHS extract was added into each well. The blank wells contained only 200 μL of the culture media. The tumor cells were then incubated with the LHS extract for a period of 24 or 48 hours when the control wells became almost confluent. The cell proliferation was then analyzed by the MTT as-

### Table 1. Tumor Cell Lines and IC₅₀ of Leonurus heterophyllus Sweet

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Culture media</th>
<th>IC₅₀ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-33A human cervix carcinoma</td>
<td>American Type Culture Collection (ATCC)ᵇ</td>
<td>Eagle’s MEM medium with Earle’s salts supplemented with L-glutamine, NaHCO₃, 10% fetal bovine serum, MEM vitamins, nonessential amino acids, and sodium pyruvate</td>
<td>8</td>
</tr>
<tr>
<td>A-549 human lung carcinoma</td>
<td>ATCC</td>
<td>RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum, MEM vitamins, nonessential amino acids and sodium pyruvate</td>
<td>20</td>
</tr>
<tr>
<td>MCF7 human breast adenocarcinoma</td>
<td>ATCC</td>
<td>Same as A-549 medium</td>
<td>40</td>
</tr>
<tr>
<td>MDA-MB-453 human breast carcinoma</td>
<td>ATCC</td>
<td>Same as A-549 medium</td>
<td>25</td>
</tr>
<tr>
<td>DU 145 human prostate carcinoma</td>
<td>ATCC</td>
<td>Same as A-549 medium</td>
<td>20</td>
</tr>
<tr>
<td>LN CaP human prostate carcinoma</td>
<td>ATCC</td>
<td>Same as A-549 medium</td>
<td>20</td>
</tr>
<tr>
<td>TsuPr1 human prostate carcinoma</td>
<td>ATCC</td>
<td>Same as A-549 medium</td>
<td>12.5</td>
</tr>
</tbody>
</table>

ᵃMilligram of raw material/per milliliter of culture media.

ᵇATCC, Manassas, VA.
MEM, modified Eagle’s medium.
say (Mosmann, 1983). The percentage of cell growth inhibition (I%) was calculated as:

\[ I\% = \frac{(A_c - A_s)}{(A_c - A_b)} \times 100 \]

where \( A_c \) is the absorbance of the control well, \( A_s \) is the absorbance of the treated well, and \( A_b \) is the absorbance of the blank well.

**Apoptosis assay.** Microscopic examination of the LN CaP and other cancer cell lines after treatment with LHS was performed. LN Cap cell apoptosis induced by LHS was also examined by the DNA ladder assay (Dai, 1999).

**Apoptotic mechanism studies.** Apoptotic mechanism studies included:

- **Treatment with the LHS extract**—Subconfluent proliferating LN CaP cells in 100-mm plastic dishes were sham-treated (control) or treated with the herbal extract (50 mg/mL for 48 hr, at 37°C) in the complete culture medium. The cultures were then washed twice with PBS to remove the herbal extract and harvested with help of trypsin for Western blot analysis.

- **Mitochondrial separation**—Cells were harvested using trypsin and washed two times with PBS. Cell fractionations were performed as described before (Bialik et al., 1999). Briefly, the cell pellet was resuspended in digitonin lysis buffer (75 mmol NaCl, 1 mmol NaH₂PO₄, 8 mmol Na₂HPO₄, 250 mmol sucrose, 190 μg/mL digiton; 150 μL per 100-mm dish) and incubated on ice for 5 minutes. The cells were spun for 5 minutes at 14,000 rpm at 4°C, and 1.5 μL of protease inhibitor (Protease Inhibitor Cocktail Set I, CalBiochem, San Diego, CA) was added. The supernatant was saved and designated as the cytosolic fraction. The pellet was resuspended in 80 μL of RIPA buffer (1% Nonide P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], in PBS) with protease inhibitor (1:100 dilution), incubated on ice for 30 minutes, and then spun for 15 minutes at 14,000 rpm at 4°C. The supernatant fraction, which contains mitochondrial proteins, was designated the “pellet fraction.”

- **Whole cell lysates.** Cells were collected using trypsin, washed two times with PBS, and then pelleted by centrifugation. The pellet was resuspended in RIPA buffer, incubated on ice for 30 minutes, and spun for 15 minutes at 14,000 rpm at 4°C to remove the insoluble material.

- **Western blot analysis.** Equal aliquots of whole-cell lysate protein (50 μg per lane) or cytosolic versus pellet fraction (20 μg protein per lane) were electrophoresed on 4%–13% SDS-polyacrylamide gradient gel or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore). The membranes were blotted using the primary antibodies and then with the appropriate secondary antibodies. The blotted proteins were visualized using the enhanced chemiluminescence detection system (Amersham), with prestained protein markers (BioRad) as size standards.

