Research Paper

Immunosuppressive effect of extracts from leaves of Fraxinus Mandshurica Rupr.

Yujuan Chen*, Gang Xuea, Feizhou Liub, and Xiuling Gongc

aSchool of Life Science and Technology, Changchun University of Science and Technology, Changchun, China; bChangchun Changsheng Life Sciences Limited, Changchun, China; cShanghai Zijiang Enterprise Group Co., LTD, Changchun Branch, Changchun, China

ABSTRACT Plants provide a rich resource of medicinal material for research and development of new medicine. To discover new compounds as immunosuppressant from plants, we evaluated the immunosuppressive effect of different fractions and particularly one compound (Calceolarioside A) that were extracted from the leaves of Fraxinus Mandshurica Rupr. The fractions and the compound were tested on the ability to reduce Immunoglobulin E (IgE) secretion by human U266 multiple myeloma cells (U266 cells) and to reduce interleukin-2 (IL-2) secretion by mouse spleen cells. Our results showed that both the butanol extract fraction and the compound of Calceolarioside A inhibited the IgE and IL-2 production in U266 cells and mouse spleen cells respectively, and no cytotoxicity was observed within the effective dose range. These results suggest that Calceolarioside A could potentially serve as an immunosuppressant.

KEYWORDS Calceolarioside A; Fraxinus Mandshurica Rupr.; Immunosuppressive effect; spleen cells; U266 cells

Introduction

Fraxinus Mandshurica Rupr. is a member of the family of Oleaceae, which is one of the most widely used hardwood trees in the Northeast China, especially in the area of Changbai Mountain. The wood is an excellent choice for furniture due to its moderate hardness and beautiful texture.1 When the bark of Fraxinus Mandshurica Rupr. is dried, it is sold as an alternative for “qinpi” (Cortexfraxini) in folk. It has been widely used in traditional Chinese medicine (TMC), such as in the TMC formulas for treatment of inflammatory, urinary retention, fever, and so on, especially for the treatment of rheumatism arthritis (RA) in Chinese folk medicine. Which is also widely used in other countries in Asia, for example, South Karea and Japan.2 Studies showed that Fraxinus Mandshurica Rupr. produces compounds such as hydroxycoumarins, a monolignol, lignans, phenylethanoids (such as Calceolarioside A), and secoiridoids.3-5 Most of these compounds have activities in vitro or in vivo. Natural compounds from plants were a rich resource of material for developing new medicines; a notable example would be Arteannuin, the developer of which as a treatment for malaria, Tu Youyou, was awarded the Noble Prize in 2015. Immunosuppressive drugs are widely used in transplanted organs, RA, chronic inflammatory diseases and so on.6-7 But there are some side effects when most of these drugs are used in clinic. So people are trying to find new medicine from the natural herbs.

To discover new compounds that could serve as an immunosuppressant for allergies and organ transplantation, we evaluated extracts from leaves of Fraxinus Mandshurica Rupr. for their immunosuppressive effect in Human U266 multiple myeloma cells (U266 cells) and mouse spleen cells.

Results and discussion

Prepare Calceolarioside A

198.9 mg Calceolarioside A was purified from 5 kg of fresh of Fraxinus Mandshurica Rupr. through organic solvent extraction and HPLC separation. It was a pale yellow amorphous powder, the melting point was 186–188°C, and the formula is C24H28O11, 1H-NMR spectrum (δ, DMSO-d6, 300 Hz): 4.21, 4.58, 6.20, 6.45, 6.54, 6.56, 6.71, 6.91, 6.97, 7.41.13C-NMR (δ, MSO-d6): 36.13, 61.92, 71.24, 72.38, 74.68, 75.15, 75.66, 103.85, 115.08, 115.89, 116.54, 116.88, 117.27, 120.64, 122.46, 126.61, 130.32, 144.56, 146.03, 146.43, 146.63,
149.47, 166.99. $^1$H-NMR and $^{13}$C-NMR data were similar to those that have been published.\textsuperscript{2,8} Glycosides were very difficult to extract using ether or ethyl acetate, but could be easily extracted by using butanol from water. Therefore, in this paper, we use butanol to extract glycosides such as Calceolarioside A, and without low polarity compounds.

**The cytotoxic activities of Calceolarioside A**

The cytotoxic activities of the extract fractions from the leaves of *Fraxinus Mandshurica* Rupr. and the purified Calceolarioside A, were evaluated by the using cytometer and counted the cells number. The butanol fractions and Calceolarioside A showed dose dependent cytotoxicity in mouse spleen cells and U266 cells within the concentrations ranged of 0–800 \( \mu \text{g/mL} \) (Fig. 1).

