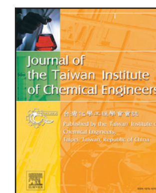
Journal of the Taiwan Institute of Chemical Engineers 66 (2016) 27–32

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice



In vitro and *in vivo* antitumor effects of the extract of *Sapindus* spp.

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ARTICLE INFO

Article history:
Received 21 May 2016
Accepted 12 June 2016
Available online 28 June 2016

Keywords:
Sapindus
Water extraction
Fraction
Inhibition
Breast cancer

ABSTRACT

This study evaluated the antitumor effect of some fractions extracted from *Sapindus* spp. on breast cancer. Four water-extracted fractions (I, II, III, IV) were isolated from the pericarp of *Sapindus* spp. using X-5 and AB-8 macroporous resin columns and MCI gel column. The *in vitro* effect was determined using the MTT assay. Our results indicated that fractions I-IV did not exhibit significant cytotoxic effects towards the normal cell MDA-kb2; Fraction II and IV inhibited the growth of MCF-7 (estrogen receptor positive), MDA-MB-231 (estrogen receptor negative), and MDA-MB-435 s human breast cancer cell lines; of these, MDA-MB-435 s cell line was the most sensitive, and the IC_{50} of Fraction II and IV against this cell line were 33.11 and 30.06 μg/mL, respectively. In the *in vivo* experiment, Fraction II and IV showed a strong antitumor effect at a dose of 162.5 mg/kg/d, with inhibition rate of 40.43% and 38.77%. Fraction II and IV also induced apoptosis of tumor cells, activations of Bax (Fraction II) and inhibition of Bcl-2 (Fraction II and IV). Our results underscore the pharmacological potential of *Sapindus* spp. extracts.

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1. Introduction

Sapindus is a natural nonionic surface-active agent extracted from the fruit pericarp of trees of the genus *Sapindus* (also known as soapnut trees) and is rich in saponins [1]. The *Compendium of Materia Medica* reported that rinsing hair with *Sapindus* spp. extract could treat recurrent headache and that sapindus has whitening and freckle-removing ability when used as a face wash [2]. *Sapindus* spp. extract has been used in Chinese traditional culture for rinsing the hair and body [3]. One of the major saponins contained in *Sapindus* spp. Extract is the triterpenoid oleanolic acid, which has been reported to have potent biological activity, including anti-bacterial, anti-inflammation, and anti-tumor activity [4]. The compound has an intermediate or strong growth-inhibitory effect on human breast cancer, liver cancer, lung cancer and gastric cancer [5–8]. Moreover, it can effectively inhibit the spread of tumors and induce cell apoptosis.

Therefore, in the present study, four *Sapindus* spp. fractions were isolated from the water-soluble extract of *Sapindus* spp. Moreover, the ingredients with inhibitory activity on breast cancer cells were screened *in vitro*. The human breast cancer cell lines used in this study included the estrogen receptor-positive (ER^+) MCF-7, estrogen receptor-negative (ER^-) MDA-MB-231, and MDA-MB-435 s. *In vivo* experiments were conducted to validate the *in vitro* anti-tumor activity of the fractions. It is expected that our study will provide experimental evidence for future studies on the biological activity of saponins extracted from *Sapindus* spp.

2. Material and methods

2.1. Materials

The concentrated *Sapindus* spp. extract was provided by Fujian Yuanhua Forestry Biology Co. Ltd. The degree of purity of the saponins in the extract was 44.63%.

Female SPF BALB/c mice aged 6–8 weeks and weighing 20 ± 2 g were purchased from Shanghai Slaccas Laboratory Animal Co. Ltd.

