Short communication

Counter-current fractionation-assisted bioassay-guided separation of active composition from the edible medicinal insect *Blaps rynchopetera* Fairmaire

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**Abstract**

An efficient strategy for the selection of active components based on counter-current fractionation and bioassay-guided separation was established in the present work. *Blaps rynchopetera* Fairmaire was an edible medicinal insect. Its extract showed the potential RAW264.7 macrophage cell inhibitory activity. After extraction with different solvents, the active components were enriched in ethyl acetate. In order to further track the active compounds, the ethyl acetate extraction was divided into 14 fractions by means of HSCCC. The results showed that the activities of F6 and F7 were significant higher than the others. Two compounds, hydroxytyrosol and 4-ethylbenzene-1,3-diol, were separated from the mixture of F6 and F7 by column chromatography and their chemical structures were confirmed by MS, 1H NMR and 13C NMR. The IC50 of hydroxytyrosol and 4-ethylbenzene-1,3-diol against RAW264.7 macrophage cell were 38.24 ± 0.26 µg/mL and 103.26 ± 0.29 µg/mL, respectively, indicating that hydroxytyrosol was the major active ingredient responsible for the RAW264.7 inhibitory activity of *Blaps rynchopetera* Fairmaire.

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1. Introduction

The chemical composition of natural products is very complex. Separation of active compounds by traditional methods was often tedious and time-consuming. So the method of bioassay-guided separation was introduced to improve the separation efficiency of active compound [1,2]. The chromatographic technologies and bioactivity detection were combined to enrich the active components until a pure active compound was obtained [3–5]. Some active compounds were successfully isolated from natural products by bioassay-guided separation, such as anti-cancer cantharinidin, antimicrobial coumarins, protein tyrosine phosphatase 1B inhibitors, antioxidants, etc [4–7]. Counter-current chromatography was a favorite chromatographic technology in the bioassay-guided separation and was often used for the separation of various active compounds in the previous studies [6,7]. However, sometimes various chromatographic techniques need to be used in the process of bioassay-guided separation [4,5]. Some minor active components may be lost during the separation because of irreversible adsorption on column. Liquid-liquid extraction can avoid irreversible adsorption, and was often used to purify the crude sample preliminarily in bioassay-guided separation [8]. However, the separation efficiency of the traditional liquid-liquid extraction was low and limited due to single solvent and extensive polarity range, which caused that the composition was still very complex in the extract. Thus, it was necessary to improve the liquid-liquid extraction method in sample pretreatment to obtain eligible fractions.

High-speed counter-current chromatography (HSCCC) was widely employed to separate various compounds [9–13]. There were many classical solvent systems in HSCCC separation, and compounds were isolated according to their different partition coefficient (K) in the solvent system. More importantly, HSCCC is a continuous liquid-liquid extraction method without any solid support. If this method was used for the fractionation of complex sample, the fractions with the different K values would be obtained.

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In this way, the crude sample can be divided into more fractions rationally, and this method avoided the irreversible adsorption of trace components. The amount of impurities would be greatly reduced for the target fractions. Furthermore, the success rate of active compound separation would be greatly improved.

Medicinal insects have a long history of human utilization, and play an important role in prevention and treatment of multiple diseases. *Blaps rynchopetera* Fairmaire is an insect of the family Tenebrionidae [14]. It is frequently used in traditional Yi medicine in Yunnan province for the treatment of fever, colds, gastritis, cough, tumor, inflammatory and other diseases [15]. The defensive secretion of *Blaps rynchopetera* had cytotoxicity against the tested cell lines, including AGS, Caco-2, HepG2, U251 and Bel-7402 [15]. It had extensive pharmacological activities, including antibacterial [16], antioxidant [17] and antitumor [18]. Its extracts can obviously inhibit the growth of cancer cells such as K562, HL-60, A549 cell lines [18]. In the previous studies, sterols, fatty acids, and phenolics were isolated from *Blaps rynchopetera* Fairmaire [19]. However, the active ingredients of this insect have not been elucidated so far. In order to explore the effective substance of *Blaps rynchopetera* Fairmaire, the HSCC fractionation was used to assist bioassay-guided separation to explore the bioactive composition against RAW264.7 from *Blaps rynchopetera* Fairmaire.

