



Study on the anaphylactoid of three phenolic acids in Honeysuckle

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ABSTRACT

Ethnopharmacological relevance: Chlorogenic acid (CA) is one of the major ingredients in Honeysuckle which exhibits anticancer, antibacterial, antiviral, hypoglycemic and anti-HIV activities. However, with the frequent emergence of anaphylactoid reactions of traditional Chinese medicine (TCM) injections which contains Honeysuckle in recent years, many researchers found that CA exhibited allergenicity.

Aim of the study: To explore the changes of content of CA, neochlorogenic acid (NCA) and cryptochlorogenic acid (CCA) during the preparation of Honeysuckle and evaluate the anaphylactoid of these three ingredients.

Materials and methods: Two extracts of Honeysuckle were prepared by different methods and the content of CA, NCA and CCA were determined by high performance liquid chromatography (HPLC) analysis. Guinea pig and mast cells RBL-2H3 were utilized as the animal and cell model to investigate the anaphylactoid of these three ingredients.

Results: The content of CA decreased while CCA and NCA increased during the preparation of Honeysuckle. In vitro and in vivo studies showed that CA and CCA could significantly increase the plasma serotonin and β -hexosaminidase levels in guinea pigs and induce the degranulation of RBL-2H3 cell, while NCA did not show such properties. In addition, CCA had a stronger effect than CA.

Conclusion: We believed that both CA and CCA could cause anaphylactoid reaction while NCA could not. Moreover, the anaphylactoid of CCA is higher than CA. Our result demonstrated that CA is not the only cause of anaphylactoid reactions of TCM injections which contains Honeysuckle.

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1. Introduction

Honeysuckle is the flowers of *Lonicera japonica* Thunb. (Wang et al., 2014). It is one of the most famous traditional Chinese medicinal herbs which have been widely utilized in China. It has the function of relieving fever and usually appears in a number of antipyretic preparations of traditional Chinese medicine (TCM). Chlorogenic acid (CA) is a secondary metabolite of plant aerobic respiration which belongs to the phenylpropanoids family (Huang et al., 2010; Lin et al., 2010) and it is one of the richest ingredients in Honeysuckle (Zhang et al., 2008). It is known as a natural antioxidant and serves as anti-inflammatory, anti-tumor, anti-mutagenic and anti-carcinogenic agent (Ozcelik et al., 2011; Gugliucci et al., 2009). CA is usually considered as an indicative component in some TCM preparations which contains Honeysuckle since it has a variety of pharmacological activities. However, in the past decade, some studies had found that it has

allergenicity (Xiao et al., 2013; Qin et al., 2010). They believed that CA might be the cause of the allergic reaction and anaphylactoid reaction of some TCM injections which contains Honeysuckle such as shuanghuanglian injection and yinzhihuang injection (Ye et al., 2010; Chen et al., 2014; Zeng et al., 2014; Ji et al., 2009; Huang et al., 2010).

During the production procedure of TCM injections, the raw herbs go through a series of complex physical or chemical process. Some ingredients in the herbs may change their forms or converted to other substances. Previous studies had revealed that CA might hydrolyze under specific pH condition. Bright light, high temperature and prolonged heating also accelerate its decomposition. Such conditions may exist in many process of production of TCM injections. Despite the high content of CA in Honeysuckle, it may convert to other compounds such as neochlorogenic acid (NCA) and cryptochlorogenic acid (CCA) during the production procedure. They are isomers and the only difference between them was the way caffeic acid and quinic acid connect (Fig. 1). Therefore, we speculated that CA may not be the only incentive of anaphylaxis and anaphylactoid reactions of some TCM injections which contains Honeysuckle. In this study, we determined the content of CA, NCA and CCA in Honeysuckle which treated with

