Inhibitory activities of Lignum Sappan extractives on growth and growth-related signaling of tumor cells

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[ABSTRACT]
AIM: To investigate the active constituents of Lignum Sappan (Caesalpinia sappan L.) on growth-related signaling and cell mitosis.

METHOD: The influence of the ethyl acetate (EtOAc) extract of Lignum Sappan and its constituents on growth-related signaling were evaluated by a luciferase assay in cells stably transfected with NF-κB, STAT1, or STAT3 responsive luciferase reporter plasmid. The inhibitory effect on the cell cycle was determined by flow cytometric analysis. The anti-tumor activities were assessed in vitro and in vivo.

RESULTS: The EtOAc extract of Lignum Sappan had inhibitory activities on growth-related signaling and cell mitosis. Three major active compounds were sappanchalcone, brazilin, and butein. Sappanchalcone blocked cell cycle progression in the G2/M phase, brazilin inhibited TNFα/NF-κB signaling, while butein inhibited IL-6/STAT3 signaling, as well as TNFα/NF-κB signaling. The three compounds all demonstrated cytotoxic activities against human tumor cells in vitro. In a S180 tumor cell-bearing mice model, the anti-tumor efficacy of the EtOAc extract was better than the individual compounds acting alone.

CONCLUSION: These results indicate that Lignum Sappan contains multiple active compounds with different antitumor activities, which act synergistically to enhance their anti-tumor effects. The EtOAc extract of Lignum Sappan may be better than individual active constituent as a novel medicine for the treatment of cancer.

[KEY WORDS] Caesalpinia sappan L.; Lignum Sappan; Anti-tumor activity; Cell cycle blocker; Signaling pathway inhibitor; Synergy

[CLC Number] R965

Introduction
Lignum Sappan is derived from the heartwood of Caesalpinia sappan L. and is used as dyestuff or in traditional medicines. Studies of Lignum Sappan have revealed several pharmacological activities, including anti-inflammatory [1-2], anti-allergic [3], antifungal [4], antiviral [5], antioxidation [6-7], and vasorelaxant activities [8]. In addition, Lignum Sappan has also been used in traditional Eastern medicine as an anti-tumor agent. Kim et al. reported that the chloroform extract of Lignum Sappan induced cell death and growth inhibi-
signaling mechanisms. Once activated, NF-κB and STATs control the expression of anti-apoptotic, pro-proliferative, and immune response genes.

The extracts of Lignum Sappan have anti-inflammation and anti-tumor activities. Sappanchalcone and brazilin are anti-inflammation constituents in Lignum Sappan. It was found in these laboratories that the EtOAc extract of Lignum Sappan had inhibitory activities on growth-related signaling and mitosis. In this report, three active compounds (sappanchalcone, brazilin, and butein) have been identified from the EtOAc extract of Lignum Sappan, their inhibitory effects on cell growth and growth-related signaling evaluated, and the anti-tumor activities of the EtOAc extract and individual active compound in vitro and in vivo were examined.

Materials and Methods

Plant material

Lignum Sappan (Batch number: 101118) was purchased from Shanghai Yang-he-tang Chinese Herbal Medicine Co., Ltd and identified by LIU Jing-Li. A voucher specimen from Shanghai Institute of Materia Medica (AP0721).