2. Materials and methods

2.1. Apparatus

A TBE-300C HSCC, a TBP5002 constant-flow pump and a model UV2000D monitor were obtained from Tauto Biotech Company, Shanghai, China used in the present study. The internal diameter of polytetrafluoroethylene multilayer coils was 1.9 mm and the total capacity was 300 mL.

A 230 P pump, a model UV230II detector and a SinoChrom ODS-BP-C18 column (5 μm, 4.6 mm × 200 mm) was obtained from Yilitne Company, Dalian, China and used in the present.

2.2. Chemicals and reagents

The macrophage cell line (RAW264.7) was obtained from National Infrastructure of Cell Line Resource (Beijing, China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *Blaps rynchopetera* Fairmaire was collected and authenticated by Kajin Li, Kunming Institute of Zoology, Chinese Academy of Sciences (Voucher No. 1886).

2.3. Preparation of crude samples

500 g of *Blaps rynchopetera* Fairmaire was extracted with 85% ethanol (2L) at 50 °C in a model X0-5B pulse ultrasound apparatus (Nanjing Atio Instruments Manufacturer, Jiangsu, China) for three times (1 h for each time). The extracts were combined and evaporated to dryness as the crude sample (27.78 g). The crude sample was dissolved in water and extracted with 500 mL light petroleum, ethyl acetate and 1-butanol for three times, respectively. After concentration in a vacuum, 0.69 g of light petroleum extraction, 2.19 g of ethyl acetate extraction, 6.15 g of 1-butanol extraction and 18.75 g of water-soluble extraction were obtained.

2.4. Selection of two-phase solvent system

In the present study, different volume ratios of light petroleum, ethyl acetate, methanol and water were prepared and equilibrated in a separatory funnel at room temperature. A suitable amount of crude sample (2 mg) was dissolved in 1.0 mL of preequilibrated upper and lower phase. The solvent system was selected based on the sample color of upper phase and lower phase. When the colors of upper phase and lower phase were similar, the solvent system can be selected to divide the crude extract into different polarity fractions.

2.5. HSCC fractionation procedure

HSCC separation was performed on a preparative HSCC instrument, and a two-phase solvent system composed of light petroleum-ethyl acetate-water (2:1:1, v/v) was used for the HSCC fractionation. The lower aqueous phase was used as mobile phase and the upper organic phase as stationary phase with the head-to-tail mode. The multiplayer coiled column was entirely filled with the upper phase. Then, the lower phase was pumped into the head end of the HSCC column at the flow rate of 5 mL/min, and apparatus was initiated at the speed of 900 rpm. After the equilibration between the upper phase and lower phase was established in the column, the sample solution was loaded into the column and the effluents were monitored by an UV detector at 254 nm. After separation, the column contents were collected into a graduated cylinder by pressured air to determine the volume of the stationary phase retained in the column. The retention of stationary phase was calculated by dividing the volume of the retained stationary phase with the total column volume [20].

2.6. HPLC analysis and identification of isolated fractions

The crude sample, liquid-liquid extracts, counter-current fractions and isolated compounds were analyzed by HPLC (Fig. 1). The mobile phase was a linear gradient of methanol (A) and 0.2% formic acid (B) as follows: A–B (15:85, v/v) to A–B (100:0, v/v) in 50 min. The flow rate was 1.0 mL/min and the detection wavelength was 254 nm.

Identification of the isolated compounds was carried out by MS, 1H NMR and 13C NMR.

2.7. RAW264.7 inhibitory activity assay

The effects of the crude sample, liquid-liquid extracts, counter-current fractions and isolated compounds on cell growth were evaluated by MTT assay in cultured cell lines [21]. The mouse mononuclear/macrophage cells (RAW264.7) stored in liquid nitrogen were thawed rapidly at 37 °C in a water bath. After centrifugation, the cells were gradually diluted with in Dulbecco’s modified Eagle medium (10% fetal bovine serum). The RAW264.7 cells were divided into 3 × 103 cells/well on a 96-well plate and incubated overnight. Then the different concentrations of samples were added to the wells and incubated at 37 °C for different hours. After that, 10 μL MTT solutions was added into the wells, and incubated at 37 °C for 4 h. The active cells can reduce the MTT to blue formazan crystals, which can be dissolved in DMSO. The absorbencies of the solutions were detected at 490 nm.