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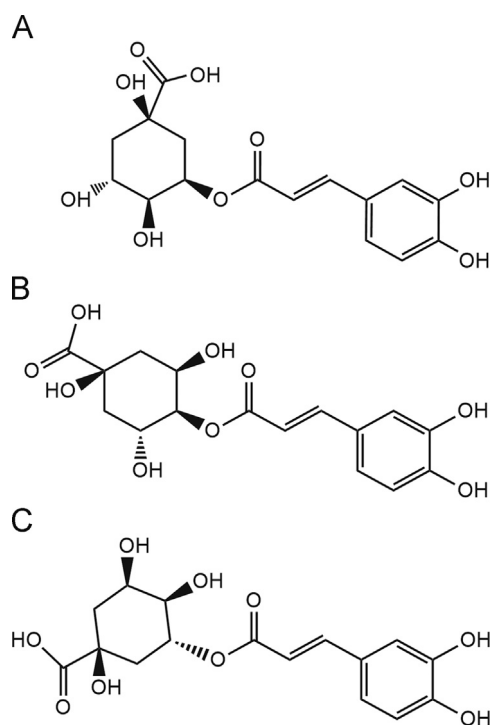


Fig. 1. Chemical structures of three compounds: (A) NCA; (B) CCA; (C) CA.

different preparation methods and explored the anaphylactoid of these three ingredients using guinea pig and mast cells RBL-2H3 as the animal and cell model.

2. Materials and methods

2.1. Reagents

CA was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). CCA and NCA were purchased from National Institutes for Food and Drug Control of China (Beijing, China). Triton X-100 and Compound 48/80 (C48/80) were purchased from Sigma-Aldrich (St Louis, USA). 4-Nitrophenyl-N-acetyl- β -D-glucosaminide was also purchased from Sigma (St Louis, USA). Dulbecco's Minimum Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Preparation of extract of Honeysuckle

The Honeysuckle was weighed (200 g) and divided into two equal parts. One part was boiled with water twice for 1 h each time (Sample A). The other part was boiled with water twice for 1 h each time and then filtered and concentrated to the density ratio of 1.20–1.25 with rotary evaporator. The concentrated solution was cooled to 40 °C and dissolved with ethanol to the ethanol content of 75% and standing for 12 h. After that, the solution was filtrated and the supernatant liquid was evaporated to dryness. The residue was dissolved with water and regulated the pH to 7.0 and sterilized at 105 °C (Sample B). All the experiments were followed the instruction of Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee 2010).

2.3. Standard preparation

Standards of the analytes were prepared in methanol at concentrations of 3000.00 μ g/mL CA, 3000.00 μ g/mL CCA, and 3000.00 μ g/mL NCA. The stock was diluted with methanol to

prepare seven different concentrations for calibration. The standards were prepared in dark brown volumetric flasks and stored at 4 °C.

2.4. HPLC analysis of CA, CCA and NCA

The analysis was performed on an Agilent 1100 LC system equipped with a photodiode array detector, an online degasser, a quaternary pump, an automatic sampler system. The separation was performed on a Hadera C₁₈ column (250 mm $\times$ 4.6 mm, 5 μ m). The mobile phase was a mixture of acetonitrile (A) and water containing 0.1% phosphoric acid (B) at flow rate of 1 mL/min. The detection wavelength was set at 327 nm, and analysis was performed at 25 °C.

2.5. Animals

Fifty male guinea pigs weighing 250–350 g were obtained from the Laboratory Animal Center in Nanjing University of Chinese Medicine. All the animals were housed with a 12 h light/dark cycle at 22 °C and 55 $\pm$ 5% relative humidity and had free access to food and water. Animal experiment protocols in this study were carried out according to the guidelines of the Animal Care Committee of Nanjing University of Chinese Medicine.

2.6. Animal experiments

Fifty guinea pigs were randomly assigned to weight-matched groups. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). C48/80, CA, CCA and NCA were dissolved in normal saline at the concentration of 0.5 mg/mL and continuously injected via the jugular vein (0.16 mg/kg). The dose of CA, NCA and CCA used in this experiment was determined according to the clinical dose of some common traditional Chinese medicines which contain Honeysuckle and converted to the dose used in guinea pigs. Anti-coagulant blood (1 mL) was collected before (blood sample A) and after (blood sample B) the stimulation through the anesthetic animals' carotid artery, and the samples after the stimulation were collected at 30 min. Serum was obtained through centrifugation (4000 rpm, 5 min) and then put into storage at –80 °C.

