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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Available online 24 April 2016

**KEYWORDS**

*Galium aparine* L.; Purification; K562; MTT

**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.

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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 turbid 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
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 Huaxia 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 (Bio-Rad 3350 microplate reader). With blank zero, perform parallel experiments twice and take average value. Calculate cell viability with the following formula:

cell viability = (absorbance of experimental group /absorbance of control group) × 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 C29H50O. Calculation obtains that degree of unsaturation is 5, which indicates that there may be double bond and ring in compound structure. In infrared 1R (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 62.27–1.03, 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. δ3.34 and δ3.51 prove the existence of an 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 alkyn carbon atoms): 140.7 ppm, 121.7 ppm, which should be one 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.
Specific NMR data are as follows:

\[ ^1H \text{ NMR (300 MHz, CDCl}_3 \] = 5.34 (1H, t, \( J = 5.2 \) Hz, H-6), 3.51 (1H, m, H-3), 0.68 (3H, s, CH\(_3\)-18). 13C NMR (75 MHz, CDCl\(_3\)) \[ \delta: 140.7 \text{ (C-5), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.8 (C-24), 42.3 (C-13), 42.2 (C-4), 39.8 (C-12), 37.3 (C-1), 36.5 (C-10), 36.2 (C-20), 33.9 (C-22), 31.9 (C-7), 31.9 (C-8), 31.6 (C-2), 29.1 (C-25), 28.3 (C-16), 26.1 (C-23), 24.3 (C-15), 23.1(C-28), 21.1 (C-11), 19.5 (C-19), 19.4 (C-26), 19.1 (C-27), 18.9 (C-21), 12.0 (C-29), 11.8 (C-18). After consulting the literature, it is found that the aforesaid data are basically consistent with \( \beta \)-sitosterol data reported in the literature (Ewa et al., 2013). Therefore, compound I is identified as \( \beta \)-sitosterol.

3.1.2. Compound II (daucosterol)

As white powder solid, it is positive in Molish reaction and shown fuchsia after heated in 10% sulfuric acid–methanol solution. ESI-MS shows molecular ion peak \( m/z: 575 \) [MH] \(+\), elemental analyzer test results: C element content is 72.83%, H element content is 10.39%. Combined with NMR data, it is speculated that the compound formula is \( \text{C}_{35}\text{H}_{60}\text{O}_{6} \). Calculation obtains that degree of unsaturation is 6, which indicates that the compound structure may contain double bond and ring. In infrared IR (cm\(^{-1}\)) spectrum: stretching vibration absorption peak at 3416 cm\(^{-1}\) shows the presence of hydroxyl, methyl signal at 2958 cm\(^{-1}\) and 2869 cm\(^{-1}\), methylene signal at 2933 cm\(^{-1}\); relatively weak absorption peak at 1627 cm\(^{-1}\) confirms the presence of double bond. 1255 cm\(^{-1}\) represents characteristic absorption peak of C–O stretching vibration in ether bond, while 1075 cm\(^{-1}\), 1025 cm\(^{-1}\) serve as acetal characteristic signal of pyranose.

Nuclear magnetic resonance hydrogen spectrum shows 0.91 shows two methyl of symmetric structure, 1.44, 1.72 show four symmetrical methylene, 4.31 shows two symmetrical oxygen methylene; \( ^13\)C NMR at 132.3 ppm, 130.9 ppm, 128.8 ppm show aromatic carbon information, 167.7 ppm shows the presence of carboxylic acid. Specific NMR data are as follows:

Specific NMR data are as follows:

\[ ^1H \text{ NMR (300 MHz, CDCl}_3 \] = 4.11–2.14 continuous peak envelope, which may be numerous methyl, methylene and sub-methylene signals on steroid skeleton and side chain. Nuclear magnetic resonance spectroscopy shows 35 carbon signals, with three C chemical shift information for unsaturated carbon atom region (except alkyne carbon atoms): 142.2 ppm, 123.2 ppm should be ene carbon signal, 103.6 ppm should be sugar anomeric carbon signal; 63.9–79.5 are sugar oxygen containing substituted aliphatic carbon signal. Except sugar carbon signal, signal of nuclear magnetic resonance spectroscopy of compound II is very close to that of compound I.

Specific NMR data are as follows:

\[ ^13\text{C NMR (100 MHz, Pyr-d5) } \delta: 142.2 \text{ (C-5), 123.2(C-6), 103.6 (C-10), 79.6 (C-3), 79.5(C-3'), 79.4 (C-5'), 76.3 (C-2'), 72.8 (C-4'), 63.9 (C-6'), 58.1 (C-14), 57.5 (C-17), 51.6 (C-9), 47.3 (C-24), 43.8 (C-13), 40.5 (C-12), 38.8 (C-4), 38.2 (C-1), 37.6 (C-10), 35.5 (C-20), 33.4 (C-22), 33.3 (C-7), 31.4 (C-8), 31.3 (C-2), 30.7 (C-25), 29.8 (C-16), 27.6 (C-23), 25.8 (C-15), 24.6 (C-28), 22.5 (C-11), 21.3 (C-27), 20.8 (C-19), 20.5(C-26), 20.3 (C-21), 13.2(C-29), 13.2(C-18). After consulting the literature, it is found that the aforesaid data are basically consistent with daucosterol data reported in the literature (Mohammad and Mahdi, 2014). Therefore, compound II is identified as daucosterol.

3.1.3. Compound III (dibutyl phthalate)

In light yellow oil, it shows molecular ion peak \( m/z: 279 \) [M+H\(^+\)], 301 [M+Na\(^+\)], elemental analyzer test results: C element content is 68.96%, H element content is 7.89%, and thus the compound formula is \( \text{C}_{16}\text{H}_{22}\text{O}_{4} \). Calculation obtains that degree of unsaturation is 6, which indicates that the compound structure may contain benzene and two double bonds or ring. In infrared IR (cm\(^{-1}\)) spectrum: 2960 and 2874 cm\(^{-1}\) are methyl signal, 2934 cm\(^{-1}\) is methylene signal; 1728 cm\(^{-1}\) is carbonyl absorption, 1600, 1580, and 1466 cm\(^{-1}\) are benzene ring skeleton vibration.

Nuclear magnetic resonance hydrogen spectrum shows 64.11–2.14 continuous peak envelope, which may be numerous methyl, methylene and sub-methylene signals on steroid skeleton and side chain. Nuclear magnetic resonance spectroscopy shows 35 carbon signals, with three C chemical shift information for unsaturated carbon atom region (except alkyne carbon atoms): 142.2 ppm, 123.2 ppm and 103.6 ppm, of which
Dibutyl phthalate has excellent in vitro anti-proliferative activity of leukemia cell K562.

Dibutyl phthalate with excellent activity and β-sitosterol with rich contents and moderate anti-tumor activity should be material basis for catchweed bedstraw herb with 60% ethanol to extract biological activity from petroleum ether phase.

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