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Anti-tumor activity of *Annona squamosa* seeds extract containing annonaceous acetogenin compounds

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ABSTRACT

Ethnopharmacological relevance: Seeds of *Annona squamosa* L. have been used in the south of China as a folk remedy to treat "malignant sores" (cancer).
Aim of the study: To investigate the chemical constituents and the anti-tumor activity of the standardized *A. squamosa* seeds extract *in vitro* and *in vivo*.
Materials and methods: Annonaceous acetogenin profiles of the standardized extract were determined by using Fourier transform infrared (FT-IR) and high performance liquid chromatography (HPLC) techniques. The anti-tumor activity of the extract was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity *in vitro* and H_{22} hepatoma cells transplantation tumor model *in vivo*.
Results: The FT-IR spectroscopy showed the presence of annonaceous acetogenin compounds in the extract. Two major annonaceous acetogenins: 12, 15-*cis*-squamostatin-A and bullatacin were identified and quantified by HPLC. The seed extract showed significant anti-tumor activity against four human tumor cell lines, especially for MCF-7 (IC_{50}. 0.25 μg/ml) and Hep G2 (IC_{50}. 0.36 μg/ml) cells *in vitro*. The extract inhibited the growth of H_{22} tumor cells in mice with a maximum inhibitory rate of 69.55% by oral administration.
Conclusion: A. *squamosa* seed extract showed significant anti-tumor activities against human hepatoma cells *in vitro* and *in vivo*, indicating a potential for developing the extract as a novel anti-liver cancer drug.

1. Introduction

Cancer is a major problem which still remains unresolved. Therapies developed along with the principles of modern medicine are often limited in their efficacy, carry the risk of adverse effects and are often very costly, especially for the developing world (Sabir et al., 2012). Botanical extracts have been used as a source of medicinal agents for thousands of years. The seeds extract of *Annona squamosa* was used in the south of China as a folk remedy to treat "malignant sores" (cancer) (Guangdong Food and Drug Administration, 2004). Recently, phytochemical and pharmacological studies on *A. squamosa* seeds have shown that the major bioactive compounds are annonaceous acetogenins, which have a strong antitumor activity (Bermejo et al., 2005; Liaw et al., 2010; Chen et al., 2011a; Li and Fu, 2004; Liu et al., 2007). Our previous work showed that the total content of annonaceous acetogenins was higher in the seeds of *A. squamosa* than in four other species (*A. glabra*, *A. muricata*, *A. reticulate* and *A. bullata*) (Yang et al., 2009a). However, few standardized seeds extracts of *A. squamosa* have been available so far as compared with those of other *Annona* species. For example, an encapsulated extract of *A. triloba* has been effectively used as a botanical supplement to treat certain cancers (McLaughlin, 2008).

In the present work, seeds extract of *A. squamosa* was prepared with a simple method. Annonaceous acetogenin profiles and anti-tumor activity of the extract were also investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

12,15-*cis*-squamostatin-A (Yang et al., 2009b) and bullatacin (Chen et al., 2011b) (Fig. 1) were isolated from the seeds of *Annona squamosa* L. in our laboratory. Both molecular structures were characterized based on spectroscopic analysis. Each compound was determined by HPLC analysis and confirmed by LC-MS, NMR spectroscopy. The purity of each compound reached above 98%. 5-Fluorouracil (5-Fu) and cyclophosphamide (CTX)

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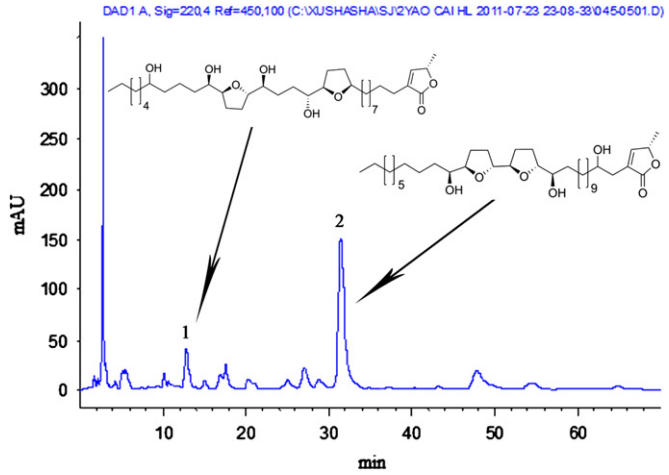

Fig. 1. Representative HPLC of annonaceous acetogenins in *A. squamosa* seeds extract. Peaks: (**1**) 12,15-*cis*-squamostatin-A and (**2**) bullatacin. The mobile phase was: 83% solvent A (methanol) and 17% solvent B (deionized water).

were used as anti-tumor agents *in vitro* and *in vivo*, respectively, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (Shanghai, China). HPLC grade methanol was purchased from Hanbang Science and Technology Company (Nanjing, China) and the deionized water was obtained from the Milli-Q Plus 185 system (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade.

