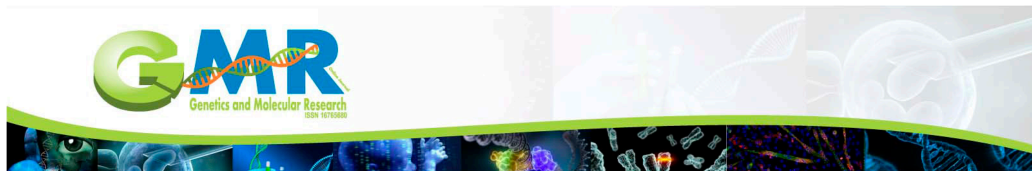

Effect of the extract from leaves of *Liquidambar formosana* Hance on S180 cells

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ABSTRACT. We examined the effects of the extract from leaves of *Liquidambar formosana* Hance on S180 cells and screened for antitumor active sites in the plant. Solvent extraction was conducted to prepare extracts from the leaves of *L. formosana* Hance and conduct preliminary separation, an MTT assay to determine the effect of leaf extract on the proliferation of S180 cells, and inverted microscopy to observe the effect of chloroform extract on the morphology of S180 cells. Double-staining (Annexin V/propidium iodide) with flow cytometry was conducted to determine the effect of the chloroform extract on S180 cell apoptosis. At some concentrations, the different extracts from the leaves of *L. formosana* Hance dose-dependently inhibited the proliferation of S180 cells. Among all extracts, the chloroform extract showed the strongest inhibitory effect on S180 cell proliferation. The IC_{50} values for the chloroform extract, ethyl acetate extract, *n*-butanol extract, and water layer were 0.238, 0.471, 0.844, and 0.411 mg/mL, respectively. We observed cell shrinkage, volume reduction, and varying sizes by inverted microscopy. Additionally, with increasing drug concentration, the number of cells decreased and debris

increased. The cells showed typical apoptotic morphological changes. The chloroform extract induced the apoptosis of S180 cells in a dose-dependent manner. Different extracts from the leaves of *L. formosana* Hance inhibited the proliferation of S180 cells, and the chloroform extract was the main antitumor component. This extract from the leaves of *L. formosana* Hance inhibited the proliferation of S180 cells in part by inducing apoptosis.

Key words: S180 cells; Leaves of *Liquidambar formosana* Hance; Active sites; Apoptosis

INTRODUCTION

Liquidambar formosana Hance is also known as maple, nienie, and acer buergerianum, among other names. It belongs to the Hamamelidaceae liquidambar deciduous trees and is common in warm, humid climates. This species is mainly distributed in the Qinling Mountains, Huaihe, south of the Pearl River Basin. All parts of liquidambar can be used as medicinal materials. The leaves are fragile, have a clear aroma when rubbed, and have weinxin and a slightly bitter flavor (Chen, 2000; Huang et al., 2000).

The leaves of *L. formosana* Hance are used as heat antidotes, to arrest diarrhea, and to treat dysentery, treat pain, heatstroke, postpartum wind, and pediatric tetanus neonatorium (Jiangshu New Medical College, 1977). The leaves of *L. formosana* Hance are a very safe and natural source of melanin, which is not only a pigment, but also important in medicine and health care. The leaves of *L. formosana* Hance are often used as a food coloring pigment material. Additionally, *L. formosana* Hance leaves can be boiled and mashed for use in the soaking of glutinous rice, which is black and shiny with good taste (Chen, 1998). Previous studies showed that the leaves of *L. formosana* Hance have many pharmacological activities; they can be used to treat diabetes and blood pressure and contain essential trace elements for humans (Xie et al., 2000; Huang and Song, 2001; Zhong et al., 2010). They also have antibacterial effects (Zheng et al., 2005; Zhong et al., 2007) and can inhibit the proliferation of human leukemia K562 cells (Xie et al., 2015) as well as improve non-specific immune function and cellular immune function in normal mice (Zhong et al., 2012). In this study, we evaluated the effects of different solvent extracts prepared from the leaves of *L. formosana* Hance on S180 cells to identify its anti-tumor active sites.