3. Results and discussion

3.1. RAW264.7 cell inhibitory activity assay of the crude sample and liquid-liquid extractions

To screen for the compounds with RAW264.7 inhibitory activity from *Blaps rynchopetera* Fairmaire, the procedures of extraction and isolation were guided by the RAW264.7 macrophage cell inhibitory bioassay. As shown in Fig. 2A, the crude sample can not inhibit the RAW264.7 cell growth at a low concentration of 25 μg/mL. However, with the increase of the extracts concentration, the inhibitory rate was increased remarkably. When the concentration increased

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Fig. 1. HPLC chromatograms of ethyl acetate extraction, counter-current fraction and isolated compounds. Column: SinoChrom ODS-BP-C18 column (4.6 mm i.d. × 250 mm, 5 μm); the mobile phase: methanol-0.2% formic acid (15:85, v/v) to (100:0, v/v) in 50 min; detection wavelength: 254 nm; flow rate: 1.0 mL/min. (A) ethyl acetate extraction; (B) F6 and F7; (C) hydroxytyrosol; (D) 4-ethylbenzene-1,3-diol.

Fig. 2. Effects of the crude sample, liquid-liquid extracts, counter-current fractions and isolated compounds from Blaps rynchopetera Fairmaire on RAW264.7 cells. (A) Cells were treated by crude sample (25–200 μg/mL) and then incubated for 24 h and 48 h. (B) Cells were treated by four liquid-liquid extracts (200 μg/mL), respectively, and then incubated for 24 h and 48 h. (C) Cells were treated by counter-current fractions (200 μg/mL), respectively, and then incubated for 48 h. (D) Cells were treated by two isolated compounds (5–200 μg/mL), respectively, and then incubated for 48 h. Data shown are the mean ± SD (n = 3).

to 200 μg/mL, the inhibitory rate was 66.60% and 76.42% after 24 h and 48 h, respectively. The result indicated that the crude sample may contain the RAW264.7 cell inhibitory compounds.

Then, the crude sample was extracted with light petroleum, ethyl acetate, and 1-butanol successively. Fig. 2B shows the inhibitory rate of the extracts against RAW264.7 cell at the concentration of 200 μg/mL after 24 h and 48 h. The results indicated that the light petroleum extract, 1-butanol extract and water-soluble extract can promote the growth of RAW264.7 cell. However, ethyl acetate extraction showed significant inhibitory effect on RAW264.7 cell, which was selected for the further bioassay-guided separation.

3.2. HSCCC fractions and their activity

In the present study, HSCCC was used to divide the ethyl acetate extract into different polarity fractions, which was different from its traditional usage. The two-phase solvent system can not be selected
according to the K value, because we do not know which compound was the active compound in the complex extract. However, the solvent system for HSCCC fractionation can still be selected according to the distribution of the sample in mutually immiscible solvents. A series of solvent systems with different volume ratio were prepared, and a suitable amount of ethyl acetate extract was dissolved in the mixture of the upper phase and lower phase. The result showed that the colors of upper phase and lower phase were similar when the solvent system was light petroleum-ethyl acetate-water (2:1:1, v/v), so it was used as HSCCC fractionation solvent system. Because the aim of HSCCC separation was to fractionate the ethyl acetate extract, the flow rate was set at 5 mL/min to shorten the separation time.

The HSCCC chromatogram of ethyl acetate extract was showed in Fig. 3. The retention of stationary phase was 66.67%. The peak fractions (F1-13) were collected manually according to the chromatogram, and the column contents were pushed out by pressured air as F14.