The reagents ethyl acetate, 95% ethanol, and glacial acetic acid were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. AB-8 and X-5 macroporous adsorption resins were purchased from the Chemical Plant of Nankai University, in Tianjin. MCI-gel^{TM} CHP-20P columns were purchased from Mitsubishi Kasei Co. GF254 silica gel plates were purchased from Yantai Zhi Fu Chemical Co. Ltd. RPMI-1640 cell culture media and fetal bovine serum were purchased from GIBCO Inc. Penicillin, streptomycin, and trypsin were purchased from Sangon Biotech (Shanghai) Co., Ltd. The reagent 3–[4,5–dimethylthiazol–2–yl]–2,5 diphenyl tetrazolium bromide (MTT) was purchased from Aladdin Industrial Corporation. Normal human breast cell line MDA-kb2, human breast cancer cell lines MCF-7, MDA-MB-231 and

Abbreviations: MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan; IC_{50}, 50% inhibitory concentration; SPF, specific pathogen free; PBS, phosphate buffered saline; CTX, cyclophosphamide; DMSO, dimethylsulphoxide; HE, hematoxylin and eosin.

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http://dx.doi.org/10.1016/j.jtice.2016.06.010
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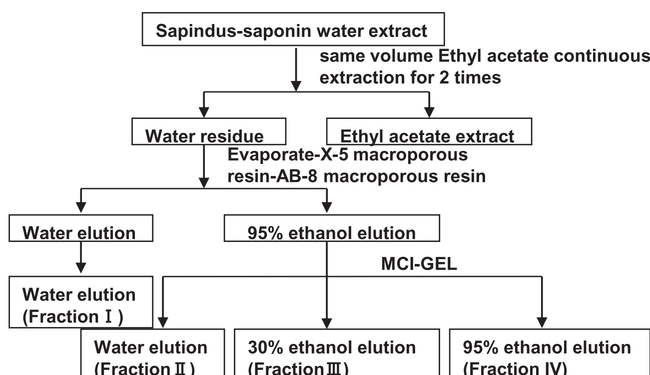

Fig. 1. Isolation and purification of Sapindus spp. extract fractions.

MDA-MB-435 s were provided by Fudan University Cell Bank (Shanghai, China).

The experimental instruments used in this study included a falling-film evaporator (Changshu Pharmaceutical Machinery Factory Ltd.) at a flow rate of 50–120 kg/h, PL2002 electronic scale (Mettler Toledo Instruments (Shanghai) Co. Ltd.), adjustable pipettes (Thermo Labsystems), SHB-III water-circulating vacuum pump (Zhengzhou Great Wall Science and Trade Co. Ltd.), R-501 rotary evaporator (Shanghai Shin Biological Technology Co. Ltd.), LS-B 50-L sterilized pots (Shanghai Medical Nuclear Instrument Factory), Forma^{TM} Series 3111 CO_2 cell culture incubator (Thermo Scientific, USA), Multiskan MK3 automated enzyme immunoassay analyzer (Thermo Scientific), CKX41 inverted microscope (Olympus), low-speed, large capacity, benchtop centrifuge (Wuxi Ruijiang Co., Ltd.), 96-well plates and cell culture flasks (Corning Inc.), and a hemocymeter (Shanghai Qiujing Biochemical Reagent and Instrument Co., Ltd.).

2.2. Methods

2.2.1. Isolation and purification of fractions in Sapindus spp. extract

The modified workflow for the isolation and purification of the extract fractions is shown in Fig. 1 [9]. Briefly, ethyl acetate (100 L) was used to remove impurities from the extract, and AB-8 (10 cm × 150 cm) and X-5 (5 cm × 150 cm) macroporous adsorption resins were used to separate the *Sapindus* spp. fractions. Water (50 L) and 95% ethanol (50 L) were used for elution. Fraction I was obtained by drying the solution eluted with water. The fraction eluted with 95% ethanol was applied onto a MCI gel column for further separation. Ethanol solutions of different concentrations were used for elution. Fraction II was obtained by drying the fraction eluted with water (7 L); Fraction III was obtained by drying the fraction eluted with 30% ethanol (5 L); and Fraction IV was obtained by drying the fraction eluted with 95% ethanol.