Extraction and isolation

The dried heartwood was crushed into a powder, and the powder (100 g) was extracted with 95% EtOH (reflux, 1 h, × 3) to give an EtOH extract (13.05 g). The extract was partitioned between EtOAc and water to give an EtOAc-soluble fraction (9.269 g). The EtOAc-soluble fraction was subjected to silica gel (200−300 mesh, 310 g) column chromatography (eluent: petroleum ether: ethyl acetate, 4:3, 1.5 L) to give eighty three sub-fractions (Fr. A1 to B83). The combined Fr. B53 was subjected to silica gel (200−300 mesh, 60 g) column chromatography (eluent: petroleum ether: ethyl acetate, 4:3, 1.5 L) to give thirty three sub-fractions (Fr. B54 to B86). The combined Fr. B45−B53 was evaporated under reduced pressure to give a yellow compound, sappanchalcone (97%, 11.1 mg). Fr. 11−16 (361, 250 nm; 1H NMR (400 MHz, CD3OD), 7.18 (1H, d, J = 8.4 Hz), 6.47 (1H, dd, J = 8.4, 2.4 Hz), 6.29 (1H, d, J = 2.8 Hz), 6.70 (1H, s), 6.60 (1H, s), 3.68 (1H, d, J = 11.6 Hz), 3.92 (1H, dd, J = 11.2, 1.2 Hz), 3.96 (1H, s), 2.76 (1H, d, J = 15.6 Hz), 3.02 (1H, d, J = 15.6 Hz); 13C NMR (100 MHz, CD3OD) δ: 156.7 (C-4a), 154.5 (C-3), 144.5 (C-9), 141.1 (C-10), 136.2 (C-11a), 131.1 (C-1), 130.1 (C-7a), 114.3 (C-1a), 111.7 (C-8), 111.2 (C-11), 108.8 (C-2), 103.1 (C-4), 76.9 (C-6a), 69.6 (C-6), 49.8 (C-12), 39.2 (C-7).

Cell lines and reagents

Gifts of 293/NF-κB cells, HepG2/STAT1, and HepG2/STAT3 cells were provided by Prof. FU Xin-Yuan (National University of Singapore, Singapore). The 293/NF-κB cells were 293 cells stably transfected with a NF-κB responsive firefly luciferase reporter plasmid. HepG2/STAT1 or HepG2/STAT3 cells were HepG2 cells stably transfected with a STAT1 or STAT3-responsive firefly luciferase reporter plasmid. Human tumor cell lines HepG2, H522, and COLO 205 were obtained from the American Type Culture Collection. The murine sarcoma S180 cell line was purchased from Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China. TNFα, IFNγ, interleukin-6, DMSO, taxol (97%), butein (98%) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). The luciferase assay system was purchased from Promega (Madison, WI, USA).

Animals

Male Balb/c mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China. Animals were housed in a controlled conditions of temperature (23 °C ± 2 °C), humidity (50% ± 5%), and a 12 h light/dark cycle. The animals had free access to sterile food and water. All the protocols of the animal experiments were approved by the Ethics Committee of Shanghai Institute of Materia Medica, and the research complied with the Guide for the Care and Use of Laboratory Animals.

Luciferase assay

The 293/NF-κB cells (2 × 10⁴ per well) were seeded into 96-well cell culture microplates (Corning, New York, NY, USA) and allowed to grow for 48 h and then treated with samples for 1 h, followed by stimulation with 25 ng/mL TNF-α for 5.5 h. Luciferase activity was determined using the Promega luciferase kits according to the manufacturer’s instruction. HepG2/STAT1 or HepG2/STAT3 cells were stimulated with 100 U/mL IFNγ or 10 ng/mL interleukin-6. The cell number was counted at seeding and was controlled by equal seeding. All luciferase assay experiments were repeated at least three times to minimize the difference caused by cell number.

Flow cytometry assay

HGC-27 cells were incubated with samples for 24 h. The cells were harvested and washed with PBS, and re-suspended in ice-cold 75% ethanol (1 mL). After being left to stand...
overnight, cell pellets were collected by centrifugation, resuspended in PBS (500µL, 50 µg·mL⁻¹ RNase in PBS), and incubated at 37 °C for 30 min. Then propidium iodide solution (25 µL, 25µg·mL⁻¹) was added, and the mixture was allowed to stand in ice for 1 h. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry. Taxol was used as a positive control in this experiment.

