

# Antitumor Activity of Polysaccharides and Saponin Extracted from Sea Cucumber

Xiurong Su<sup>1\*</sup>, Caiyun Xu<sup>1</sup>, Yanyan Li<sup>1</sup>, Xiang Gao<sup>1</sup>, Yanru Lou<sup>2</sup> and Jinfeng Ding<sup>1</sup>

<sup>1</sup>Faculty of Life Science and Biotechnology, Ningbo University, Fenghua Road, Ningbo, Zhejiang Province 315211, P.R China

<sup>2</sup>Key Laboratory of Ningbo No.1 Hospistal, Liuting Street, Ningbo, Zhejiang Province 315010, P.R China

## Abstract

The density gradient of S180 cells were treated with various extracts at a concentration of polysaccharides (P1 and P2) and saponins (S1 and S2) measured after 22h, 44h, 68h and 92 h post-treatment. P1, P2, and saponin S1 were extracted from *Acaudina leucoprocta*, and S2 was extracted from the concentrated liquid of *Stichopus japonica*. The cytotoxic effects of S1 and S2 on S180 cells was demonstrated dose and time dependent, using MTT assay. Saponin S2 exhibited the highest potency against S180 cells, with an IC<sub>50</sub> of 41.04μg/ml at 44 h. Additionally, by Annexin V/PI staining, we observed more viable cells under polysaccharides treatment compared to the saponins. These results indicate that the *in vitro* anti-tumor effects of saponins S1 and S2 are more potent than those of polysaccharides P1 and P2.

**Keywords:** *Acaudina leucoprocta* and *Stichopus japonica*; Polysaccharide; Saponin; Anti-tumor activity

## Introduction

An ACE inhibitory peptide isolated from *Acaudina leucoprocta* was shown to exhibit anti-hypertensive effects in spontaneously hypertensive rats (SHR) when administered at a dosage of 3 mM/kg [1]. Moreover, proteins isolated from *A. leucoprocta* harbored hypolipidemic and antioxidative properties, which reduced oxidative damage caused by a diet rich in fat [2].

Previous studies reported that polysaccharides have anti-tumor, immuno-stimulatory, and anti-oxidative effects. Recently, the non-starch polysaccharides have emerged as an important class of bioactive natural products [3-6]. In many Asian countries, several pharmaceutical agents derived from polysaccharides have been extracted such as lentinan, schizophyllan and krestin [7,8]. Saponins are natural products discovered in sea cucumbers and sponges, and possess a variety of biological and pharmacological activities [9,10], including anti-tumor, anti-bacterial, anti-microbial, anti-fungal, anti-yeast, and anti-inflammatory activities [11].

In this study, we isolated and investigated the effects of polysaccharides and saponins isolated from *A. leucoprocta* on S180 cells, and test whether saponins S1 and S2 had differential *in vitro* anti-tumor activity. We also extract saponins from the concentrated liquid of boiled *S. japonica* and demonstrate that these saponins possess potent *in vitro* tumor suppressive activity towards HeLa, A-549 lung cancer, SGC-7901 stomach cancer, and Bel-7402 liver cancer cells. A previous study also indicated that saponins can inhibit tumor growth *in vivo* of mouse S180 solid tumors [12].

## Materials and Methods

### Materials

*A. leucoprocta* was obtained from the Sumiyama Island of Zhejiang Xiangshan (Ningbo, China). Concentrated liquid of boiled *S. japonica* were from Dalian BangChui Dao Seafood Enterprise Group Co., Ltd (Dalian, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT) was purchased from Sigma (St.Louis, MO, USA). Mouse ascites tumor cells lines (S180) was purchased from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China). RPMI Medium 1640 was purchased

from GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from Shanghai Lanji Co., Ltd. (Shanghai, China). Neutral protease (20 U/g) was purchased from Nanning Pangbo Biological Engineering Co., Ltd (Nanning, China).

### Extraction of polysaccharides and saponins

*A. leucoprocta* was digested with 2% neutral protease. Insoluble material was removed by filtration, and polysaccharide P1 from the digest were precipitated by adding 30% EtOH (v/v) of 95% at 4°C overnight, followed by centrifugation at 4500 rpm for 10 min. After filtration, the filtrate (I) and the precipitate (I) were separated. After sequential washing with ethanol, acetone, and ether, the precipitate (I) was dried in a vacuum to yield the crude polysaccharide P1, and denoted as P1. The filtrate (I) was then mixed with 60% EtOH (v/v) of 95% at 4°C overnight, followed by centrifugation at 4500 rpm for 10 min. After filtration, the filtrate (II) and the precipitate (II) were separated, and the precipitate (II) was sequentially washed with ethanol, acetone, and ether. The precipitate (II) was dried in a vacuum to yield the crude polysaccharide P2, and was denoted as P2 [13].