2.2.2. In vitro *anti-tumor effects of fractions isolated from* Sapindus *spp. extract*

MCF-7, MDA-MB-231, MDA-MB-435 s and MDA-kb2 cells were grown until the logarithmic growth phase and harvested. The cell concentration was adjusted to 1×10^5 cells/mL. A 100-µL aliquot of the cell suspension was transferred to each well (sterile PBS was added to the outer wells as control). The cells were incubated at 5% CO_2 and 37°C for 24 h and the extract fractions were added. The final concentrations of the fractions were 200, 100, 50, 25 and 12.5 µg/mL. A blank control and negative control (without the extract fractions) were also included. The reaction volume in each well was 100 µL. Triple duplicates were performed for each concentration. After a 24-h incubation at 5% CO_2 and 37 °C, the cells were analyzed under an inverted microscope. A volume of 20 µL of MTT solution (5 mg/mL, for a final concentration of 0.5%) was added to each well and the cells were cultured for another 4 h. Subsequently, the medium was discarded and 150 µL of dimethyl sulfoxide was added to each well. The plate was shaken for 10 min to solubilize the crystals [9]. The optical density was detected at a wavelength of 570 nm using an enzyme immunoassay analyzer. The inhibitory rate of the drug for tumor cells was calculated.

Inhibition Rate (%) = Absorbance of sample (treated cells)/Absorbance of correspondingcontrol (untreated cells)

2.2.3. In vivo *anti-tumor effects of* Sapindus *spp. extract fractions*

2.2.3.1. Animal model. We used 4T1 cells in the logarithmic growth phase. When the number of viable cells reached 95–100% using the trypan blue (0.4%) exclusion method, the cells were detached from the flask wall by gently pipetting. The cell suspension was washed twice with serum-free media and counted under a microscope. The cell concentration was adjusted to 1×10^7 cells/mL using serum-free culture media (RPMI-1640). The tumor cell suspension was harvested using a syringe and inoculated in the right axillary breast tissue of BALB/c mice. Each mouse was inoculated with 0.1 mL of the suspension (1×10^6 cells). Subcutaneous tumors could be palpated approximately 7 days later, which suggested the success of this tumor model in mice. Random grouping began when the diameter of the tumors reached approximately 2–3 mm.

2.2.3.2. Sample grouping and drug administration. Fifty mice were randomly divided into five groups, with 10 mice in each group, as follows: negative control group, 0.2 mL of distilled water per day; positive control (cyclophosphamide) group, 0.2 mL of cyclophosphamide at 120 mg/kg/d; experimental groups that received Fraction II, III and IV, 0.2 mL of each corresponding fraction at a dose of 162.5 mg/kg/d. The drugs were administered by the intragastric route for 21 days.

2.2.3.3. Analysis of tumor growth.

(1) Tumor growth
After the development of the tumors, the long and short diameters of the tumors were measured using a Vernier caliper every two days. Tumor volume was calculated according to the formula: tumor volume = 0.5 × long diameter × short diameter^2 and the tumor growth curve was plotted.

(2) Changes in body and tumor weight and calculation of the tumor inhibition rate
After the development of the tumors, the animals were weighed every two days using an electronic scale. Subsequently, the animals were euthanized, and the tumors were removed, dried in filter paper, and weighed.
The average weight of the tumors and the tumor inhibition rate were calculated as follows:

$$\text{Tumor inhibition rate} = \frac{\text{Average tumor weight of the control group} - \text{Average tumor weight of the treatment group}}{\text{Average tumor weight of the control group}} \times 100\%$$

(3) Count of metastatic foci in the lungs
The histological examination of the tumors and organs indicated the presence of visible metastatic foci in the lungs. The lung tissues of the mice were isolated and fixed for 24 h using a solution of saturated picric acid, formic acid and glacial

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