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Anti-tumor properties of anthocyanins from *Lonicera caerulea* 'Beilei' fruit on human hepatocellular carcinoma: *In vitro* and *in vivo* study

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ABSTRACT

In this study, the anthocyanin from *Lonicera caerulea* 'Beilei' fruit (ABL) was extracted and purified. The purified component (ABL-2) was then evaluated for its anti-tumor properties on human hepatoma cells (SMMC-7721) *in vitro* and the murine hepatoma cells (H22) *in vivo*. *In vitro*, ABL-2 not only significantly inhibited the growth of SMMC-7721 cells, but also remarkably blocked the cells' cycle in G2/M phase, inducing DNA damage and eventually leading to apoptosis. *In vivo*, ABL also killed tumor cells, inhibited tumor growth, and improved the survival status of H22 tumor-bearing mice. These effects were associated with an increase in the activities of antioxidase and a decrease in the level of lipid peroxidation, as evidenced by changes in SOD, GSH-Px, GSH, and MDA levels. In addition, ABL-2 also regulated the levels of immune cytokines including IL-2, IFN-γ, and TNF-α. These results revealed that ABL-2 exerts an effective anti-tumor effect by dynamically adjusting the REDOX balance and improving the immunoregulatory activity of H22 tumor-bearing mice. High performance liquid chromatography (HPLC) analysis revealed that cyanidin-3,5-diglucoside (8.16 mg/g), cyanidin-3-glucoside (387.60 mg/g), cyanidin-3-rutinoside (23.62 mg/g), and peonidin-3-glucoside (22.20 mg/g) were the main components in ABL-2, which may contribute to its anti-tumor activity.

1. Introduction

Hepatocellular carcinoma (HCC) represents a growing worldwide health crisis with rising incidence, limited effective therapies, and persistently poor prognosis. Five-year survival remains less than 20% despite decades of research [1]. Surgery and chemotherapy, the main therapeutic methods of currently treating patients with HCC [2], showed limited effects and often caused severe side effects [3]. Therefore, a great urgency exisits to develop effective therapeutic strategies to improve the current status of HCC therapy [4]. A number of studies have shown that active substances from food sources can inhibit the proliferation of cancer cells by inducing the apoptosis of cancer cells [5–8]. As a normal mode of cell death, apoptosis has become the main mean to inhibit the proliferation of cancer cells.

Blue honeysuckle (*Lonicera caerulea* L.) is a member of the Caprifoliaceae. It is traditionally used in folk medicine in China, Russia, and Japan, and recently gaining use as a healthy berry in Europe and North America with potential bioactive properties [9–12]. But it is less known as an edible fruit because of its bitterness and astringency [13]. Fruits of blue honeysuckle are not only rich in nutrients, such as vitamins, minerals, organic acids and so on, but also contain a number of anthocyanins and flavonols that potentially contribute to their color and human health-promoting properties [14–17], including protection against cancer [18,19] and ischemic heart disease [20], and other properties [10,21–24].

Lonicera caerulea 'Beilei' is the first approved cultivar of blue honeysuckle in China, and the area in cultivation of this cultivar in northeastern China continues to increase. Its fruit is bigger than wild

Abbreviations: HCC, hepatocellular carcinoma; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, glutathione; IL-2, interleukin-2; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor; bw/daily, body weight daily; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; FM, fluorescence microscopy; ABL, anthocyanins from 'Beilei' fruit; LAK, interferon (IFN) by inducing the production of lymphokine activated killer cells

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blue honeysuckle and orbicular-ovate. The flesh of the fruit is atropurpureus with thin, silvery-white, waxy layer of “fruit frost” or “bloom” on the epidermis. *Lonicera caerulea* 'Beilei' has no significant difference in anthocyanin content and antioxidant activity compared with wild varieties, but it has better taste and higher yield than wild varieties [25].

In cellular immunity, T lymphocytes also play an important role, where they exert many biological effects, such as modulating antibody production by B cells, responding to specific antigens and mitogens, and cytokine productions [26]. Cells can also be separated into two major functional subpopulations (Th1 and Th2 cells) according to the cytokines they secrete. Th1 cells produce cytokines (including IL-2, IFN-γ, and TNF-β) that activate T cells and macrophages to exert anti-tumor function [27]. IL-2 mainly induces the production of tumor necrosis factor (TNF) and interferon (IFN) by inducing the production of lymphokine-activated killer cells (LAK), thereby playing an important role in anti-tumor immunity [28]. TNF-α, produced by macrophages, can directly kill tumor cells and has no obvious toxicity to normal cells [29]. IFN-γ can inhibit the proliferation of tumor cells, alter the surface of tumor cells, and induce new antigen, so as to achieve anti-tumor effects [30]. According to the literature [31], IFN-γ and TNF-α exerted synergistically direct anti-tumor activity through apoptosis on some negative neuroblastoma (NB) cells.

At present, most research related to blue honeysuckle mainly focuses on the wild fruit as a raw material, although a few reports discuss the biological activity of L. *caerulea* 'Beilei'. In this study, ethanol extracts of L. *caerulea* ‘Beilei’ fruit were evaluated for their anti-tumor properties on SMMC-7721 cells *in vitro* and human tumor H22 cells *in vivo*. We also investigated the effects of the extracts on proliferation, viability, cycle, and apoptosis on cancer cells. Moreover, anti-tumor activity and the possible mechanism of the anthocyanin against transplanted H22 cells were also evaluated.