**MTT assay**

About 5 000 cells of HepG2, H522, COLO 205, or S180 per well were seeded into 96-well plates. Twenty-four h later, cells were treated with vehicle control (DMSO) or samples for 72 h. The MTT assay was performed by adding MTT solution (20 µL, 5 mg·mL⁻¹) to each well and incubated for 3 h at 37 °C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO (150 mL). The absorbance was measured by a microplate reader at a wavelength of 570 nm. IC₅₀ values (50% concentration of inhibition) were determined through non-linear regression analysis.

**In vivo tumor model**

BALB/c mice were inoculated subcutaneously with 2 × 10⁶ S180 cells/0.2 mL PBS. After 24 h, the mice were randomly assigned to five groups (n = 8, excluding taxol group): (a) model, (b) taxol (10 mg·kg⁻¹), (c) brazilin (400 mg·kg⁻¹), (d) sappanchalcone (200 mg·kg⁻¹) and (e) EtOAc extract (200 µg·mL⁻¹). Each mouse received either vehicle or samples intraperitoneally every day for a total of seven doses. Mice were sacrificed eight days after cell inoculation, and the tumors were surgically removed for measurement.

**Statistical analysis**

Data are presented as T ± s. A one-way ANOVA determined whether the results had statistical significance. In some cases, Student's t test was used for comparing two groups. The level of statistical significance was set at P < 0.05.

**Results**

*Effects of the EtOAc extract on cell mitosis and signal transduction*

Lignum Sappan was first extracted with refluxing 95% EtOH. The EtOH extract was fractionated with EtOAc to yield EtOAc-soluble fraction. The content of brazilin and sappanchalcone in the EtOAc extract was 18.82% and 1.98% respectively by HPLC methods. A preliminary evaluation revealed that the EtOAc extract of Lignum Sappan had inhibitory activities on cell mitosis and growth-related signaling pathway. The EtOAc extract interfered with cell mitosis. The cell population was blocked at 56.62% in G₂/M phase 24 h after they were exposed to the EtOAc extract at 50 µg·mL⁻¹ (Fig. 2). The EtOAc extract also inhibited the IL-6-induced NF-κB-responsive promoter reporter gene in HepG2 cells with an IC₅₀ of (21.50 ± 0.18) µg·mL⁻¹ and (15.21 ± 0.16) µg·mL⁻¹, respectively. It did not obviously influence IFN-γ/STAT1 cell signal transduction at the same concentration (data not shown).

*Three active constituents of the EtOAc extract are identified*

In order to identify the active constituents of the EtOAc extract from Lignum Sappan, further isolation was performed. Two active compounds were isolated and identified as sappanchalcone and brazilin (Fig. 1) by comparing physicochemical and spectroscopic data with literature values [18-19]. Another active constituent, butein, was reported to be isolated from the EtOAc fraction of Lignum Sappan [20]. It was purchased from Sigma Aldrich, and its activities examined.

![Fig. 1 Structures of the active constituents identified from the EtOAc extract of Lignum Sappan](image)

*Sappanchalcone arrests cells at G₂/M phase*

Sappanchalcone induced gradual accumulation of rounded cells, which resemble the appearance of mitotic cells. The effects of sappanchalcone on cell cycle were therefore examined. Sappanchalcone (10 µg·mL⁻¹) induced G₂/M arrest (97% block) after 24 h of sample exposure (Fig. 2).

*Brazilin and butein inhibit cell signal transduction*

The active constituents from the EtOAc extract were then evaluated for activity on TNF-α/NF-κB and IL-6/STAT3 cell signal transductions. Among the isolated compounds, brazilin effectively inhibited the TNF-α-induced expression of the NF-κB-responsive promoter reporter gene in 293 cells with an IC₅₀ of (21.50 ± 0.18) µg·mL⁻¹ (Table 1). Butein effectively suppressed the IL-6-induced expression of the STAT3-responsive promoter reporter gene in HepG2 cells with an IC₅₀ of (5.47 ± 0.10) µg·mL⁻¹, as well as the TNF-α/NF-κB signal transduction (Table 1).

