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Antitumor effects and chemical compositions of *Eupolyphaga sinensis* Walker ethanol extract

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ABSTRACT

Ethnopharmacological relevance: Eupolyphaga sinensis Walker popularly known as "preferred drug to regulate blood flow" are traditionally used in folk medicine in the treatment of ecchymoma, posttraumatic wound, hepatic fibrosis and tumor.
Aim of the study: To characterize chemical compositions and to evaluate the antitumor and immunomodulatory of *Eupolyphaga sinensis* Walker ethanol extract (ESEE) in hepatocarcinoma H_{22} bearing mice.
Materials and methods: ESEE was obtained by ethanol reflux extraction and analyzed by gas chromatography–mass spectrometry (GC–MS) after methylation. ICR mice were treated with ESEE for 14 consecutive days at doses of 31 mg/kg (low-dose), 62 mg/kg (mid-dose) and 124 mg/kg (high-dose) after H_{22} tumor cells were implanted. At the end of the experiments, the tumor weight of each mouse was measured. Levels of serum TNF-α and IFN-γ was assayed by ELISA. Protein expressions of Bax, Bcl-2 and caspases-3 were detected by immunohistochemistry.
Results: Chemical analysis revealed the presence of 6 components that account for 97.55% of fatty acids, indicating the occurrence of saturated and polyunsaturated fatty acids. Oral administration of ESEE could inhibit tumor growth, promote Th1 type cytokine productions (TNF-α and IFN-γ) and induce apoptosis of hepatocarcinoma via increase of Bax/Bcl-2 ratio and activation of caspases-3. Oral administration of ESEE in a dosage of 6.2 g/kg did not lead to toxic effects in mice.
Conclusions: ESEE was effective in inhibiting tumor growth in vivo and could also serve as immunoadjuvant for tumor therapy.

1. Introduction

Insects are one of the largest resources of living organisms and have been used by human beings for a long time as food, medicine and biochemical materials. In long term practice, medicinal insects and their products have been used to treat many different diseases. In particular, it as an essential part of Chinese medicine has been used to cure cardiovascular, metabolic and neoplastic disease for thousands of years (Tsuneo et al., 1988; Dossey, 2010).

Eupolyphaga sinensis Walker belonging to the family *Corydiidae* (*Blattodea*) is the main species bred and used in traditional Chinese medicine (TCM) as a basic component of anticoagulant and vulnerary medicines. Recently it has been used as tonic and spice for its special flavor and healthy effects in Southeast Asia, such as China, Thailand, India and Malaysia (Zhang et al., 2008; Feng et al., 2009). In folk medical practices, it could promote blood circulation by removing blood stasis (Ahn et al., 2000; Qian and Wang, 2009; Yang et al., 2011) and enhance immune response (Tang et al., 2010). According to TCM theories, tumor is mainly due to the blood stasis in the organs. It is believed by TCM physicians that the drug, which can improve blood circulation and remove blood stasis, is useful for the treatment of tumor. Therefore, *Eupolyphaga sinensis* Walker is also used to inhibit tumor growth and has potential in treating leukemia, gastric carcinoma and hepatocarcinoma. Some reports showed that *Eupolyphaga sinensis* Walker, one of the main components in Dahuang zhechong Pill, inhibited proliferation of vascular smooth muscle cells by depressing PDGF expression and alleviated CCl_4-induced hepatic fibrosis by down-regulating p38 and ERK phosphorylation (Zhang et al., 2009; Cai et al., 2010). However, no studies evaluating potential roles of *Eupolyphaga sinensis* Walker have been made yet. In the present study, to verify the ethnomedical claim, ethanol extract of *Eupolyphaga sinensis* Walker

Abbreviations: ESEE, *Eupolyphaga sinensis* Walker ethanol extract; Fu, fluorouracil; NC, normal control; MC, model control, H22 tumor bearing mice; MC + Fu, H22 tumor bearing mice treated with fluorouracil; MC + ESEE, H22 tumor bearing mice treated with ESEE; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 antagonist X.

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were tested for its antitumor effects in hepatocarcinoma H_{22} bearing mice. Fatty acid compositions of the extract were also analyzed by GC–MS.

2. Materials and methods

2.1. Preparation of ESEE

The raw material used in the study was commercially available dry matter, which were purchased from Zhejiang Chinese crude drug Co., and identified by Associate Professor Bing Yu, College of Pharmaceutical Science, Zhejiang Chinese Medical University, China. The specimen (No. 20090926) was preserved at chemical laboratory, College of Pharmaceutical Science, Zhejiang Chinese Medical University, China. The raw material was soaked in 95% ethanol for 30 min and then refluxed in 10 volumes of 95% ethanol (v/w) for 1 h and extracted twice. The suspension was then centrifuged (3000 rpm, 20 min) and the supernatant was decompressed and dried in vacuum condition until a yellow oily liquid was achieved with the yield 12.9%.

