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PII: S0378-8741(18)32010-5
DOI: <https://doi.org/10.1016/j.jep.2018.10.027>
Reference: JEP11566

To appear in: *Journal of Ethnopharmacology*

Received date: 28 June 2018
Revised date: 11 October 2018
Accepted date: 21 October 2018

Cite this article as: Yujie Ye, Shuyuan Mo, Wenya Feng, Xiaochun Ye, Xiang Shu, Yeli Guan, Jiangeng Huang and Jianping Wang, The ethanol extract of *Involucrum castaneae* ameliorated ovalbumin-induced asthma in guinea pigs, *Journal of Ethnopharmacology*, <https://doi.org/10.1016/j.jep.2018.10.027>

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The ethanol extract of *Involucrum castaneae* ameliorated ovalbumin-induced asthma in guinea pigs

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ABSTRACT

Ethnopharmacological relevance:

Involucrum castaneae (IC) is used in Chinese folk medicine to treat various lung diseases, as well as for its reducing phlegm and anti-inflammatory properties.

Aim of the study:

The purpose of this experiment is to verify the effect of IC on asthma and its potential mechanism. The main chemical composition of IC was also analyzed.

Materials and methods:

The potential of the ethanol extract of *Involucrum castaneae* (EEIC) to protect against ovalbumin-induced asthma in guinea pigs was investigated. The latency of asthma in guinea pigs were recorded after the asthma induced. Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of immunoglobulin E (IgE), interleukin-5 (IL-5), nerve growth factor (NGF) and interferon- γ (IFN- γ) in asthma

allergy. Reverse transcription-PCR (RT-PCR) was used to detect the expression of IL-5 mRNA in asthmatic guinea pig lungs. Paraffin sections of lung tissue used to analyze pathological changes. The total flavonoid content was determined and the chemical components were analyzed by LC-MS/MS.

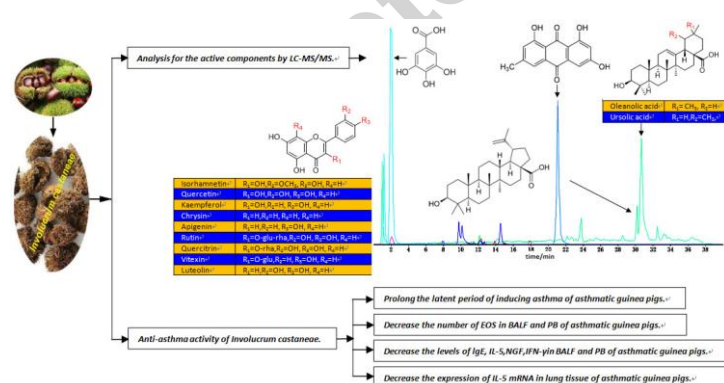
Results:

It was found that EEIC was able to reduce the number of EOS in BALF and PB of ovalbumin-induced guinea pig model of asthma. Meanwhile, it also significantly reduced the levels of inflammation-related factors IgE and IL-5, decreased the expression of IL-5 mRNA in lung tissue, and increased the level of IFN- γ factor. Pathological examination of paraffin section of lung tissue showed that EEIC can reduce the thickening of bronchial smooth muscle and reduce the infiltration damage of tissues by various inflammatory cells. The presence of flavonoids, terpenoids and phenolic compounds in EEIC might be responsible for these activities.

Conclusion:

The paper explains the traditional efficacy and material basis of IC and lays a foundation for further development.

Graphical Abstract:



1. Introduction

Asthma is a chronic airway inflammation, and difficult to completely cure (Jiao et al., 2015). Because of the environmental pollution caused by modern industry, the incidence of asthma in the world is increasing year by year. Although the pathogenesis is not yet fully understood, the current research shows that asthma is an inflammatory disease, caused by a variety of inflammatory cells and cytokines that mediate and interact with each other (Canning and Chou, 2008; Bueno-Silva et al., 2017). Eosinophil (EOS) is a key cell for asthma inflammation that damages the airway epithelium by releasing the inflammatory mediators that are produced and stored in the cell (Khatami, 2014). The increase in the number of EOS is closely related to the severity of asthma (Jiao et al., 2015). In the peripheral blood (PB), bronchoalveolar lavage fluid (BALF) and lung tissue, inflammatory cells such as eosinophils and lymphocytes, and cytokines such as interleukin-5 (IL-5), and immunoglobulin E (IgE) are significantly increased (Nirmal et al., 2012). Interferon- γ (IFN- γ) is an important cytokine produced by lymphocytes, and it exists in a variety of T cell dependent lung diseases. It inhibits the aggregation of EOS and the production of IgE in blood (Wei et al., 2014; Pu et al., 2018).