2.7. Detection of serotonin

Plasma sample (100 μ L) was thawed in the dark and extracted with 100 μ L methanol by vortexing for 3 min. The mixture was separated by centrifugation at 10,000 r/min for 15 min. After freezing for 30 min, supernatants (20 μ L) of the pretreated serums were collected for the detection of serotonin.

Serotonin in the plasma sample was measured through HPLC analysis (Yu et al., 2013). The analysis was carried out on a Waters 510 liquid chromatography equipped with a Waters 2465 electrochemical detector (Massachusetts, USA) and EC 2000 chromatographic work station (Dalian, China). Hadera ODS-2 column (150 mm $\times$ 4.6 mm, 5 μ m, Jiangsu Hanbang Science & Technology Co., Ltd.) was used at a column temperature of 25 °C. Mobile phase was 25 mmol/L sodium dihydrogen phosphate (containing 0.5 mmol/L EDTA and 3 mmol/L sodium heptane sulfonate, pH 4.6) mixed with acetonitrile at a volume ratio of 85: 15. The mobile phase was filtered through a 0.45 μ m filter membrane in vacuum, with a flow velocity of 0.8 mL/min. ISAAC (in-situ silver/silver chloride) was used as the reference electrode. The detection potential was 0.6 V and the injection volume was 20 μ L. The average increasing rate of serotonin in plasma was calculated

according to the following equation:

$$\text{Sample}(\%) = \frac{(\text{valueB} - \text{valueA})}{\text{valueA}} \times 100\%$$

where value B is the amount of serotonin in blood sample B and value A is the amount of serotonin in blood sample A.

2.8. Determination of β -hexosaminidase in guinea pigs plasma

β -hexosaminidase in the plasma sample was measured through enzyme-linked immunosorbent assay (ELISA) following the instruction of the manufacturer of the ELISA kits. The increasing rate of β -hexosaminidase was calculated according to the following equation:

$$\text{Sample}(\%) = \frac{(\text{valueB} - \text{valueA})}{\text{valueA}} \times 100\%$$

where value B is the amount of β -hexosaminidase in blood sample B and value A is the amount of β -hexosaminidase in blood sample A.

2.9. Cells culture

RBL-2H3 cells were purchased from Shanghai institutes for biological sciences. The cells were cultured in Dulbecco's minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (Gibco, Gaithersburg, MD, USA), 1% penicillin/streptomycin (Sigma, Steinheim, Germany) at 37 °C in an incubator with 95% oxygen and 5% CO₂.

2.10. Neutral red staining and degranulation measurement of RBL-2H3 cells

RBL-2H3 cells were seeded into 24-well plates at the density of 4×10^4 cells/well in 400 μ l medium and incubated overnight at 37 °C. The cells were treated with C48/80 (50 μ g/mL), CA (5 mmol/L), CCA (5 mmol/L) and NCA (5 mmol/L) for 30 min at 37 °C. The supernatants were collected and then put into storage at –80 °C. The cells were stained with neutral red (0.5%) for 3 min. Degranulated mast cells were observed by microscopy, and the degranulation index (DI) was calculated using the following formula:

$$\text{DI} = (\text{number of degranulated mast cell}/100) \times 100\%$$

2.11. Measurement of β -hexosaminidase release

RBL-2H3 cells were seeded in 96-well plates at the density of 2×10^4 cells/well in 100 μ l medium and incubated with 50 μ l substrate for 24 h at 37 °C. The cells were stimulated by C48/80 (50 μ g/mL), CA (5 mmol/L), CCA (5 mmol/L) and NCA (5 mmol/L) for 1.5 h. After stimulation, each well was gently rinsed twice with PBS. The cells were lysed with Triton X-100 solution (0.5%) and Na₂CO₃/NaHCO₃ buffer solution was added to each well to suspend reaction. The absorbance (OD) of each well was measured at 405 nm. The releasing ratio of β -hexosaminidase was calculated

using the following formula:

$$\beta - \text{hexosaminidase release}(\%) = \frac{\text{OD supernatant} - \text{OD zero pore}}{\text{OD Triton X} - 100 - \text{OD zero pore}} \times 100\% \quad (\text{B})$$