MATERIAL AND METHODS

Instruments

Continuous spectrum multi-function enzyme (Varioskan Flash, Thermo Fisher, Waltham, MA, USA), inverted phase contrast microscope (CKX 41, Olympus, Tokyo, Japan), flash extractor (HBE-50S, EYELA), rotary evaporator (N-1000v-W, Büchi, Flawil, Switzerland), atmospheric microwave/extraction reactor (WF-4000C, EYELA), flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA), clean bench (SW-CJ-2FD, Boxun, Shanghai, China), and CO_2 incubator (Forma 311, Thermo Fisher) were used in this study.

Materials

The murine sarcoma cell line S180 was obtained from the Institute of Medicine, Chinese Academy of Medical Sciences. Leaves of *L. formosana* Hance were collected at the Huangjin campus of Gannan Medical University of Jiangxi province in the month of June. Professor Li Jialin, from the Department of Pharmacy at Gannan Medical University, identified all leaves of *L. formosana* Hance. Ethanol (70%), aether petrolei, chloroform, acetic ether, and water-saturated butanol were purchased from Guoyao Corporation (Nanjing, China). Other materials included dimethyl sulfoxide (Sigma, St. Louis, MO, USA), DMEM (Gibco, Grand Island, NY, USA), fetal calf serum (Si Jiqing Co., Hangzhou, China), Annexin V/propidium iodide (PI, BD Biosciences), and 3-(4,5-dimethylthigal-2-yl)-2,5-(diphenyltetragalium) bromide (MTT, Sigma) were used.

Methods

Preparation of experimental drugs

First, 200 g of washed and dried leaves of *L. formosana* Hance was mashed and mixed with 70% ethanol twice for extraction. Each extraction was performed for 1 h. The filtrate together and decompre and recover alcohol extract, extract was mixed by water and extracted with petroleum ether, chloroform, ether acetate, and normal butanol in the same volume in turn. After evaporating the extraction and water layers, we obtained petroleum ether extract, chloroform extract, ethyl acetate extract, *n*-butyl alcohol extract, and water layer. This dry paste was dissolved in dimethyl sulfoxide and its volume fraction was less than 0.1%. Phosphate-buffered saline volumes were very low and could not be formulated as 100 mg/mL storage solution (solubility of the extract of petroleum ether extract is very low and cannot be formulated as the concentration required); therefore, it was not used in the experiment, and the samples were stored at 4°C. Samples were prepared to the required concentration when used.

Cell culture

S180 cells were inoculated into the abdominal cavity of Kunming mice; after seeing obvious ascites, we injected the cells into other mice. After stably passaging the cells for 3-4 generations, we obtained the ascites, centrifuged the washed S180 cells, and stained the cells with trypan blue. If more than 95% cells were S180 cells, we conducted the MTT experiments.

Effects of different extracts from leaves of L. formosana Hance on the proliferation of S180 cells by MTT

Cells in the logarithmic growth phase were adjusted to 1 x 10^5/mL in DMEM culture medium containing 10% fetal bovine serum and incubated in 96-well plates (180 μL per well). The samples were then placed at 37°C, 5% CO_2, and saturated humidity and incubated for 18 h. We divided the cells into a blank control group and drug experimental group, placing enough cell groups in 6 wells. We added 20 μL PBS to the blank control group and 20 μL

different concentrations (0.25, 0.5, 1, 2, 4, 8, and 16 mg/mL, all concentrations were based on preliminary test results) extract from leaves of *L. formosana* Hance in the drug experimental group, and then continued to cultivate the cells for 24 h. Inverted microscopy was used to record morphology; 20 μL MTT (5 mg/mL) was added to each well and the plate was incubated for 4 h and centrifuged at 1500 rpm/min for 10 min. The supernatant was removed and 150 μL dimethyl sulfoxide was added to each well, concussed, and mix evenly for 10 min, after which the absorbance of A (optical density, OD) was measured using a microplate spectrophotometer at 570 nm. The inhibitory rate and the median inhibition concentration of the drug to the tumor cells was calculated, and we evaluated the preliminary efficacy of the drug using the following formula: tumor cell inhibition rate (%) = (1- experimental group OD)/blank control group OD × 100%. LogC- inhibitory rate regression analysis was used to calculate the median inhibition concentration (IC_{50}).