- **Mitochondrial membrane potential.** Mitochondrial membrane potential was assessed using DePsipher Assay kits, per the manufacturer’s instructions (R & D Systems, Minneapolis, MN). Briefly, cells were cultured on an eight-chamber slide (Lab-Tek Chamber Slide, Nal Nunc International, Naperville, IL). Subconfluent cells were treated with the LHS extract and then washed twice with PBS. The cells were further incubated with DePsipher solution (200 μL per chamber) at 37°C for 15 minutes and washed once with reaction buffer. The cells were observed immediately by fluorescence microscopy, using a Nikon UFX-II microscopy with bandpass filter for fluorescein and rhodamine. In healthy cells, the mitochondria appear red after aggregation of the DePsipher within the mitochondria. The red aggregates emit visible light at 590 nm. In dying cells or cells with disrupted mitochondrial membrane potential, the dye remains in its monomeric form in the cytoplasm and will appear green with an emission wavelength of 350 nm.

**RESULTS**

The antiproliferation effects of LHS were tested against seven cancer cell lines. LHS was effective in inhibiting the growth of all the cell
lines tested. The IC\textsubscript{50} (50% inhibition concentrations, milligrams of raw material per milliliter) were in the range of 8.0–40.0 when the drug exposure time was 48 hours (Table 1).

In the case of the C-33A human cervix carcinoma (Fig. 1) all cells underwent apoptosis and no living cells were remaining after exposure to the LHS extract (25 mg/mL) for a period of 48 hours. Results with other cell lines showed the similar dose-response sigmoidal mode. For the A-549 human lung cancer cells, 93% and 76% inhibition of growth of the cancer cells was seen after a 48-hour exposure period with the LHS extract at concentrations of 50 mg/mL and 25 mg/mL, respectively. However, in the case of the breast cancer cell lines, complete inhibition of growth of the proliferating cells was not observed at the highest concentration used. The highest inhibition was 68% in the case of the MDA-MB-453 cell line and 63% in the case of the MCF7 cell line with a 48-hour exposure period with the LHS extract (50 mg/mL). In the case of the prostate cancer cell lines, 100% cell death of the TsuPr1 cell line was observed after a 48-hour incubation with the LHS extract (50 mg/mL), while 85% and 91% growth inhibition was found for the DU 145 and the LNCaP prostate cancer cell lines, respectively. The inhibitory action of the herbal extract was also time-dependent. A significant decrease in activity was seen when the drug exposure time was shortened in the time-dependent antiproliferation assay against the C-33A human cervix carcinoma (Fig. 2).

Microscopic examination of the LNCaP and other cancer cell lines after treatment with LHS revealed morphologic changes that are typical of cells undergoing apoptosis. The changes included the induction of apoptosis of the cancer cells by LHS as demonstrated by the DNA ladder assay. DNA fragmentation became obvious after the cells had been exposed to LHS for 24 hours at a concentration of 100 mg of raw material per milliliter of the culture media.

**FIG. 1.** Antiproliferation against C-33A human cervix carcinoma by *Leonurus heterophyllus* Sweet (LHS). Approximately $5 \times 10^3$ cells per 200 µL of culture media were seeded into each well on a 96-well plate. The cells were cultured for 15–20 hours before the addition of the LHS extract (50 µL per well). The cells were then incubated with the extract for 48 hours until the control wells became confluent. The cell proliferation was then analyzed by the microtiter assay. Data are reported as mean ± standard deviation from 3 plates, total 18 bits of data per concentration.

**FIG. 2.** Time-dependent antiproliferation against C-33A human cervix carcinoma cells by *Leonurus heterophyllus* Sweet (LHS). Approximately $5 \times 10^3$ cells per 200 µL of culture media (48-hour drug exposure) and $6 \times 10^3$ cells per 200 µL of culture media (24-hour drug exposure) were seeded into each well on a 96-well plate. The cells were cultured for 15–20 hours before the addition of the LHS extract (50 µL per well). The cells were then incubated with the extract for 24 or 48 hours until the control wells became confluent. The cell proliferation was then analyzed by the microtiter assay. Data are reported as mean ± standard deviation from 3 plates, total 18 bits of data per concentration. *Significant difference ($p < 0.05$) from the corresponding 24-hour exposure data.

The involvement of mitochondria in LHS-induced apoptosis

To assess the function of mitochondria in LHS-induced apoptosis in LNCaP cells, the prostate cells were harvested after treatment with the herb extract and processed to separate
the cytosol from the particulate fraction (pellet), which contained the mitochondria. Both the cytosol and pellet fraction were examined for the presence of cytochrome c, Cox-4 and α-actin. Figure 3 shows that cytochrome c could be detected in the cytosolic fraction (supernatant) from the LHS extract-treated cells but not from the control cells. Cox-4 was not detected in the cytosolic fraction. In addition, α-actin was detected in both the pellet and the cytosolic fraction and the quantities of α-actin protein were not affected by the cell treatments.

Caspase activation in the LHS-treated cells

To assess the potential role of caspases in the LHS extract-induced apoptosis, whole cell lysates were prepared after the treatment of the cells with the herb extract. Caspase-3 activity in the whole-cell lysates was examined by Western blot assay on one of its major substrate, poly[ADP (ribose)] polymerase (PARP) and the metabolite. Figure 4 shows the cleavage of the full-length PARP (112 kDa) to generate the 85-kd cleaved PARP fragment, indicating the activation of caspase-3.