2.12. Dose–effect of anaphylactoid reactions provoked by CA, CCA and NCA

RBL-2H3 cells were seeded in 48-well plate at the density of 3×10^4 cells/well and treated with different concentrations of CA (1 mmol/L, 2.5 mmol/L and 6.25 mmol/L), CCA (1 mmol/L, 2.5 mmol/L and 6.25 mmol/L), NCA (1 mmol/L, 2.5 mmol/L and 6.25 mmol/L) and C48/80 (1, 10, 50 μ g/mL) for 30 min. Then, the supernatants were collected for the detection of serotonin using HPLC analysis.

2.13. Statistical analysis

Results were presented as mean $\pm$ SD. Experiments were conducted separately at least three times. Data were analyzed by one-way ANOVA. Tukey's multiple comparison test was made for multiple comparisons among all groups if overall $P < 0.05$. Probability (P value) of < 0.05 was considered to be statistically significant.

3. Results

3.1. Content of CA, NCA and CCA in different Honeysuckle extracts

The calibration curves were constructed by analyzing seven different concentrations of standard solutions. The limits of detection (LOD) and limits of quantitation (LOQ) under the present chromatographic conditions were determined at a signal to noise (S/N) ratio of 3 and 10, respectively. LOD and LOQ of 3 marker compounds were within a range of 0.07–0.21 μ g/mL and 0.18–0.74 μ g/mL, respectively, which showed a high sensitivity under the established chromatographic condition. The calibration curves, LOD and LOQ are shown in Table 1.

The typical HPLC chromatogram of the aqueous extract solution (Sample A) and the preliminary preparation (Sample B) of Honeysuckle are shown in Fig. 2. The content of CA, NCA and CCA in different samples is shown in Fig. 3. The results showed that the content of CA reduced significantly in Sample B ($P < 0.001$) while the content of NCA and CCA was increased compared to Sample A ($P < 0.01$).

3.2. Measurement of serotonin and β -hexosaminidase in guinea pig plasmas

After guinea pigs were sensitized by injecting CCA, NCA, and CA intravenously for 30 min, the serotonin level in guinea pigs plasma was about three times and eight times higher in CA and CCA group than that of the Control group. However, the average increasing rate of serotonin in NCA group did not significantly increased compared with Control group. The average increasing rate of β -hexosaminidase

Table 1
Regression equation, concentration range, LOD and LOQ of three investigated compounds.

Peak no.	Components	Wavelength (nm)	Calibration curve	R ²	Liner range (μ g/mL)	LOD (μ g/mL)	LOQ (μ g/mL)
1	CCA	327	$y = 17419x + 24.159$	1	46.88 ~ 3000.00	0.12	0.46
2	CA	327	$y = 19955x + 37.089$	0.9999	46.88 ~ 3000.00	0.07	0.18
3	NCA	327	$y = 16386x + 33.574$	0.9999	46.88 ~ 3000.00	0.21	0.74