Morphological observation

The chloroform extract of the leaves of *L. formosana* Hance was added to S180 cells. The processes of dividing the cells into groups, concentrations, and times were the same as in the MTT experiment. The growth and changes in cell morphology of S180 cells in the experimental and control groups were observed by inverted microscopy and photos were acquired.

Detection of S180 cell apoptosis induced by chloroform extract with Annexin V-fluorescein isothiocyanate/PI double-staining method

Cells in the logarithmic growth phase were adjusted to 2 x 10^5/mL and incubated in 6-well plates (1.8 mL per well), then placed in a 37°C, 5% CO_2, and saturated humidity for 18 h. We divided the cells into a solvent control group and drug experimental group, added 200 μL experimental drug (chloroform extract was 0.25 and 0.5 mg/mL) into the experimental group, added the same volume of PBS into the control group, incubated the cells for 24 h, and then harvested the cells. We blew cells into a single cell suspension. According to the kit instructions, using V-fluorescein isothiocyanate/PI double-staining and flow cytometry to detect cell apoptosis and calculated the percentage of total cells and apoptotic cells based on Annexin V-positive staining (AV+/PI-).

RESULTS

Effect of different extracts from liquidambar leaves on S180 cell proliferation

The results are shown Table 1; these cells were treated with the extracts for 24 h at different concentrations. Different extracts from the leaves of *L. formosana* Hance inhibited the proliferation of S180 cells in a dose-dependent manner. The inhibition rate of S180 cells and IC_{50} were chloroform extract > water layer > ethyl acetate extract > N-butanol extract, with chloroform extract showing the strongest inhibitory effect on S180 cells proliferation. Thus, the chloroform extract from the leaves of *L. formosana* Hance contained the main active components with antitumor activity, and therefore we selected chloroform extract for subsequent analysis.

Table 1. Inhibition rate of the proliferation of S180 cells and IC_{50} after treatment with different extracts from the leaves of *Liquidambar formosana* Hance after 24 h.

Group	Chloroform extract	Ethyl acetate extract	N-butanol extract	Water layer
Control	0	0	0	0
0.025 mg/mL	4.15 ± 2.53	-34.39 ± 8.74	4.96 ± 4.18	2.65 ± 4.06
0.05 mg/mL	15.34 ± 2.97	3.94 ± 4.75	8.41 ± 5.11	6.70 ± 3.13
0.1 mg/mL	29.38 ± 1.06	18.84 ± 3.14	13.66 ± 13.80	26.44 ± 9.69
0.2 mg/mL	51.07 ± 0.90	31.51 ± 2.32	25.19 ± 3.49	36.17 ± 0.07
0.4 mg/mL	78.08 ± 1.12	53.90 ± 1.35	37.40 ± 2.12	55.50 ± 0.19
0.8 mg/mL	81.50 ± 0.82	64.14 ± 2.34	49.32 ± 1.24	68.09 ± 0.31
1.6 mg/mL	82.18 ± 0.96	74.89 ± 1.58	60.37 ± 0.81	75.03 ± 0.59
IC_{50} mg/mL	0.238	0.471	0.844	0.411

Effect of chloroform extract from leaves of *L. formosana* Hance on morphology of S180 cells

As shown in Figure 1, S180 cells in the control group grew rapidly as clusters; the cells were large, round, and bright, and a large number of cells were actively dividing. The growth of S180 cells was inhibited by treatment with chloroform extract for 24 h. Cells were in the growth state of dispersion and shrinkage, and some cells shrunk and were different sizes. With increasing drug concentration, the number of cells decreased and cell debris increased. The cells showed typical apoptotic morphological changes (Figure 1).