The RAW264.7 inhibitory activities of F1-14 were given in Fig. 2C. It was observed that F6 and 7 exhibited higher RAW264.7 inhibitory activity than the other fractions at the concentration of 200 μg/mL after 48 h, and the chemical compositions of the two fractions were similar by HPLC analysis. So, they were combined and concentrated, and 29 mg of the sample was obtained from 500 mg ethyl acetate extract (Fig. 1B).

### 3.3. Isolation and identification of compounds

The sample (mixture of F6 and F7) was further subjected to silica gel column, and eluted with light petroleum and acetone in a gradient mode. Two major compounds, compound 1 (13 mg) and compound 2 (8 mg), were obtained. HPLC method was optimized for the analysis of different samples from Blaps rynchopetera Fairmaire. The isolated compounds were analyzed by HPLC (Figs. 1C & 1D). Their structural identification was carried out by MS, $^1$H NMR and $^{13}$C NMR as follows:

**Compound 1:** ESI-MS: [M–H]$^-$ at m/z 153. $^1$H NMR (400 MHz, MeOD, δ, ppm, J/Hz); 6.68 (2H, m, H-2, 5), 6.53 (1H, dd, J = 8.0, 2.0, H-6), 2.68 (2H, t, H-7), 3.69 (2H, t, H-8), $^{13}$C NMR (MeOD, 100 MHz): 130.30 (C-1), 114.85 (C-2), 144.73 (C-3), 143.21 (C-4), 115.63 (C-5), 119.78 (C-6), 38.27 (C-7), 63.21 (C-8). Compound 1 was identified as hydroxytyrosol [22].

**Compound 2:** ESI-MS: [M–H]$^-$ at m/z 137. $^1$H NMR (400 MHz, MeOD, δ, ppm, J/Hz); 6.58 (2H, m, H-32, 56), 6.44 (1H, dd, H-65), 2.55 (2H, q, H-7), 1.18 (3H, t, H-8), $^{13}$C NMR (MeOD, 100 MHz): 149.73 (C-1), 115.39 (C-2), 147.50 (C-3), 131.35 (C-4), 115.16 (C-5), 112.38 (C-6), 22.87 (C-7), 13.28 (C-8). Compound 2 was identified as 4-ethylbenzene-1,3-diol [16].

The RAW264.7 inhibitory activities of identified compounds were assayed (Fig. 2D). The isolated compounds inhibited RAW264.7 in a concentration-dependent manner. The activity of RAW264.7 were inhibited by 11.82% and 8.97% respectively after 48 h by 5 μg/mL hydroxytyrosol and 4-ethylbenzene-1,3-diol. With the increase of concentration, the inhibitory rate was increased. When 25 μg/mL of two compounds were applied, the activity of RAW264.7 was inhibited by 46.27% and 31.52%, respectively. When 200 μg/mL of hydroxytyrosol and 4-ethylbenzene-1,3-diol were applied, the activity of RAW264.7 was inhibited by 77.17% and 61.33%, respectively. The IC_{50} values of hydroxytyrosol and 4-ethylbenzene-1,3-diol increased with the increase of the cell culture time. Their inhibitory rates were the best at the concentration of 200 μg/mL. Among them, hydroxytyrosol showed the highest inhibitory rates, followed by the crude sample and 4-ethylbenzene-1,3-diol. It is worth noting that the inhibitory rate of hydroxytyrosol was only slightly higher than that of the crude sample, which may be caused by the synergistic effect. Hydroxytyrosol and the other components in the crude sample bound to the different targets of RAW264.7 and produced exaggerated effect, which was better than the way they interacted with RAW264.7 alone [23].

### 4. Conclusions

In the present study, a strategy based on counter-current fractionation was established and developed for bioassay-guided separation of the active compositions from the edible medicinal insect Blaps rynchopetera Fairmaire. The contribution of this method was fractionating the complex mixtures into several sections with a small number compounds. There were only one or two compounds with limited impurities in the refined fractions. It was helpful for looking for active components and the following separation. In this experiment, two compounds with RAW264.7 inhibitory activity were successfully obtained from Blaps rynchopetera Fairmaire, which indicated that the present method was very efficient and can be applied to the isolation of the active compositions from various medicinal resources.

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### References