Filtrate (II) was concentrated to 1/5 of its original volume, washed twice with 50 ml of diethyl ether to remove the fat contents using a separatory funnel. It was further extracted three times using 150 ml of water-saturated butanol. The butanol solution was washed twice with 100 ml of distilled water to remove the impurities, thereby obtaining crude saponins. The remaining n-butanol solution was transferred to a tarred round bottom flask for evaporation using a rotary evaporator under vacuum at 55°C. The dry material obtained was saponin from *A. leucoprocta* and denoted as S1 [14].

**\*Corresponding author:** Xiurong Su, Faculty of Life Science and Biotechnology, Ningbo University, Fenghua Road, Ningbo City, Zhejiang Province 315211, P. R. China, Tel: 86-574-87608368; Fax: 86-574-87608368; E-mail: [suxiurong@gmail.com](mailto:suxiurong@gmail.com)

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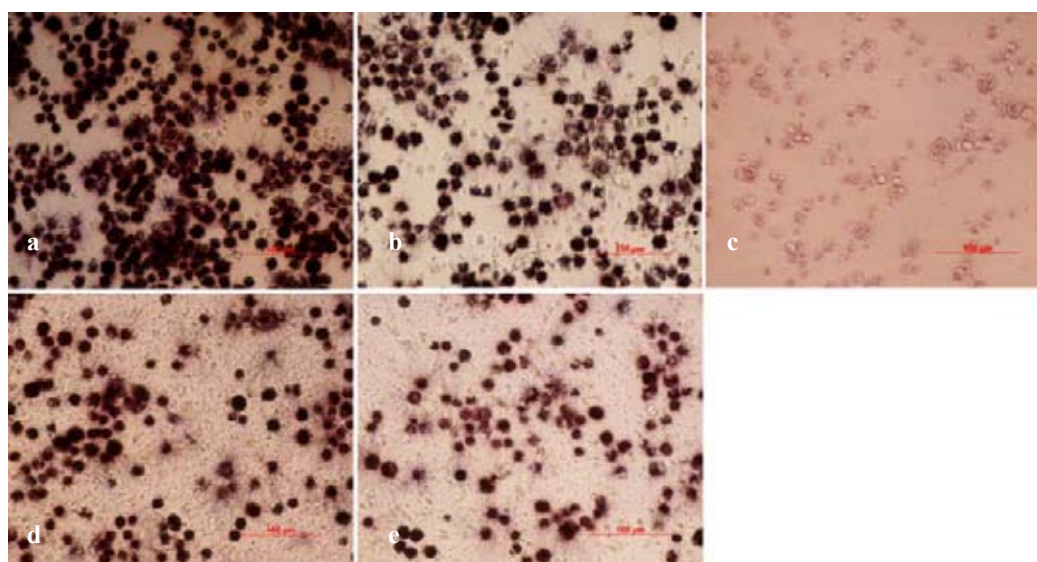
The concentrated liquid of boiled *S. japonicus* was centrifuged at 4500 rpm for 10 min and the precipitate was discarded. The filtrate was washed twice with 50 ml of diethyl ether to remove the fat contents using a separatory funnel, followed by extraction with 150 ml of water-saturated n-butanol. The extraction was performed three times and the water layer was discarded. The butanol solution was washed twice with 100 ml of distilled water to remove the impurities, thereby obtaining

V-FITC, and 10µl of propidium iodide. The mixture was incubated at room temperature for 5 min in the dark, and detected by fluorescence microscopy. Flow cytometry was used for quantification.

## Results

### Cytotoxic effects of S1, S2, P1 and P2 on S180 cells

In study, we used MTT assay to test the cytotoxic effects of S1, S2,



Note: a: control; b: the treatment of S180 with S1; c: the treatment of S180 with S2; d: the treatment of S180 with P1; e: the treatment of S180 with P2. Concentrations of P1, P2, S1 and S2 were 250 µg /ml. (As followed)

Figure 1: Images of S180 cells observed by invert fluorescence microscope of MTT assay.

crude saponins. The remaining butanol solution was transferred to a tarred round bottom flask for evaporation using a rotary evaporator under vacuum at 55°C. The dry material obtained was saponin of *S. japonicus*, and denoted as S2.