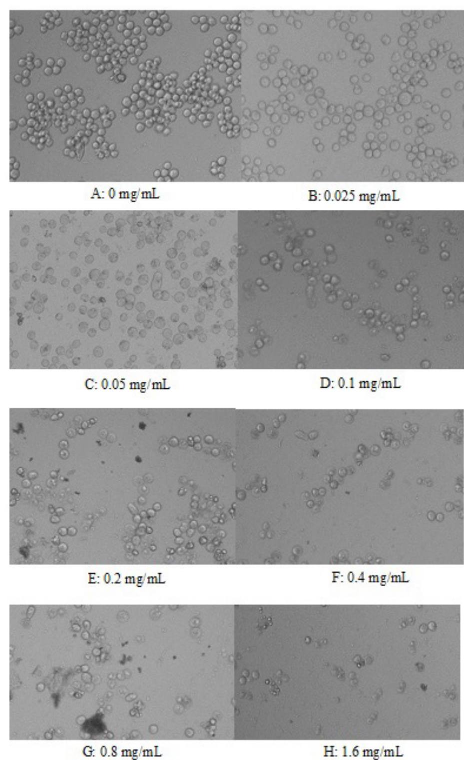

Figure 1. Effect of chloroform extract from leaves of *Liquidambar formosana* Hance on the morphology of S180 cells after 24 h (200X).

Effect of chloroform extract from leaves of *L. formosana* Hance on S180 cell apoptosis

As shown in Figure 2, we determined the effect of different concentrations of chloroform extract on S180 cells after 24 h. We detected the apoptosis rate by Annexin V/PI double-staining. The results showed that the apoptosis rate was significantly increased with increasing drug concentration. When the drug concentration was 0.025 mg/mL, the early apoptosis rate was 3.88%, while the late apoptosis rate was 3.86%. When the drug concentration was 0.05 mg/mL, the early apoptosis rate and late apoptosis rate were 4.05 and 4.21%, respectively. Compared with the control group, these differences were significant ($P < 0.05$).