2. Materials and methods

2.1. Reagents

RPMI-1640 culture medium, DMEM culture medium, dimethyl sulfoxide, Annexin V-FITC / PI apoptosis detection kit (Beyotime Biotechnology Co., Ltd., China); fetal bovine serum (Hangzhou Sijiqing Biotechnology Materials Co., Ltd., China); trypsin, PI dye (Sigma, USA); MDA kit, SOD kit, GSH-Px kit, GSH kit, IL-2 serum test kit, IFN-γ serum test kit, TNF-α serum test kit, IL-2 tissue test kit, IFN-γ tissue test kit, TNF-α tissue test kit, protein test kit (Nanjing Jiancheng bioengineering institute, China); physiological saline (Tianjin Guangfu Fine Chemical Research Institute, China).

2.2. Instrument

TGL-16G high-speed centrifuge (Shanghai Anting Science Instrument Factory, China), DK-98-1 water bath (Tianjin Taisite Instrument Co., Ltd., China), Micro 21R high speed centrifuge (Thermo, USA), SW-CJ-1F ultra clean workbench (Shanghai Yiheng Technology Co., Ltd., China), PHS-3C Rayon Precision pH Meter (Shanghai Precision Instrument Co., Ltd., China) Uv-ViSEVO300 UV–vis spectrophotometer (Thermo, USA), XSP-SG-63XF Fluorescence Microscope (Shanghai Precision Instrument Factory, China), Multiskan FC Microplate Reader (Thermo, USA), Cube8 Flow Cytometer (Partec, Germany).

2.3. Frozen powder of ABL

Ultrasonic assisted counter current extraction has been used for ABL. The ultrasonic solution of 70% (v/v) ethanol, a solid-liquid ratio of 1:10 (g/v), and a 28 min extraction time at 30 ℃ and 120 W yielded 5.22 mg/g anthocyanin. The purification conditions were as follows: XDA-6 resin was the stationary phase medium and 70% ethanol served as the mobile phase; the column (10 cm × 150 cm) had a diameter/height ratio of 1:12, the sample volume was 1/5 of the column volume, and the loading concentration was 100 μg/mL. The elution and injection flow rates were 1 mL/min. We used 2 BV water to first wash away sugar and other impurities, and then a 3 BV 70% ethanol aqueous solution for gradient elution. Each 1000 mL of eluent was collected in one bottle, and then the same type of eluent was incorporated into one bottle by UV spectroscopy. Finally, four mixture groups were obtained (ABL-1, ABL-2, ABL-3 and ABL-4) and ABL-0 (7.74 ± 0.26%) was used to express the crude extract of anthocyanin. Resultant anthocyanin purity was ABL-1 (25.35 ± 0.23%), ABL-2 (42.91 ± 0.32%), ABL-3 (31.12 ± 0.32%) and ABL-4 (24.87 ± 0.26%), respectively. Samples were vacuum concentrated and freeze dried, with the frozen powder stored at -18℃ until used.

2.4. Cell culture

Human hepatoma cell line SMMC-7721 was purchased from Beijing Yinzijing Biomedical Technology Co., Ltd. Cells were cultured on RPMI-1640 medium containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 ℃ with 5% CO_2.

2.5. Cell proliferation activity

We selected SMMC-7721 cell growth in good condition to adjust the cell concentration for planking. After the cells were adhered, cell culture solution was aspirated, and 95 μL of fresh cell culture solution was added. Based on our preliminary experiment, 5 μL of anthocyanin components (ABL-0, ABL-1, ABL-2, ABL-3, and ABL-4) with different concentrations (0, 0.1, 0.2, 0.4, and 0.8 mg/mL) was added, respectively. After 48 h of incubation, MTT and DMSO were added in order and shaken until the crystals were completely dissolved; the substance was measured at 490 nm. Anti-proliferative activity was expressed as the 50% effective concentration (IC_{50} value), the low IC_{50} value means a high anti-proliferative effect.

$S = (1\text{-}OD_s/OD_0) \times 100\%$;

S: cell growth inhibition rate (%);
OD_0: absorbance value of the blank group;
ODs: absorbance of the sample group.

2.6. Transmission electron microscopy (TEM)

Based on the cells proliferation experiment, SMMC-7721 cells were treated with 0.2 mg/mL of ABL-0 and ABL-2 (referring to the IC_{50} value of ABL-2) for about 48 h respectively, and the cells were washed with PBS buffer so that the cells could be dispersed into cell suspensions. The cells were centrifuged and added to a 2.5% glutaraldehyde fixed solution 1 mL, fixed at 4 ℃ for 12 h, followed by an embedding treatment. Then the ultra-structure of the cells was observed under TEM.

2.7. Fluorescence microscopy (FM)

SMMC-7721 cells (1×10^6 cells/mL) were inoculated into 6-well plates in the logarithmic growth-phase. The experiment was divided into either the control group or ABL-0 and ABL-2 treatment groups (0.2 mg/mL); the processing time was 48 h and each group contained 3 parallel wells. Then the culture medium was discarded and the collected cells were carefully re-suspended and added into 100 μL AO/EB solution (100 g/mL) at 25 ℃. After 15 min of staining, the cells were redispersed into PBS, and observed quickly under fluorescence microscopy.