### Cell culture

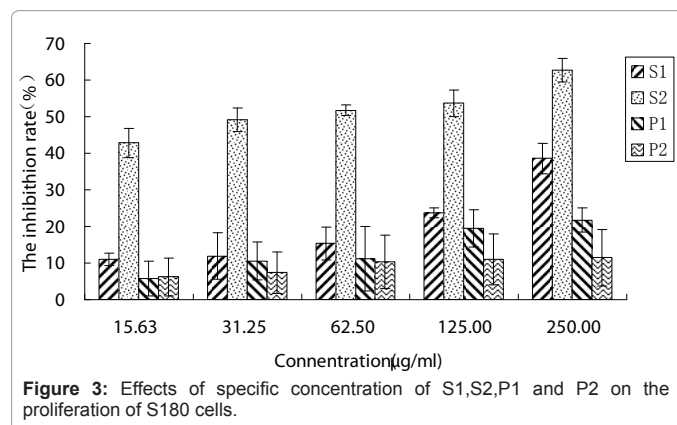
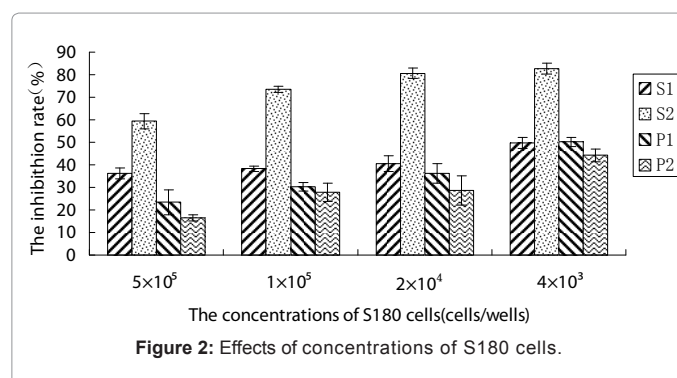
S180 cells were cultured in RPMI Medium 1640 with 10% fetal bovine serum, and 0.1% gentamicin sulfate in a 37°C humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were subcultured every 2 days, and harvested during the exponential growth phase.

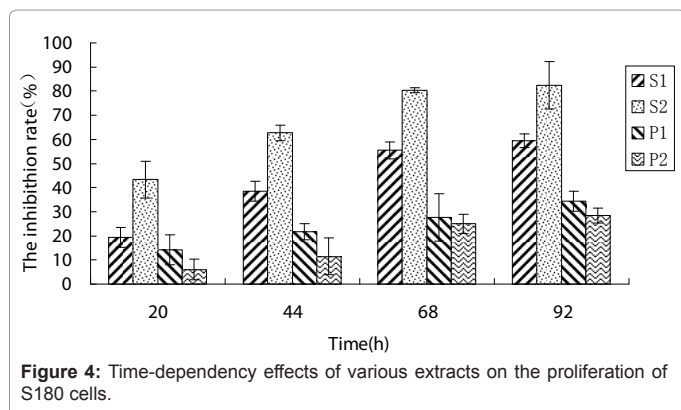
### Cytotoxicity assay

S180 cells were seeded in 96-well plates at a density of 5×10<sup>5</sup>cells/wells, 1×10<sup>5</sup> cells/well, 2×10<sup>4</sup>cells/well, and 4×10<sup>3</sup>cells/well. The cells were treated with various extracts at a concentration of 0µg/ml, 15.63µg/ml, 31.25µg/ml, 62.5µg/ml, 125µg/ml, or 250µg/ml and measured after 22h, 44h, 68h and 92 h post-treatment by MTT assay. Absorbance in control and treated wells was measured in an automated microplate reader at 550 nm [15]. The percentage of cytotoxicity on S180 cells at each sample were calculated using the following formula: the inhibition rate (%) = (OD<sub>550</sub> value of control without effector cells - OD<sub>550</sub> value of with effector cells)/OD<sub>550</sub> value of control without effector cells × 100%.

### Apoptosis assay

Cells were cultured at a density of 1-5×10<sup>5</sup> cells and treated with 250µg/ml of sample concentration for 68 h. Cells were washed twice with PBS, and resuspended in 250µl of 1× binding buffer, 5µl of annexin





**Figure 4:** Time-dependency effects of various extracts on the proliferation of S180 cells.

P1, and P2 on S180 cells. The MTT assay uses a colorimetric indicator for measuring cell proliferation and viability by reducing tetrazolium salt to formazan.