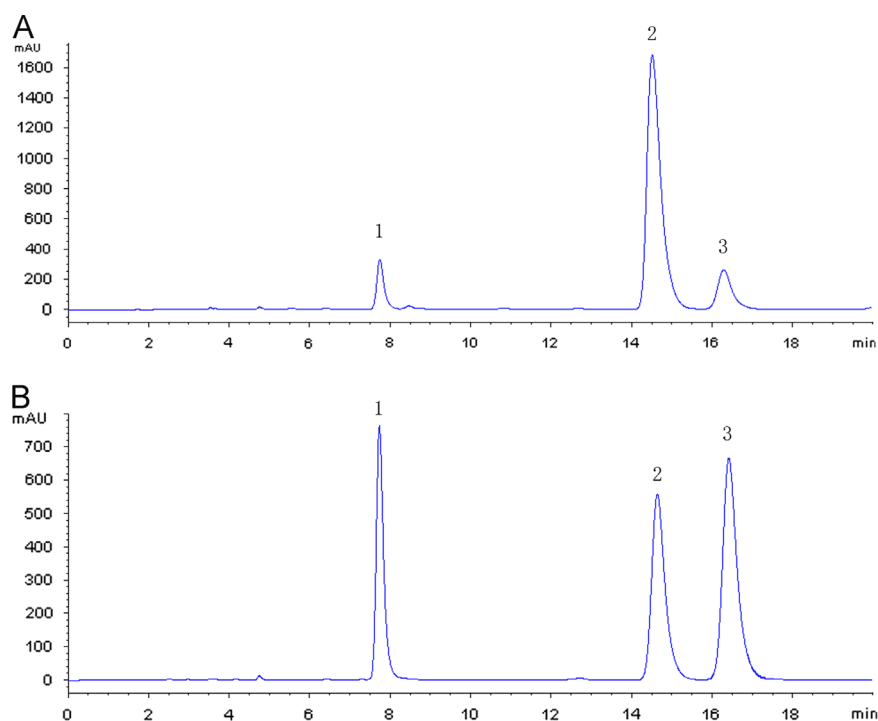


Fig. 2. Chromatogram of (A) the aqueous extract solution and (B) the preliminary preparation of honeysuckle. (1) NCA; (2) CA; (3) CCA.

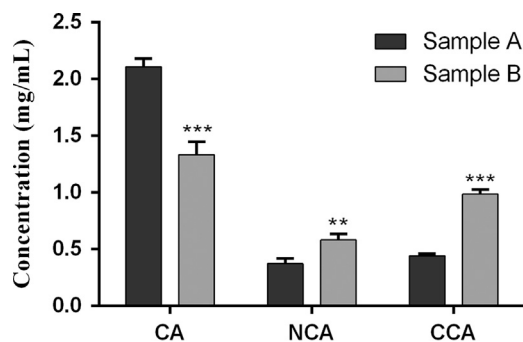


Fig. 3. The content of CA, NCA and CCA in different samples. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. Sample A.

exhibited the same trend. Typical chromatogram of serotonin and results of the average increasing rate are shown in Fig. 4.

3.3. Neutral red staining and RBL-2H3 cells degranulation

Normal RBL-2H3 cells appeared long fusiform after neutral red staining and the cellular membrane was intact. After degranulation, the cells became round, the volume became bigger and even appeared vacuolated cytoplasts (Fig. 5).

As shown in Fig. 6, CA and CCA promoted the degranulation of RBL-2H3 cells but NCA did not promoted the DI of the cells. These results suggested that CCA had a much stronger effect on RBL-2H3 cells degranulation than CA while NCA exhibited no effect on RBL-2H3 cells degranulation.

3.4. Measurement of serotonin in the culture supernatants

RBL-2H3 cells were sensitized with C48/80, CA, CCA and NCA for 30 min and the supernatants were collected for the measurement of serotonin. The content of serotonin in supernatant is shown in Fig. 7. Compared with Control group, content of serotonin in NCA group did not increase, while in CA and CCA

groups, the content of serotonin increased by 73% and 137%, respectively.

3.5. Measurement of β -hexosaminidase release

The β -hexosaminidase concentration in RBL-2H3 cells after incubating with NCA for 30 min did not increase. However, the results were opposite in cells incubating with CA and CCA. In addition, the β -hexosaminidase concentration in CCA group was much higher than that of CA group ($P < 0.01$). The results are shown in Fig. 8.