When we test the cytotoxic activities of Calceolarioside A against mouse spleen cells and U266 cells, we found that when its concentration was higher than 800 \( \mu \text{g/mL} \), it was cytotoxic against U266 cells, and when its concentration was higher than 400 \( \mu \text{g/mL} \), it was cytotoxic against mouse spleen cells. In the doses range of 0–100 \( \mu \text{g/mL} \) (see Fig. 1), Calceolarioside A was no cytotoxic to either mouse spleen cells or U266 cells.

**IgE measurement**

U266 cells were used to evaluate the anti-IgE properties of the extract fractions and the purified Calceolarioside A. The fractions were added at day 0 at the concentrations of 0, 1.25, 2.5, 5, 10, 20, 40 \( \mu \text{g/mL} \). After 6 d of incubation, IgE levels in these culture supernatants were determined by human IgE ELISA kits. The butanol extract fraction (BF) and Calceolarioside A (CA) compound strongly inhibited IgE production in U266 cells. At the concentration of 25.79 \( \mu \text{g/mL} \), BF inhibited about 50% of IgE production, so did CA at 9.25 \( \mu \text{g/mL} \). As shown in Fig. 2, the anti-IgE effects of both BF and CA were dose-dependent.

**IL-2 measurement**

The dose-dependent effect of Calceolarioside A on IL-2 production in mouse spleen cells is shown in Fig. 3A and Fig. 3B. When Calceolarioside A was added at more than 10 \( \mu \text{g/mL} \), IL-2 production is significantly lower in the cells culture supernatant compared with the control. The IC\textsubscript{50} is 14.95 \( \mu \text{g/mL} \).

Calceolarioside A reduced 50% IgE production by the U266 cells at a concentration of 9.25 \( \mu \text{g/mL} \), and reduced 50% IL-2 production by the mouse spleen cells at a concentration of 14.95 \( \mu \text{g/mL} \). This result demonstrates that, under 100 \( \mu \text{g/mL} \), Calceolarioside A not only has no toxicity to cells but also has potential anti-IgE effect. This is consistent with the report that showed extract of *Fraxinus Mandshurica* Rupr. had anti-inflammatory effects.\textsuperscript{4} However, the mechanism of how Calceolarioside A inhibits IgE production by the U266 cells and IL-2 production by the mouse spleen cells is not known. Animal model studies are needed in the future to test the in vivo anti-IgE activity of this compound.

Allergy to food and pollen of various plants is a worldwide health concern, especially for children. For example, peanut allergy is affecting 1% to 3% of children in the whole world, especially in some western countries.\textsuperscript{9} Allergy medicines are very limited; therefore developing new medicine for allergy, potentially from plants, is of great importance. The results in this paper suggest that Calceolarioside A could be a potential target. In the future, in vivo studies are needed to show the effects of Calceolarioside A in anti-food allergy, for example, the IgE-mediated type I hypersensitivity reactions in humans.

**Materials and methods**

**Reagents and materials**

Fetal bovine serum (FBS), 2-mercaptoethanol (2-ME), RPMI-1640 medium, penicillin/streptomycin (P/S),

![Figure 1. Cytotoxic activities of BF and CA against mouse spleen cells (A) and U266 cells (B). BF: butanol fraction; CA: Calceolarioside A.](image-url)
dimethylsulfoxide (DMSO), trypan blue, and other reagents, which were used for cell culture, were purchased from Shanghai Jianglai Biology Life Technology, U266 cells were obtained from Chinese Academy of Science Cell Bank. Sephadex LH-20, IgE Immunocap and reagents obtained from sigma (USA), silica gel and GF254 panels were purchased from Qingdao Haiyang Chemical Co., Ltd. Ethanol, ethyl acetate and other chemical reagents were obtained from Beijing Chemical Works. The leaves of Fraxinus Mandshurica Rupr. were obtained from Changbai Mountain in 2013.

**Extraction and isolation of Calceolarioside A**

Five kilogram of fresh leaves of Fraxinus Mandshurica Rupr. were extracted with ethanol (4×1 L) by boiling for about 180 min, 376.9 g (7.54%) extract was yield. The ethanol extract (200 g) was dissolved with water (1.0 L), then shaken with ether for 3 times (each time with 500 mL), after extract with ether, ether ethyl acetate was used to extract as the same method as ether (3×500 mL), then extract with n-butanol (3×500 mL), successively. The solvents were removed under reduced pressure and 3 fractions were obtained, they were ether extract (EE: 97.9 g), ethyl acetate extract (EAE: 12.5 g), and n-butanol extract (BE: 53.4 g). Calceolarioside A was identified by High Performance Liquid Chromatography (HPLC) with standard of Calceolarioside A. The HPLC condition as: the Agilent C18 column (4.6×150 mm, 5 μm) was used; the detection wavelength was 330 nm; the flow rate was 1 mL/min; methanol and water were used as

**Figure 2.** Activities of extract fraction and Calceolarioside A suppress IgE production in U266 cells. (A) and (B) were the result of Calceolarioside A to U266 cells; (C) and (D) were the results of butanol fraction to U266 cells.