*Sappanchalcone, brazilin, and butein inhibit growth of human tumor cells*

The cytotoxic effects of sappanchalcone, brazilin, and

Fig. 2 Effects of the EtOAc extract and sappanchalcone on cell cycle distribution. HGC-27 cells were incubated with indicated concentration of samples for 24 h. The cells were then fixed and stained with PI, and analyzed by flow cytometry

Table 1 Effects of the EtOAc extract and compounds on signaling pathway and on growth of tumor cells (IC50 (X ± s))

<table>
<thead>
<tr>
<th>Sample</th>
<th>IL-6/STAT3</th>
<th>TNFα/NF-κB</th>
<th>S180</th>
<th>HepG2</th>
<th>H522</th>
<th>COLO 205</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc extract (μg·mL⁻¹)</td>
<td>6.50 ± 0.07</td>
<td>15.21 ± 0.16</td>
<td>12.22 ± 0.06</td>
<td>13.51 ± 0.12</td>
<td>12.78 ± 0.16</td>
<td>8.81 ± 0.07</td>
</tr>
<tr>
<td>Sappanchalcone (μg·mL⁻¹)</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>6.63 ± 0.05</td>
<td>0.91 ± 0.18</td>
<td>1.31 ± 0.16</td>
<td>21.76 ± 0.10</td>
</tr>
<tr>
<td>Brazilin (μg·mL⁻¹)</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>21.50 ± 0.18</td>
<td>11.91 ± 0.19</td>
<td>3.70 ± 0.09</td>
<td>6.47 ± 0.13</td>
</tr>
<tr>
<td>Butein (μg·mL⁻¹)</td>
<td>5.47 ± 0.10</td>
<td>10.17 ± 0.10</td>
<td>4.60 ± 0.16</td>
<td>1.78 ± 0.13</td>
<td>10.40 ± 0.14</td>
<td>3.95 ± 0.08</td>
</tr>
<tr>
<td>Taxol (μg·mL⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.13 ± 0.02</td>
<td>0.20 ± 0.03</td>
<td>2.00 ± 0.04</td>
<td>0.05 ± 0.03</td>
</tr>
</tbody>
</table>

N.D., not detected.

Brazilin and butein were examined using human tumor cell lines of different tissue origins, including the hepatocellular carcinoma cell line HepG2, the lung adenocarcinoma cell line H522, and the colorectal adenocarcinoma cell line COLO 205. The data indicated that the EtOAc extract showed similar cytotoxic effects on different tumor cells, but the three compounds exhibited differential antiproliferative activities (Table 1). Effects of the EtOAc extract and single compound on tumor growth in vivo

To test the antitumor effects in vivo, a mouse xenograft model bearing S180 mouse sarcoma cells was used. The EtOAc extract and its active constituents significantly inhibited S180 growth both in vitro (Table 1) and in vivo (Fig. 3). The in vivo tumor inhibition rate was 64.84% for the EtOAc extract, 39.22% for brazilin, and 57.61% for sappanchalcone respectively. The body weight of the mice was also decreased in the EtOAc extract and sappanchalcone treated groups (Fig. 3A). One animal died on the fourth day of the treatment of sappanchalcone. Taxol was used as the positive control. These data suggest the EtOAc extract has better antitumor effects than the individual active constituents from Lignum Sappan.
Fig. 3 Effects of the EtOAc extract and individual active constituents on S180 tumor cell growth in vivo. (A) The body weights of S180 bearing mice were measured after treatment with samples. (B) S180-bearing mice were sacrificed eight days after cell inoculation, and the tumors were surgically removed for measurement. ***P < 0.001, sappanchalcone, brazilin, or the EtOAc extract vs model group; ##P < 0.01, brazilin vs the EtOAc extract; (C) S180 tumor tissues