We studied the morphological effects of extract-treated S180 cells. As shown in Figure 1, S180 cells in the control treated group were bright and transparent, and exhibited an increase in cell proliferation. However, S180 cells treated with saponins S1 and S2 inhibited proliferation, and exhibited a fragmented phenotype. The P2- and H-treated S180 cells did not significantly effect their proliferation when compared to S1 and S2-treated S180 cells.

Apoptosis was observed when S180 cells were treated by S1, S2, P1, and P2 (250µg/ml) for 68h compared to control-treated cells (Figure 2). This observation was also seen in S1, P1 and P2-treated S180 cells, indicating that these extracts can induce apoptosis (Figure 2).

We also used the MTT assay to test the effects of using various concentrations of S1, S2, P1, and P2 on S180 cells. As shown in Figure 3, the S180 cells treated by 250µg/ml for 48 hrs S180 cells treated by the various extracts at 250µg/ml for 48 h were Cytotoxic using S1, S2, P1, and P2. This indicated that the cytotoxicity of S1, S2, P1, and P2 on S180

cells is concentration-dependent. In addition, 44 hrs post-treatment, the inhibition rate of S1, S2, P1, and P2 to S180 cells was 38.58 %, 62.68 %, 21.711%, and 1.46%, respectively. After 92 hrs of post-treatment, the inhibition rate of S1, S2, P1, and P2 to S180 cells was 59.49%, 82.42%, 34.27%, and 28.35%, respectively. These results indicate that treatment with the various extracts also had a time-dependent effect. We observed that S2-treated S180 cells showed the highest inhibition rate at 62.70%, in which the IC<sub>50</sub> value was 41.04µg/ml.

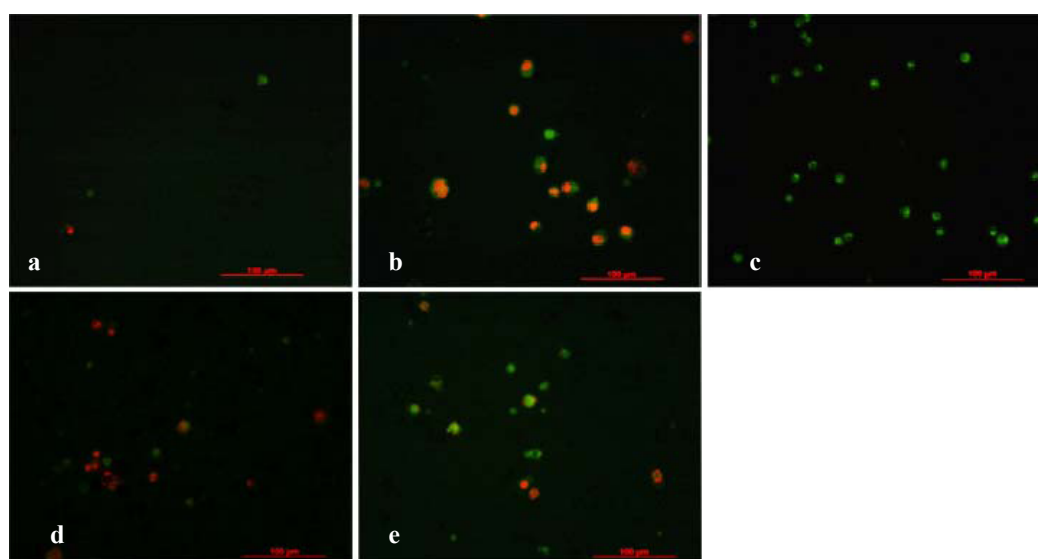
### Analysis of apoptosis by Annexin V/PI

To study whether S1, S2, P1, and P2-treated S180 cells undergo apoptosis, we stained for annexin V and PI 68 h post-treatment. Cells that have lost membrane integrity will show red staining (PI) throughout the nucleus and a green staining (FITC) around the cell surface (Figure 5). Using flow cytometry, we demonstrate that 95.6%, 1.8%, and 2.6% of S2-treated S180 cells were apoptotic, necrotic, and viable, respectively. Similarly, for S1-treated S180 cells, we observed that 73.2%, 21.1%, and 5.7% were apoptotic, necrotic, and viable, respectively. For P2-treated S180 cells, we observed that 12.2%, 2.5%, and 0.5% were apoptotic, necrotic, and viable, respectively. For P1-treated S180 cells, 10.6% were apoptotic, 14.5% were late apoptotic, 1.2% were necrotic, and 73.7% were viable (Figure 6).