3.6. Dose–effect relationship serotonin with anaphylactoid reactions provoked by CA, CCA and NCA

After stimulated successively by different concentrations of CCA and CA, serotonin production in the culture supernatants increased with the increased doses. At the concentration of 2.5 and 6.25 mmol/L, the serotonin production in CCA group was much higher than that of CA group ($P < 0.05$). However, after sensitized successively by different concentrations of NCA, serotonin production showed insignificant difference. The results are shown in Fig. 9.

4. Discussion

For the past years, with the rising usage of herbal injections in clinical applications, reports of their adverse reactions were also increasing (Li, 2008; Jia and Hui, 2006; Han and Guo, 1999; Peng, 2005). Most of the researches regard the adverse reaction of herbal injections as anaphylactic reaction, but in fact, the adverse reaction of herbal injections occurred at the first time of drug administration and closely related to the dose. All these characteristics were in keeping with anaphylactoid reaction. According to the recent studies, about 77% adverse reaction of TCM injections were anaphylactoid reaction (Zhao et al., 2010), indicating that the incidence rate of anaphylactoid reaction is much higher than

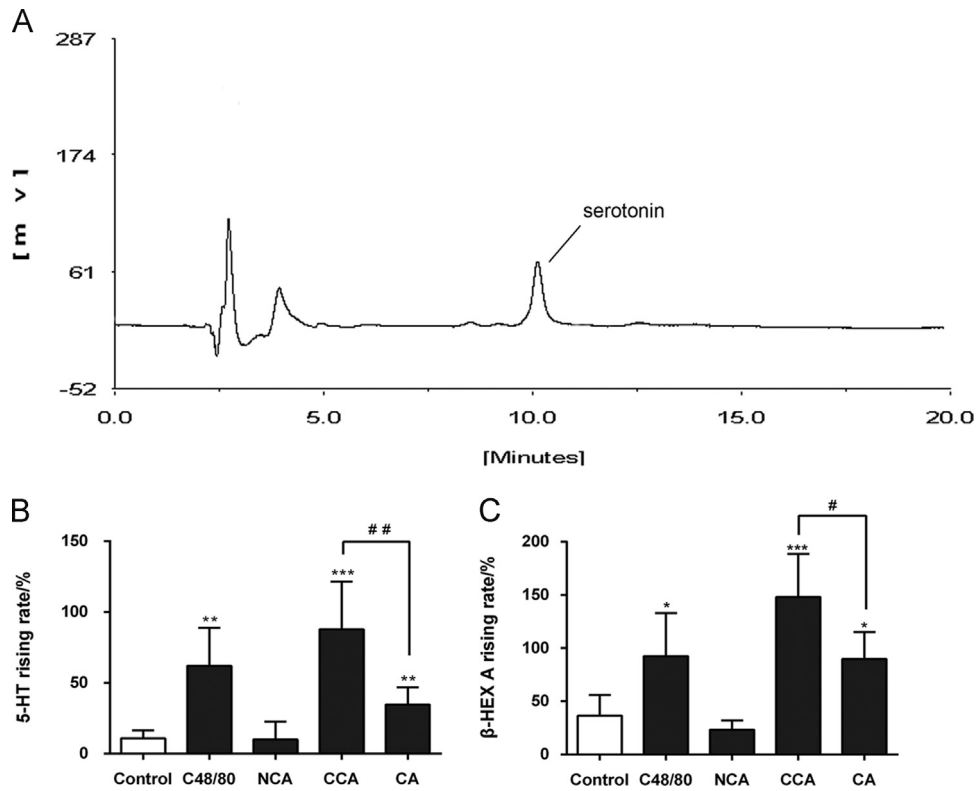


Fig. 4. Typical chromatogram of (A) serotonin and increasing rate of (B) serotonin and (C) β -hexosaminidase in guinea pig plasmas after treating with normal saline, C48/80, NCA, CCA and CA. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. Control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CCA.