Inhaled corticosteroids combined with long-acting β_2 -agonist are often used in the treatment of asthma (Wang et al., 2017; Cooper et al., 2018). Inhaled corticosteroids is the most effective drug at present, however, patients who use steroidal anti-inflammatory drugs for a long time are prone to side effects such as osteoporosis and immunosuppression, and the long-term use of β_2 -agonist may lead to drug resistance in patients (Koch et al., 2017). Therefore, there is an enormous need to find effective and less toxic drugs for the treatment of asthma.

Castanea mollissima Blume (CMB) is derived from the genus of *Castanea* in the family of *Fagaceae*. Its root, bark, leaf, flower, exocarp (*Involucrum castaneae*), inner peel and total bracts of CMB have been recorded in the old medical works of all ages. At the same time, its fruit is rich in nutrition and has the reputation of "the king of nuts" (Chen and Jian, 2018; Brus et al., 2018).

Involucrum castaneae (IC) is the exocarp of CMB, which has a properties of tonic and astringent in traditional Chinese medicine, and has the effect of relieving cough through reducing phlegm and anti-inflammatory (Sorice et al., 2016). It is used in clinic to treat various lung diseases. eg. Chronic bronchitis, pertussis and other cough-related diseases, which was recorded in "Chinese Pharmacopoeia" (1977 version) and "Specification for processing of Chinese Herbal Medicine in Gansu Province" (1980 version). "Qingre antitussive syrup", "Sanwei Zhike tablet" and "Huatan Xiaoke tablet", recorded in "Chinese Pharmacopoeia" or "Prescription set of Chinese patent medicine", which all use the IC as the main medicine to treat cough (Cheng et al., 2016; Li et al., 2009).

Some literatures confirmed the various pharmacological activities of IC, and the literature studies confirmed the high clearance rate of polyphenol in IC on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical system (Cheng et al., 2017; Xiao et al., 2017). The pigment from IC is typical of brown (chocolate), which belongs to water soluble flavonoids, and the published literature also confirms that it has strong anti-oxidative and antibacterial ability. All of these indicate its role in asthma (Yu et al., 2014; Chen and Jian, 2018).

The purpose of this experiment is to verify the effect of IC on asthma and its potential mechanism. The main chemical composition of IC was also analyzed.

2. Materials and methods

2.1. Plant material

IC was collected from Luotian, Hubei, China, in autumn 2016. A voucher specimen (No. 160902) for reference was kept in our laboratory. The air-dried IC was powdered using a high speed grinder for future use.

2.2. Preparation of extract and preliminary phytochemical screening

2.2.1. Preparation of extract

IC (3.0 Kg, dry weight) was extracted three times with 70% ethanol (solid (g)-

liquid (mL) ratio = 1:8) reflux extraction in a water bath for 3 h, each for 1 h. The extract was filtered by suction filtration and the filtrate was concentrated under vacuum through a rotator evaporator to remove the solvent. The ethanol extract of IC (EEIC) was lyophilized and then stored at low temperature for later use. EEIC was weighed and the percentage yield was calculated at 8.93% (w/w).

2.2.2. Analysis for the chemical components and determination of total flavonoid content

LC-MS/MS analyses of the ethanol fraction of IC were carried out using Shimadzu Prominence Ultra fast liquid chromatography (Shimadzu Corporation, Kyoto, Japan)-tandem API4000 QTrap® triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved on a Shimadzu Inertsil ODS-3 column (100 × 3 mm, 3 μm). The flow rate was kept at 0.5 mL/min and the sample volume injected was set at 10 μL. The column temperature was maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient program was set as follows: 10%B (0-1 min), 10%B ~ 95%B (1-35 min), 95%B (35-36.5 min), 95%~10% B (36.5-36.51 min), 10% B (36.51-40 min).

The ion pairs were monitored in negative mode with multiple reactions monitoring (MRM) and nitrogen was used for both nebulizer and collision gas for electrospray ionization with negative ion polarity. The following parameters were used: curtain gas: 12 psi, collision gas (CAD): medium, ion-Spray voltage: -4500V, temperature: 500 °C, nebulizer gas: 35 psi, auxiliary gas: 0 and interface heater: on. The optimum compound dependent parameters including decluttering potential (DP), collision energy (CE), entrance potential (EP) and cell exit potential (CXP) are shown in Table 1.

The total flavonoid content of EEIC was determined by the aluminium chloride colorimetric method (Patel et al., 2010). In brief, 50 μg of crude extract was dissolved in 1 mL ethanol completely, and mixed with 4 mL of distilled water; 0.3 mL of 50g/L

(5%) NaNO_2 solution was then added and the mixture was allowed to stand for 5 min; 0.3 mL of 100g/L (10%) AlCl_3 solution was added and incubated for 6 min. Then, 2 mL of 1 mol/L NaOH solution was added, and the mixture was brought to a final volume of 10 mL with double-distilled water. The mixture was allowed to incubate for 15 min. The measured absorbance wavelength was 510 nm. Quercetin (12.5, 25, 37.5, 50 and 62.5 $\mu\text{g/mL}$) was used to make a standard curve for total flavonoids. the sample was analyzed in triplicate. As a result, the total flavonoid content of EEIC, calculated from the calibration curve ($R^2 = 0.999$), was 58.72 ± 8.25 mg quercetin equivalents/g EEIC.