Discussion

Lignum Sappan has been used in traditional Chinese medicine to treat cancer. These studies found that the EtOAc extract of Lignum Sappan inhibited growth-related signaling and mitosis of tumor cells. The chemical constituents were isolated and their effects on cell cycle and cell signal transduction analyzed. Three active constituents from the EtOAc extract were identified: sappanchalcone blocked cell cycle progression in the G2/M phase, brazilin inhibited TNFα-induced NF-κB signal transduction, and butein inhibited the IL-6-induced STAT3-luciferase activities, as well as NF-κB and STAT3 play key roles in many physiological processes, such as innate and adaptive immune responses, cell proliferation, cell death, and inflammation. It has become clear that aberrant regulation of the NF-κB and STAT3 signaling pathways that control its activity are involved in cancer development and progression, as well as in resistance to chemo- and radiotherapy. [25-29]. NF-κB and STAT3 are a critical link between inflammation and cancer [14-15, 17]. Much effort has been put into strategies to inhibit NF-κB and STAT3 activation for the treatment of inflammation and cancer [29-32]. Therefore, brazilin and butein would be beneficial for immunotherapy of transplantation, autoimmune, and allergic diseases, as well as for cancer treatment.

In these studies, it was found that the anti-proliferative effects of EtOAc extract were as effective as that of sappanchalcone in vivo, but that sappanchalcone caused the death of mice. The anti-proliferative effect of brazilin was better than that of the extract in vitro, but the tumor inhibitory rate of brazilin was lower than that of the extract in vivo, because of the fast metabolism of brazilin (data not shown). It has been reported that the inhibition rate of butein on hepatocellular carcinoma is 53.90% after mice receive an intraperitoneal injection of 2 mg·kg⁻¹ butein for three consecutive weeks [24]. But the EtOAc extract contains much less than 1% butein. Therefore, the EtOAc extract is superior to the individual active constituents as an antitumor drug, considering both efficacy and safety. The extract contains many active constituents with different antitumor activities. These active constituents may synergistically enhance antitumor effects by targeting different proteins. Some other constituents in the extract may also influence the metabolism of individual antitumor constituents. Further studies are needed to systematically explore these possibilities. 

These results indicate that Lignum Sappan contains different types of compounds with distinct cytotoxic activities, and their molecular targets may be different.

Sappanchalcone or brazilin, as anti-inflammatory constituents from Lignum Sappan, could inhibit the growth of human tumor cells. In these studies, brazilin inhibited the TNFα/NF-κB signaling. Sappanchalcone, as a biosynthetic precursor of brazilin [21], did not show inhibitory effects on TNFα/NF-κB signal transduction. It has been reported that brazilin inhibits lipopolysaccharide-induced DNA binding activity of NF-κB and AP-1 in RAW 264.7 macrophage cells [22]. Butein is another active constituent identified from Lignum Sappan. It suppresses many protein activities. Such as NF-κB, cyclin D1, c-Myc, STAT3, ICAM-1, EGFR, and INK, etc. [23-24]. In the luciferase assay, butein inhibited IL-6/STAT3 and TNFα/NF-κB signal transduction. NF-κB and STAT3 play key roles in many physiological processes, such as innate and adaptive immune responses, cell proliferation, cell death, and inflammation. It has become clear that aberrant regulation of the NF-κB and STAT3 signaling pathways that control its activity are involved in cancer development and progression, as well as in resistance to chemo- and radiotherapy. [25-29]. NF-κB and STAT3 are a critical link between inflammation and cancer [14-15, 17]. Much effort has been put into strategies to inhibit NF-κB and STAT3 activation for the treatment of inflammation and cancer [28-32]. Therefore, brazilin and butein would be beneficial for immunotherapy of transplantation, autoimmune, and allergic diseases, as well as for cancer treatment.

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In summary, these findings provide evidence that sappanchalcone, brazilin, and butein isolated from Lignum Sappan heartwood inhibit the growth of human tumor cells. Sappanchalcone are cell cycle blockers, while brazilin and butein
are signal transduction inhibitors. The data also suggest that the EtOAc extract of Lignum Sappan may be better than the individual active constituent as a novel treatment for cancer.

References