**Figure 3.** Effect of Calceolarioside A on the level of IL-2 in mouse spleen cell culture supernatant.
the mobile phase, and the ratio is 45: 55. The butanol fraction (20 g) was applied to D101 macroporous resin column chromatography to obtain 4 sub-fractions by using different concentrations of ethanol from 0 to 95% (0 – 30 – 50 – 70 – 95%). Following the bioactivity guided isolation procedure; immunoglobulin E (IgE) inhibition effects of the extract fractions were determined. Fraction collected with 70% ethanol, showed the best activity. Then the fraction obtained with 70% ethanol was continued on isolation and this fraction was applied to sephadex LH-20 using methanol-dichloromethane as the solvent system. Five new fractions were obtained, and the third fraction has the highest IgE inhibition effects.

**Cytotoxic activity**

Mouse spleen cells and U266 cells were culture with growth medium at the concentration of 1×10^5 cells/mL individually, and seeded into 96-well plates for 0.1 mL/well, in a humidified 5% CO2 incubator at 37°C. After 24 h, added test sample into the wells with cells incubated for 48 h, the doses of each extract as following: 0 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL and 800 µg/mL. After incubation, take 10 µL of cell suspension from each culture well, and mixed with 10 µL of typan blue, then load 10µL of the mixed suspension into a cytometer and count the cells under a microscope. The dead cells were blue and the viable cells were not. The cells viability was defined as ratio (expressed as percentage) of total number of viable cells to total number of cells.10,11

**U266 cells cultures**

For U266 cells, started cultures at 5×10^5 cells/mL and maintain between 5×10^5 and 10×10^5 cells/mL with a medium replaced every 2 or 3 d. The growth medium was: RPMI-1640 with 10% fetal bovine serum + 1×10^-5 M 2-ME + 0.5% penicillin and streptomycin +1 mM sodium pyruvate. When determined the IgE inhibition effects of the fractions, started cultures at 2×10^5 cells/mL and maintained 6 d at 37°C under 5 % CO2 in a 24-well plate. All the test fractions and compounds were added at day 0. After culturing for 6 d, the supernatants will be harvested for IgE determination by Immunocap.12,13

**Spleen cells cultures**

Female BALB/c mice (6 weeks old, about 22 ± 2 g) were obtained from the Experimental Animal Center of Jinlin University. Killed these mice, harvested spleens then did as following: (1) place one spleen in one well of the 6-well plate. (2) Wash it with 5 mL PBS with P/S twice transferring the spleen to the next well. (3) Place spleen with 5 mL PBS with P/S and grind through cell filter with 5 mL syringe plunger. (4) Place Splenocytes in 50 mL tube and used 5 mL PBS with P/S to wash remainder of well for cells. (5) Spun at 1000 rpm×10 min at 4°C. (6) Add 5 mL RBC lysis buffer per spleen and incubated at room temperature for 5 min. (7) Add 40 mL PBS with P/S to stop reaction. (8) Span at 1000 rpm × 10 min at 4°C. (9) Wash the cells with RPMI with P/S × 2 and counted cells. (10) Resuspend in Splenocytes culture medium to final concentration of 8×10^6 cells/mL. (11) In 24-well plate, put 0.5 mL of splenocytes (8×10^6 cells /mL) + 0.5 mL of medium + extract fractions. (12) After 72 h, collected supernatants and stored at −80°C until analyzed.13-15

**Statistics**

The data were analyzed by Microsoft Office Excel 2007 and GraphPad Prism 5.

**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| BF | butanol fraction |
| CA | Calceolarioside A |
| DMSO | dimethylsulfoxide |
| FBS | fetal bovine serum |
| HPLC | High Performance Liquid Chromatography |
| IgE | immunoglobulin E |
| IL-2 | interleukin-2 |
| 2-ME | 2-mercaptoethanol |
| PBS | phosphate buffered saline |
| P/S | penicillin/streptomycin |
| RA | rheumatism arthritis |
| RBC | red blood cell |
| TMC | traditional Chinese medicine |
| U266 cells | human U266 multiple myeloma cells |

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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