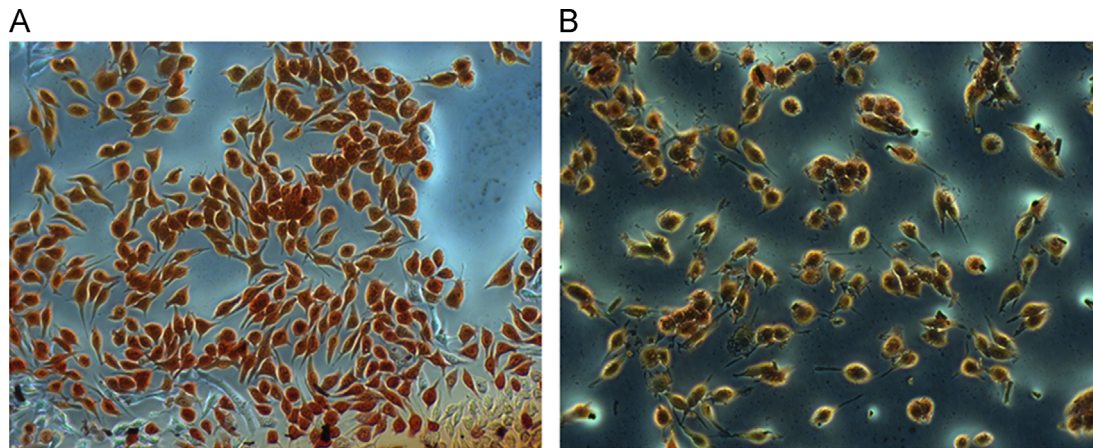


Fig. 5. RBL-2H3 cells stained with neutral red. (A) Normal RBL-2H3 cells, (B) Degranulated RBL-2H3 cells.

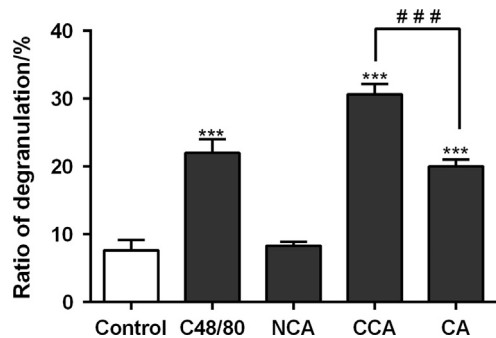


Fig. 6. Ratio of degranulation in RBL-2H3 cell after treating with C48/80, NCA, CCA and CA. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. Control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CCA.

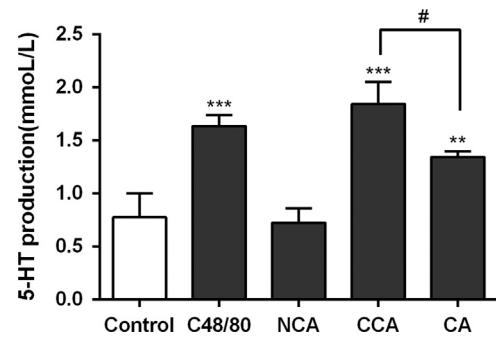


Fig. 7. Serotonin production in the culture supernatants after RBL-2H3 cells sensitized with C48/80, NCA, CCA and CA. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. Control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CCA.

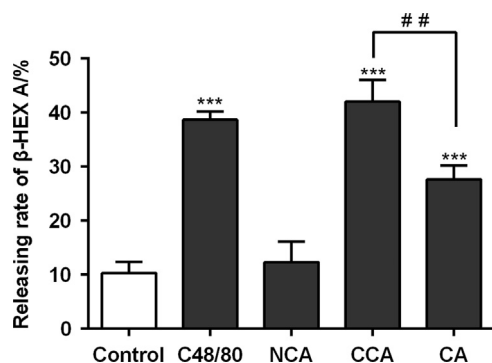


Fig. 8. The releasing rate of β -hexosaminidase in RBL-2H3 cells after incubating with C48/80, NCA, CCA and CA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CCA.

