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ORIGINAL ARTICLE

Separation and purification and in vitro anti-proliferative activity of leukemia cell K562 of *Galium aparine* L. petroleum ether phase

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Abstract To explore material basis of in vitro anti-proliferative activity of leukemia cell K562 of petroleum ether phase of product resulting from *Galium aparine* L. 60% ethanol extraction, the experiment adopts column chromatography combined with thin layer preparation, isolates and purifies petroleum ether, conducts structural identification of obtained single compound and applies MTT method for viability assay of in vitro anti-proliferative activity of leukemia cell K562. Experimental results show that *G. aparine* L. petroleum ether contains mainly β-sitosterol, daucosterol and dibutyl phthalate and other substances. Under experimental conditions, the three could inhibit the proliferation of leukemia cell K562 with dose-effect and time-effect relationship, of which dibutyl phthalate has strongest activity. Dibutyl phthalate with excellent activity, β-sitosterol with rich content and moderate effect should be the main contributor to its biological activity.

1. Introduction

Galium aparine L. is a common weed in wheat and barleyfields, also known as *Rubia cordifolia*, *Scutellaria tuberifera*, *G. aparine*, catchweed bedstraw herb. As herbaceous plant of gallium of rubiaceae, it was first recorded in *Yunnan Materia* as Chinese herbal medicine. Traditional medicine holds that *G. aparine* L. can clear away damp-heat, eliminate stasis, dissipate detumescence and detoxify, which can be used for treatment of stranguria with trubid urine, hematuria, traumatic injury, acute appendicitis, furuncle, otitis media, etc. In modern clinical medicine, it is also used for treatment of cancer, especially leukemia (Yang and Yang, 1975). Among these traditional Chinese medicine prescription and Chinese medicinal formulae for treatment of tumors, *G. aparine* L. serves as principal drug, which indicates that it has ingredients to eliminate evil and can suppress tumor cells. However, its material basis for antitumor activity has not been mentioned

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in current domestic and international research (He and Zheng, 1994; Health Center in Sandian District, 1972; Chen, 2002; Peng et al., 2015; Wang, 2005; Zhao and Zhao, 2006; Li and Chen, 2004; Ling, 2009; Bojko, 1995; Kirillov et al., 2002).

We once adopted ethanol of different concentrations to extract catchweed bedstraw herb. Activity tracking results show that petroleum ether phase of material extracted by 60% ethanol can well inhibit leukemia cell viability. In this paper, column chromatography combined with thin layer preparation and recrystallization method is adopted to separate monomer compound, and in vitro anti-proliferative activity of leukemia cell K562 detection of the resultant monomer compound is done in order to find antitumor activity substances and lay a foundation for further development and utilization.

2. Experimental method

2.1. Materials, equipment and reagents

G. aparine L. whole plant which was collected from Maozhuang, Zhengzhou, in May 2009, and identified by Associate Professor Yang Huaixia of Henan University of Traditional Chinese Medicine as herb *G. aparine* L. of *G. aparine* of rubiaceae.

Flash EA1112 element analyzer from US Thermo Electron SPA company; Nexus470 intelligent Fourier transform infrared spectrometer from US Nicolet company; Avance-300 superconducting NMR spectrometer, Avance-400 superconducting NMR spectrometer, from Germany Bruker; Agilent 1100 LC-MSD-Trap-XCT, from US Agilent company; carbon dioxide incubator from Shanghai Yiheng company; clean bench from Suzhou purification equipment plant; BIO-RAD680 microplate reader from US Bio-Rad company; micro-injector from Germany eppendorf company; high-speed centrifuge from US sigma company; culture plate from US costar company.

MTT from US merck; RPMI1640 medium from Gibco Company; fetal bovine serum from Chinese Academy of Medical Sciences; other reagents are domestic analytical pure.

2.2. Methods

2.2.1. Extraction

Weigh 200 g dried crushed *G. aparine* L. whole plant, conduct 2 h reflux extraction of 1000 mL of 60% ethanol, extract twice, filtrate with vacuum concentration and obtain extract, 3 kg materials are used and 624.5 g extract is obtained.

2.2.2. Extraction

Disperse extract with appropriate amount of distilled water, extract with petroleum ether, conduct vacuum concentration, evaporate petroleum ether and obtain 20.8 g petroleum ether phase.

2.2.3. Preparation

For obtained petroleum ether phase, use petroleum ether: ethyl acetate as eluent for column chromatography, conduct gradient elution, test sample with thin layer analysis board, conduct color detection with UV fluorescence combined with sulfuric acid – methanol and acetic anhydride – concentrated sulfuric acid (Liebermann–Burchard reaction); for eluted components, use petroleum ether: ethyl acetate, petroleum ether: chloroform and chloroform: methanol as the developing solvents, repeatedly prepared with silica gel GF254 preparative thin layer, 3 single compounds are obtained, with concrete results shown in Table 1.

2.2.4. Anticancer activity assay (MTT method)

Formulate different monomer compounds into DMSO solution with concentration at 0.1 mol/L, draw 10 μL and dilute with medium to 1 mL and obtain liquid to be measured. Take K562 cells of logarithmic growth phase to be inoculated in three 96-well plates with density at $1 \times 10^5/cm^2$, each well with 100 μL. Add 4, 8, 12, 16, and 20 μL soup into each well of sample set, and supplement volume to 200 μL with medium. Blank group (only added with culture solution) and control group (without drug) are set, with six parallel for each group. After cultured for 48 h, 36 h and 72 h, add MTT, culture for 4 h, centrifuge, discard supernatant, and add 150 μL DMSO to each well to fully dissolve the crystals. Measure absorbance at 570 nm wavelength with microplate reader (Bio-Rad 3350 microplate reader). With blank zero, perform parallel experiments twice and take average value. Calculate cell viability with the following formula:

$$\text{cell viability} = (\text{absorbance of experimental group} / \text{absorbance of control group}) \times 100\%.$$

3. Results and analysis

3.1. Structural analysis of compound

3.1.1. Compound I (β-sitosterol)

As white powder solid, it is positive in sulfuric acid – methanol and acetic anhydride – concentrated sulfuric acid reaction, and negative in Molish reaction, which indicates that the compound is a steroid or triterpenoids aglycone; ESI-MS shows molecular ion peak m/Z: 413 $[MH]^-$, elemental analysis result: C element content is 83.92%, H elemental content is 12.06%. Combined with NMR data, compound formula is speculated as $C_{29}H_{50}O$. Calculation obtains that degree of unsaturation is 5, which indicates that there may be double bond and ring in compound structure. In infrared IR (cm^{-1}) spectrum: stretching vibration absorption peak at 3425 cm^{-1} shows the presence of hydroxyl, methyl signal at 2960 cm^{-1} and 2866 cm^{-1}, and methylene signal at 2935 cm^{-1} and 2851 cm^{-1}; absorption peak relatively weak at 1641 confirms the presence of double bond.

Nuclear magnetic resonance hydrogen spectrum data indicate the presence of continuous peak envelope at δ2.27–1.03, which may be because of steroid nucleus backbone and side-chain hydrogen information generated due to overlap of numerous methylene and sub-methylene signal on steroid skeleton. δ5.34 and δ3.51 prove the existence of a unsaturated CH and hydrogen on one carbon monoxide; nuclear magnetic resonance spectroscopy only shows two C chemical shift information in unsaturated carbon atom region (except alkyne carbon atoms): 140.7 ppm, 121.7 ppm, which should be ene carbon signal; C NMR data show a total of 29 carbon in compounds. According to dept, it can be seen that there are a total of 6-methyl, 3 quaternary carbon, 11 methylene, 9 methine.