The effect of LHS treatment on mitochondrial depolarization

DePsipher dye was used to determine the depolarization of the inner mitochondrial membrane (loss of Δψ). Punctate extranuclear red staining indicates mitochondria with normal membrane potential, while cells with depolarized mitochondria show loss of the red fluorescence. The green fluorescence shows the cellular morphology. At 48 hours after the treatment with LHS extract, more than 75% of the cells showed mitochondrial depolarization (Fig. 5).

DISCUSSION

LHS has been implied in ancient Chinese medical texts to have anticancer activities. Our studies on the in vitro anticancer activities of the aqueous extract of LHS demonstrate that the extract was effective in inhibiting the growth of all seven cancer cell lines tested. The antiproliferation effect was found to be dose-dependent and also dependent on the drug exposure time. The antiproliferation activity of the extract was also dependent on the type of cancer cell line and it also varied among the cancer cell lines having the same type of organ origin. Among the cancer cells tested were three prostate cancer cell lines, DU 145, TsuPr1, and LN CaP. The former two are androgen-independent while LN CaP is androgen-depen-
dent. From the \( \text{IC}_{50} \) values it can be concluded that both androgen-dependent or -independent cell lines are sensitive to the herb extract. Microscopic examination of the cancer cells and the apoptotic DNA ladder assay demonstrated that LHS induced apoptosis of all the cancer cell lines tested.

Our studies also show that mitochondria is involved in the apoptotic activity of LHS. It is known that mitochondria undergo a number of profound changes early within the apoptotic program and appear to play a central role in apoptosis (Green et al., 1998; Kroemer et al., 1997). These mitochondrial changes include a loss of the inner transmembrane potential, which may lead to the release of cytochrome c (normally localized in the space between the outer and inner mitochondrial membranes, and is only released into the cytosol in the setting of apoptosis) or a temporally independent event (Bossy-Wetzel et al., 1998; Kluck et al., 1997) and disruption of electron transport and energy metabolism. Cytochrome c release has been implicated in the activation of caspase-3, linking mitochondrial events with caspase activation (Liu et al., 1996; Zou et al., 1997). Studies have further demonstrated the release of cytochrome c from the inner mitochondrial membrane space to the cytosol, may cause serious events such as the formation of a high molecular weight complex of cytochrome c/Apaf-1/procaspase-9, caspase-9 activation, caspase-3 activation, and cleavage of key cellular proteins such as PARP and lamins (Budimir, 1999).

In the present studies, cytochrome c release, mitochondrial membrane depolarization, and caspase-3 activation were all observed with the cells treated with the herb extract. These results strongly indicated the involvement of mitochondria in the apoptosis process. To confirm the integrity of the cell fractionation procedure, Cox 4 and \( \gamma \)-actin were also assayed. The absence of Cox 4, which is localized in the inner mitochondrial membrane, in the cytosolic fraction, indicates there was no contamination of the inner mitochondrial membrane in the cytosolic fraction. The similar quantities of \( \gamma \)-actin detected in both the pellet and the cytosolic fraction show the integrity of the fractionation process.

It is well known that one of the most important problems faced with anticancer drugs is their severe toxicity. This is where herbal medicine has a definite advantage over conventional chemotherapy. LHS has low toxicity; multiple and long-term oral doses have produced no toxic reactions. Intramuscular injection solution of the herb did not cause any side effects except dry mouth and shortened sleep. The LD\(_{50} \) value of the injection solution of the herb was 30–60 g/kg in mice by intravenous administration. The intravenous LD\(_{50} \) of the total alkaloids of the herb in mice was 572.2 \( \gamma \)
37.2 mg/kg. Daily intraperitoneal dose of 2 mg of leonurine (one of the major components) for 4 days did not produce any adverse effects in rats. In rabbits, daily subcutaneous administration of 30 mg/kg of leonurine for 2 weeks did not affect food intake, fecal and urinary excretions, and body weight. Male rats that consumed feed containing 50% of the powdered herb for 80 days did not exhibit toxic reactions nor was fertility affected (Zhu, 1998).

Considering the low toxicity of LHS and its apoptotic effect on cancer cells, it would be meaningful to further investigate this herb for potential herbal treatments for chemoprevention.

**CONCLUSIONS**

LHS was able to induce apoptosis of all the tumor cell lines tested. The antiproliferation effect was dose- and time-dependent. The mitochondrion was found to be involved in the apoptosis induced by the LHS extract. Mitochondrial depolarization-cytochrome c release-caspase-3 activation is one of the pathways for the apoptosis induced by LHS.

**REFERENCES**


Address correspondence to:
Jun Shao, Ph.D.
Department of Pharmacy and Administrative Sciences
College of Pharmacy and Allied Health Professions
St. John’s University
8000 Utopia Parkway
Jamaica, NY 11439

E-mail: shaoj@stjohns.edu