2.3. Experimental design and treatments

2.3.1 Experimental animals

Male guinea pigs (SPF, weight 250-300 g) were obtained from the Research Center of Laboratory Animals, Tongji Medical Center, Huazhong University of Science and Technology, Wuhan, China (SCXK 2016-0009). The animals were maintained under a 12 h light/dark cycle at 22°C, relative humidity of 55%, and free access to water and food. All animals were allowed to acclimate for at least 7 days prior to the first treatment. All animal experiments followed the guidelines for the care and uses of animals established by Tongji Medical College, Huazhong University of Science and Technology, and were approved by the Ethics Committee of Tongji Medical College (SYXK 2016-0057).

2.3.2 Guinea pig model of ovalbumin-induced airway inflammation

EEIC and dexamethasone (Dex) are dissolved in a small amount of DMSO respectively, and then was diluted to less than 1/100 with saline. The animals were divided into 6 groups (n=8): normal control group (treated with saline), model control group (induced with OVA and treated with saline), positive control group (induced with OVA and i.p. injected with DEX 1 mg/kg body weight (bw)), EEIC-0.25 group (induced with OVA and treated with EEIC 0.25 g/kg bw), EEIC-0.5 group (induced with OVA and treated with EEIC 0.5 g/kg bw), and EEIC-1 group (induced

with OVA and treated with EEIC 1 g/kg bw).

The guinea pig airway inflammation was induced by chicken ovalbumin (OVA, Grade III; Sigma-Aldrich, US) using the method described previously (Xue et al., 2017). Briefly, guinea pigs were injected intraperitoneally (i.p.) with 1 mL of sensitized solution, containing 100 µg of OVA and equal weight of aluminum hydroxide adjuvant on day 1st and day 14th. The OVA-induced guinea pigs were exposed to inhalation with 1% OVA solution aerosolized using an ultrasonic nebulizer (Oumulong NE-C28, Dalian, China) for 1 h per day from days 21th to 27th after the second sensitization.

Three groups of asthma-induced animals were orally administrated once daily with 0.25, 0.5 and 1 g/kg bw of EEIC, respectively, and positive control group were i.p. injected with 1 mg/kg of Dex from the day 15th to 27th. The model control group were sensitized and treated with saline by gavage.

2.3.3 Effects on the latent period of inducing asthma of asthmatic guinea pigs

During the guinea pigs received last airway inhaled excitation, the time when they appeared symptoms of the whole body rhythmic of abdomen-like dyspnea was recorded as inducing asthma latent period. If more than six minutes it still did not appear the above performance then the latent period was recorded as 360 seconds.

2.3.4 Collection of guinea pigs bronchoalveolar lavage fluid (BALF) and peripheral blood (PB)

Two hours after the last administration, the guinea pigs were given an anesthetic with intra-peritoneal (i.p.) 2% pentobarbital 2.5 mg/kg bw, and then cut the skin along the abdominal mid-line, drew blood using a 5.0-mL syringe with EDTA from the inferior vena cava, sub-packaged as follows: (1) Used for EOS counting: pack in the anticoagulant tubes (EDTA-K2), mixing; (2) Used for the determination of cytokines: put in the ordinary tube and kept in the refrigerator at 4°C for three hours, then centrifuged at 4000rpm at room temperature, last the supernatant was aliquoted and

stored at -20°C.

Meanwhile, the left trachea was exposed, cutting a V-shaped incision at the bottom of the cricoids cartilage for 1 cm and using a blunt syringe needle for endotracheal intubation. Bronchoalveolar lavage operation was carried out with 4°C saline 2 mL and stayed for 1 min, the recovery BALF was collected together and preserved in a single-use human venous blood samples container, after two above lavages, 2-4 mL of lavage fluid was collected in total, blending, and sub-packed as before.

2.3.5 Effects on the number of EOS in BALF and PB of asthmatic guinea pigs

The above BALF and PB were stilled for 15min, and the number of EOS was measured on the fully automatic five-class blood analyzer.

2.3.6 Effects on the levels of IgE, IL-5, NGF, IFN- γ in BALF and PB of asthmatic guinea pigs

Taking the above already sub-packaged BALF supernatant and PB serum, respectively, and the levels of IgE, IL-5, NGF and IFN- γ were measured by enzyme-linked immune-sorbent assay (ELISA), strictly following the various kit instructions.