### Discussion

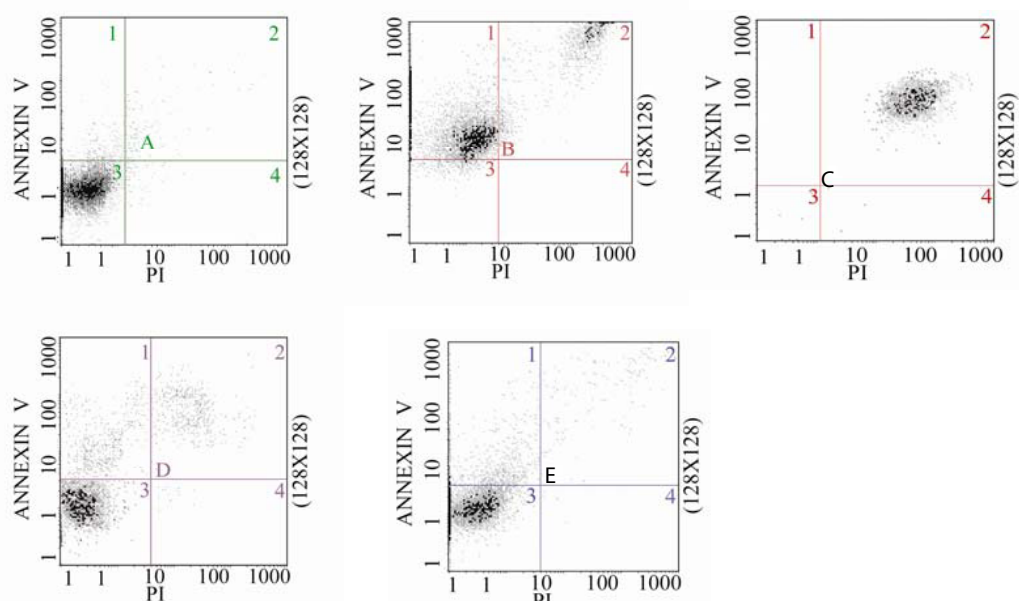
We demonstrate for the first time that polysaccharides and saponins can be extracted simultaneously from *A.leucoprocta*. Polysaccharides are generally extracted using an alcohol precipitation method, but extracting saponins is based on their physical properties of being soluble in diluted alcohol. Therefore, we were able to extract saponin S1 from filtrate with EtOH at 60% (v/v), which then allowed for precipitation of the polysaccharides. This extraction method not only achieves simultaneous extraction from *A.leucoprocta*, but also improves the extraction efficiency and reduces production costs.

The primary objective of this study is to assess the anti-tumor activity of polysaccharides and saponin isolated from *A. leucoprocta*,



Note: a: control group ; b: the treatment of S<sub>180</sub> with S1; c: the treatment of S<sub>180</sub> with S2; d: the treatment of S<sub>180</sub> with P1; e, the treatment of S<sub>180</sub> with P2.

**Figure 5:** Images of S180 cells observed by invert fluorescence microscope of annexin V/PI staining .



Note: 1: Early apoptotic cells; 2: Medium-term apoptotic cells ;3: Living cells; 4: Necrotic cell

Figure 6: Images of flow cytometry of S180 cells

and to study whether there is differential anti-tumor activity between S1 and S2-treated S180 cells.

Previous studies indicate that polysaccharides and saponins may have anti-tumor activity. A water-soluble polysaccharide, known as HPS-1, can significantly inhibit the proliferation of human hepatocellular carcinoma HEP-G2 cells and human gastric cancer MGC-803 cells *in vitro*, indicating HPS-1 could be a potential cancer therapeutic. Hu et al. [16] studied the inhibitory effects of *S. japonicus* acid mucopolysaccharide (SJAMP) in combination with cortisone on murine solid tumors and demonstrated that the treatment could inhibit the proliferation of a number of cancer cell lines. The study on anti-tumor activity of saponins sea cucumber began in 1990. Currently, more than 10 types of saponins have been isolated and purified. All types has been shown to have displayed significant anti-tumor activity on cancer cell lines including on murine leukemia, human gastric cancer, liver cancer, lung cancer, and renal cancer, oral epithelial cancer, nasopharyngeal carcinoma, melanoma, breast cancer, ovarian cancer, the former column adenocarcinoma, and HeLa cells [17-19] found three types of sea cucumber, *Intercedensides* A, B, and C, and observed that *Intercedensides* S2 possess *in vivo* cytotoxic activity against various tumor cells.

We demonstrate that the *in vitro* anti-tumor effect of saponins S1 and S2 were more potent than those of polysaccharides P1 and P2. However, further studies are needed to assess the anti-cancer properties of saponins S1 and S2 in an *in vivo* model.

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