2.2. Preparation of fatty acid methyl esters (FAMEs)

By preliminary chemical screening in standard procedures, the sample present orange by sudan 3 staining method while other chemical test failed to response positive results, indicating that the extract mainly consisted of fatty acids. Therefore, the oily liquids were converted to corresponding FAMEs by methylation reacted with MeOH containing 10% (w/v) KOH as previously described (Basconcillo and McCarry, 2008). FAMEs were extracted three times using hexane (1:1, v/v, 15 mL). The organic layers were combined, dried by anhydrous sodium sulphate to remove the moisture and stored in a refrigerator until analysis.

2.3. GC–MS analysis

Analyses were performed on a HP 5973A MSD (full scan mode) equipped with HP 6890 Series II GC, a cool on-column injector and a CP-Wax 52 CB column (30 m × 0.32 mm × 0.5 μm) using helium as the carrier gas with the flow rate of 0.8 mL/min. The oven temperature was held at 50 °C for 3 min, then programmed at 4 °C/min to 220 °C and held at 220 °C for 10 min. The injector and detector temperatures were both 260 °C. Electron impact ionization (EI^+, 70 eV) in the scan range of 50–550 m/z was used for all samples. The FAMEs sample (0.1 mL) was dissolved in 4.9 mL of methanol. All injection volumes of sample solutions were 10 μL. Qualitative analysis was based on the comparison of retention times and the computer mass spectra libraries using Wiley GC–MS Library. The percentage composition was computed from the GC peak areas.

2.4. Preparation of model

Male ICR mice, weighing 20–24 g, were purchased from Animal Experimental Center, Zhejiang Chinese Medical University, China. Experiments reported in this study were carried out in accordance with local guidelines for the care of laboratory animals and approved by the ethics committee for research on laboratory animal use of the institution.

The murine tumors H_{22} (5×10^6) cells were diluted with 0.9% saline to 1×10^6 cells. Each mouse was injected 0.2 mL tumor cell suspensions in the right flank subcutaneously as reported before (Sreelatha et al., 2011). Twenty four hours after inoculation, mice were divided randomly into five groups orally receiving water, fluorouracil (Fu, 20 mg/kg) and ESEE extract (31, 62 and 124 mg/kg), respectively. All the drugs were orally administered once daily, sustaining until the end of the experiment, while the normal control group was treated with the vehicle (water). On the 14th day, diets were removed from the cages 12 h before the mice were sacrificed and tumors were peeled off and weighed after washing with PBS.

2.5. Biochemical analysis

Blood samples were collected and centrifuged at 3500 × g for 15 min to obtain serum. Levels of IFN-γ and TNF-α in serum were determined using commercially available ELISA kits (Boster Bioengineering Institute, Wuhan, China) and levels of ALT in serum were determined using colorimetric kits (Nanjing Jiancheng Bioengineering Institute, China).

2.6. Apoptosis assay

Tumors cells were gently resuspended, collected by centrifugation, washed twice with PBS and then resuspended in 500 μL binding buffer. Five microlitres of Annexin-FITC and 5 μL of PI solution were added, and then the cells were incubated in the dark at room temperature for 15 min. Annexin V-FITC/PI apoptosis detection kit (KeyGEN, Nanjing, China) was used for apoptosis detection. Cell apoptosis was analyzed on a FACScan flow cytometry (Becton Dickinson, USA). Annexin V-positive, PI-negative cells were scored as early apoptotic, and double-stained cells were considered as late apoptotic.

2.7. Immunohistochemistry analysis

Protein expressions of Bax, Bcl-2 and caspase-3 were detected using corresponding immunohistochemistry detection kit and the enhanced kit (Boster Bioengineering Institute, Wuhan, China) following the manufacturer's instructions. The positive cells showed yellow or brown articles or clumps in the cytoplasm with a blue colored cellular nucleus. Using Image pro plus software version 6.0 (Media Cybernetics, USA), the optical density values were measured under 400× objective lens of each slice for semiquantitative analysis.

2.8. Acute toxicity

This protocol was performed according to the Organization for Economic Cooperation and Development (OECD) Test Guidelines (OECD, 2008). After overnight fasting, groups of ten mice were administered ESEE extract in graded doses up to 6.2 g/kg, while the normal control group received only water. Animals were observed for general behavioral and body weight changes, hazardous symptoms and mortality for 14 days after treatment.

2.9. Statistical analysis

All parameters were recorded for individuals within all groups. All data were shown as mean ± SD. Statistical comparisons of data were carried out using the ANOVA and t-test of the SPSS 18.0 system. A value of $P < 0.05$ was considered significant.

3. Results and discussion

The oily extract contained the significant amount of saturated and unsaturated fatty acids. A total of 6 compounds were identified, representing 97.55% of the total oil as shown in Table 1. The major constituents were palmitic acid (21.70%), cis-9-oleic acid (40.78%), cis-9,12-linoleic acid (21.86%), cis-9-palmitoleate (9.86%), cis-9,12,15-linolenate (1.69%) and myristate (1.67%). Since oleic and polyunsaturated fatty acids produced antiinflammatory, antitumor and cardioprotective effects (Ito et al., 1982; Gil, 2002),