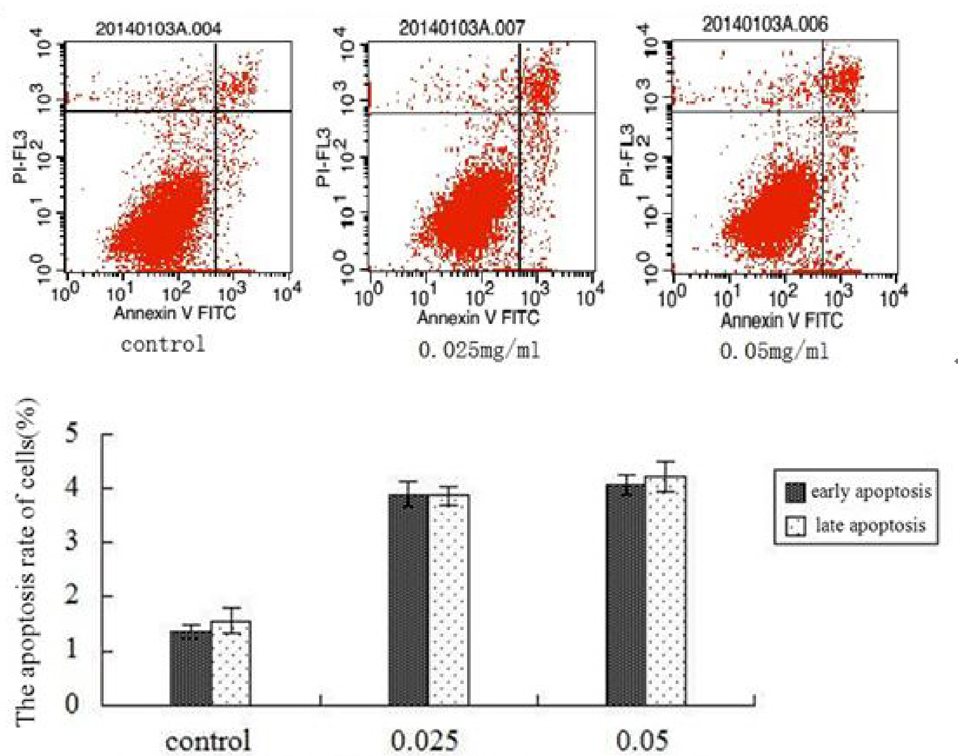

Figure 2. Effect of chloroform extract from leaves of *Liquidambar formosana* Hance on S180 cell apoptosis.

DISCUSSION

In recent years, Chinese herbal medicine and natural plants have played important roles in tumor therapy. Herbal medicine and natural plant medicine have relatively minor toxic side effects, unique antitumor effects, and good curative effects. They have received increased attention and have been examined for their ability to prevent and cure tumors and for the development of antitumor drugs (Wang et al., 2010).

The leaves of *L. formosana* Hance contain terpenoids, flavonoids, tannins, and volatile oil (Rong, 2003). Previous studies reported that terpenoids, flavonoids, and tannins have anti-tumor effects (Xu et al., 2008). The antitumor mechanism of terpenoids occurs mainly through the inhibition of tumor cell proliferation and the induction of tumor cell apoptosis, anti-angiogenesis, and anti-invasion (Lee et al., 2011). Flavonoids resist tumors by inhibiting tumor cell proliferation, inducing apoptosis, regulating the cell cycle, interfering with cell signal transduction, and inhibiting angiogenesis (Cao et al., 2004).

According to the principle of similarity law, chloroform can be used to extract terpenoids, while the ethyl acetate layer can extract alkaloids, flavonoids, and tannins. These substances inhibit the proliferation of tumor cells. However, the content and activity of the extract affect the antitumor effects of the extract. We found that at certain concentrations, the extract of chloroform, ethyl acetate, *n*-butanol, and water from the leaves of *L. formosana* Hance could inhibit the proliferation of S180 cells in a dose-dependent manner. The inhibition rate increased with increasing drug concentration, and the chloroform extract showed the strongest inhibitory effect on S180 cell proliferation, which was the principal antitumor active fraction. The chloroform extract from the leaves of *L. formosana* Hance was used to treat S180 cells for 24 h, and cell proliferation was shown to be inhibited. Cells were in the growth state of dispersion and shrinkage, and the cells shrunk and were different sizes. With increasing drug concentration, the number of cells decreased and cell debris increased. The cells showed typical apoptotic morphological changes. The apoptosis rate was detected by Annexin V/PI double-staining. The results showed that the apoptosis rate was significantly increased with increasing drug concentration. When the drug concentration was 0.025 mg/mL, the early apoptosis rate was 3.88% and the late apoptosis rate was 3.86%. When the drug concentration was 0.05 mg/mL, the early apoptosis rate and the late apoptosis rate were 4.05 and 4.21%, respectively. Compared to the control group, these differences were statistically significant ($P < 0.05$). These results demonstrate that the chloroform extract from the leaves of *L. formosana* Hance induced S180 cell apoptosis. One of the mechanisms of inhibition of the leaf extract of *L. formosana* Hance on S180 cell proliferation is to induce cell apoptosis.

The chemical constituents and antitumor mechanism of Chinese herbal medicine are very complex. Single components and mechanisms can play a role, but a variety of components and mechanisms are also involved. The effect of crude extracts from the leaves of *L. formosana* Hance on S180 cells was explored. The exact chemical composition and antitumor mechanism require further analysis.

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