2.2. Preparation of seeds extract

Seeds of *A. squamosa* were collected from Guangdong Province in October 2009 and identified by Prof. Jian-wei Chen (Nanjing University of Chinese Medicine, Jiangsu, China). The sample was authenticated and deposited at the herbarium of the Pharmaceutical College of Nanjing University of Chinese Medicine, Jiangsu (No. 083). Dried seeds (50 g, 40-mesh) were soaked in ethanol (1000 ml) for 3 days and filtered through filter paper. The residue was further extracted twice. The combined filtrates were concentrated using a rotary evaporator at 45 °C. The ethanol extract was dissolved in water and partitioned with equal volume of ethyl acetate twice. The ethyl acetate portion was concentrated using a rotary evaporator at 45 °C, giving a yield of 10–12%. The extract was diluted to a series of desired concentrations for the experiment.

2.3. FT-IR and HPLC analysis of annonaceous acetogenin compounds

FT-IR spectra were recorded from the dried seeds extract (2 mg) in KBr pellets on a NEXUS-470 FT-IR spectrophotometer in the range of 4000–500 cm^{-1}. The dried seeds extract was dissolved in HPLC grade methanol (3 mg/ml), filtered through a 0.45 μm membrane filter, and subjected to qualitative and quantitative analysis by using Agilent 1200 liquid chromatography system (Agilent technologies, CA, USA) consisting of double pump (G1312A), auto-sampler (ALS G1329A), diode-array detector (G1315A) and Agiletn LC 3D ChemStation Software (B.03.01). Chromatographic analysis was carried out under the conditions described previously (Yang et al., 2009a) with minor modifications. The column configuration consisted of an Agilent Zorbax Extend RP-C_{18} column (250 mm × 4.6 mm, 5 μm). Detection wavelength was set at 220 nm. The mobile phase consisted of methanol (83%) and deionized water (17%). The flow rate was 1.0 ml/min. The column temperature was maintained at 30 °C. The standard solutions of bullatacin and 12,15-*cis*-squamostatin-A were prepared with HPLC grade methanol. Working standard solutions were prepared by diluting standard solution with methanol to give six different concentrations in the range of 0.224–2.24 mg/ml (bullatacin) and 0.0524–0.524 mg/ml (12,15-*cis*-squamostatin-A) for calibration curves. The standard solutions were filtered through a 0.45 μm membrane prior to injection. Chromatographic peaks were confirmed by comparing their retention times with the reference standards and by DAD spectra (200–600 nm). Quantification was performed by peak integration using the external standard method. The calibration curve for bullatacin was: $y = 4.66 \times 10^3 c + 56.97$ ($R^2 = 0.9995$) and 12,15-*cis*-squamostatin-A: $y = 5.891 \times 10^3 c - 24.71$ ($R^2 = 0.9993$). All chromatography was performed at room temperature and in triplicates. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.4. Cell lines and animals

A-549 (human lung carcinoma), Hela (human cervix carcinoma), MCF-7 (human breast carcinoma) and HepG2 (human hepatoma carcinoma) cell lines were provided by the Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica and cultured with DMEM medium (HyClone Laboratories Inc., Logan, UT) containing 10% fetal calf serum (Sijiqing, Hangzhou, China) and penicillin and streptomycin 100 U/ml, at 37 °C with 5% CO_2. Male Kunming mice [SCXK (Shanghai) 2007-0005] weighting 18–22 g were purchased from Slac laboratory animal Co. Ltd. (Shanghai, China). All the procedures were approved by Animal Ethical Council of Nanjing University of Chinese Medicine. The mice were maintained under laboratory conditions at 25 °C under a normal 12 h/12 h light/dark cycle with humidity of 55% and fed with food and water *ad libitum*. The mice were allowed, at least, 5 days to adapt to the laboratory environment before experiments.

2.5. In vitro anti-tumor activity

Five-day *in vitro* MTT cytotoxicity tests (Mosmann, 1983; Scudiero et al., 1988) against human tumor cell lines were carried out at the Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, using A-549, Hela, MCF-7 and HepG2 cell lines, with 5-Fu as a positive control. Briefly, cells were incubated in 96-well plates (1×10^4cells/well) containing 100 μl of the culture medium. After overnight growth, cells were treated with various concentrations of the seeds extract for 5 days. The cells were harvested and washed with PBS and incubated with 20 μl MTT (5 mg/ml) for 4 h, and finally, 150 μl of DMSO were added to each well. The maximum absorbance was detected at 570 nm by an ELISA plate reader (Model680, BIO-RAD, USA). The inhibition rate was calculated and IC_{50} values were calculated by Probit analysis (Finney, 1980).

2.6. In vivo anti-tumor activity

At least 10 mice were used for each sample and each dose. Mean values and S.D. were determined by standard methods (Suzuki et al., 1986). The significant difference was estimated by the standard student t-test.

Anti-tumor effect on H_{22} was observed in normal male Kunming mice. The test was performed by observing the effect on the growth of the tumor as described previously (Qin et al., 2004). A dose of 0.2 ml of aseptic H_{22} cells (about 1.0×10^7/ml) was implanted subcutaneously at the right groin of mice. 24 h