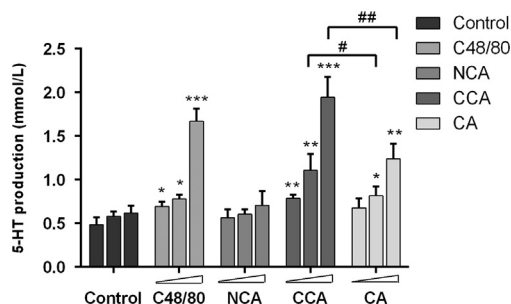


Fig. 9. Serotonin production in the culture supernatants after RBL-2H3 cells were stimulated with different concentrations of the compounds (C48/80: 1, 10, 50 $\mu\text{g/mL}$; CA, CCA and NCA: 1, 2.5, 6.25 mmol/L). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CCA.

allergic reaction. There are multiple ways to induce anaphylactoid reaction, mainly by the direct stimulation of target cells (mast cells or basophils) or through the complement activation mechanism and then releasing allergic medium, such as histamine, serotonin and β -hexosaminidase (Aketania et al., 2001; Huang et al., 2008; Jutel et al., 2001).

During the production process of TCM preparation, raw herbs need to go through a series of processes, such as heating, boiling with water or other solvent, alcohol precipitation and sterilization. These methods may lead to some changes of the ingredients of TCM. Some may change their form; others may hydrolyze or convert to their isomers. CA is widely distributed in plants with a variety of isomers. It is a very important active ingredient and indicator of the quality control of TCM such as *Lonicerae Japonicae* Flos. and *Lonicerae Japonicae* Caulis. CA is caffeic acid and quinic acid ester; previous studies have found that CA can be hydrolyzed both by alkali and acid. The aqueous solution of CA is most stable at pH3.0. In this study, Sample B was prepared by water extraction and alcohol precipitation and then regulated pH to 7.0 which is detrimental to the stability of CA. CA is also thermal instability. Sample B was sterilized at 105 °C for 30 min. In this process, the content of CA was decreased while the content of its isomers, NCA and CCA was increased.

There are a lot of controversies about whether CA is a potential allergen. In our study, we found that CA could convert to NCA and CCA after a series of processing. Therefore, we studied the anaphylactoid of CA, NCA and CCA. Evidence from animal studies has suggested that CA and CCA could induce the increase of serotonin and β -hexosaminidase in guinea pig plasma. The results indicated that CA and CCA have anaphylactoid while NCA does not. In vitro study showed that, CA and CCA could induce the degranulation of RBL-2H3 cells and significantly increased the release rate of β -hexosaminidase and the concentration of serotonin. Dose–effect

experiment revealed that CA and CCA could increase the serotonin in a dose-dependent manner. NCA did not exhibit such effect. All these results indicated that CA and CCA could induce anaphylactoid reaction while NCA has no anaphylactoid.

There is a close relationship between the pharmacological actions and molecular structure of the drug. The significant discrepancy of the anaphylactoid between three compounds in this study might be concerned with their structural differences. In vitro and in vivo experiment showed that CCA exhibited stronger anaphylactoid than CA. In some herbal injections, the concentration of CCA is higher than CA. These may remind us that CCA might be an important precipitating factor of anaphylactoid reaction in some herbal injections. Because of the dose-dependent effects in anaphylactoid reaction, we should strictly control the content and transformation of CCA and CA during the course of production in order to reduce the risk of anaphylactoid reaction.

5. Conclusions

In this study, we explored the conversion of chlorogenic acid in Honeysuckle during the proceeding of TCM injection. We demonstrated that chlorogenic acid could convert into cryptochlorogenic acid and neochlorogenic acid. We also evaluated the allergenicities of these three ingredients and found that both chlorogenic acid and cryptochlorogenic acid could cause anaphylactoid reaction while neochlorogenic acid could not. Such findings might provide the guidance for the quality control of TCM injection.

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