2.3.7 Effects on expression of IL-5 mRNA in lung tissue of the asthmatic guinea pigs

The specimens were collected on two hours after the last administration. After the guinea pigs were treated as above, the guinea pigs were sacrificed by air embolism. Their right trachea was exposed, their trachea and lungs were separated, and the lung tissues were removed. After sucking blood, they were stored in a refrigerator at -80°C.

The expression of IL-5 mRNA was detected by reverse transcription-PCR (RT-PCR). Using β -actin as an internal reference, quantitative PCR was used to detect the expression of IL-5 mRNA. Primers (IL-5 mRNA primer: upstream primer: CTGCTTAAAGGCAATGAGACTC; downstream primer: ATCCAGGAAGTCTTCACTCTC) were designed based on the GenBank sequence and total RNA was extracted from the lung homogenate using the Trizol reagent. The

cDNA was synthesized via 2 µg of RNA under the action of Oligo (dT) 20 primers and AMV reverse transcriptase. After PCR amplification of 2 µl of cDNA, the product was electrophoresed on a 1.5% agarose gel and the gel was analyzed on the Eagle Eye Image Processing System for the absorbance (A) value of each PCR amplified DNA band, with the A value of the β-actin PCR product as the internal control. The IL-5 mRNA expression was determined as the ratio of the PCR product A value and the β-actin A value.

2.3.8 Histopathological examination of lung tissue of the asthmatic guinea pigs

Paraffin sections of the left lung tissue of sacrifice guinea pigs were used for histopathological observation. Lung tissue was cut into 5µm slices and stained with hematoxylin and eosin (H&E) prior to observation with an optical microscope.

2.3.9 Statistical analysis

All statistical analysis were conducted by the software of Origin 9.0 and the measurement data were expressed as mean ± standard deviation ($\bar{X} \pm S$), the t-test was used to compare the mean value of two samples, the bivariate linear regression and correlation analysis were employed for correlation analysis.

3. Results

3.1 Analysis for the chemical components by LC-MS/MS

In order to clarify some of the substances in EEIC, a preliminary qualitative test of the sample was performed using the MRM-ER-IDA-EPI method and compared with the results of standard solution test. The peaks of the compounds in the standard solution (Fig.1.A) and the sample solution (Fig.1.B) are shown in the Fig.1. (Chromatograms of each standard and the corresponding substance extracted from the sample are reported in the Supplementary Material B, Fig. S2 -S15).

Fig.1. LC-MS/MS chromatogram of the standard solution (Fig.1.A) and the sample

solution (Fig.1.B) in negative mode with multiple reactions monitoring (MRM).

According to the ion pair information and the retention time of each compound, it can be initially confirmed that the sample may contain all the compounds in Table 2. Among them, the contents of betulinic acid, oleanolic acid (and/or ursolic acid), gallic acid, and emodin were significantly higher than those of other compounds. The peak retention time for each standard substance and its counterpart in EEIC was shown in Table 2.

3.2 Effects on the latent period of inducing asthma of asthmatic guinea pigs

Figure 2 shows that the model group significantly shortened the latency of asthma ($p < 0.01$) compared with the normal group, indicating that the asthma induction model was successful. The entire treatment group prolongs the latency of allergic asthma in different degrees compared with the model group. And it suggested that EEICB can significantly increase the threshold of allergic asthma and have a protective effect on allergic asthma guinea pigs (Supplementary Material A, Tab. S1).

Fig. 2. Effects on the latent period of inducing asthma of asthmatic guinea pigs induced by ovalbumin. Results are presented as means $\pm$ S.D. (n=8). The Dex-group was treated by injection with 1 mg/kg bw of dexamethasone. $\#p \leq 0.05$ or $\##p \leq 0.01$ compared with model-group; $*p \leq 0.05$ or $**p \leq 0.01$ compared with normal control-group. The EEIC-group were treated by gavage with 0.25, 0.5, 1 g/kg bw of EEIC, respectively.

3.2 Effects on the number of EOS in BALF and PB of asthmatic guinea pigs

The number of EOS in BALF (Fig.3.A) and PB (Fig.3.B) in the model control group was significantly higher than that in the normal control group ($p < 0.05$). It shows that asthma causes the increase of inflammatory cells in BALF and PB, and has the pathological basis of inflammatory reaction.

Compared with the model control group, in the asthmatic guinea pig BALF and PB,

the treatment group significantly reduced the number of EOS, EEIC high dose group was statistically significant ($p<0.05$), the model group has a very significant statistical significance ($p<0.01$). The results showed that after asthmatic guinea pigs were administered, the inflammation in BALF and PB of asthmatic guinea pigs was reduced (Supplementary Material A, Tab. S2).

Fig.3. Effects on the number of EOS in BALF (Fig.3.A.) and PB (Fig.3.B.) of asthmatic guinea pigs induced by ovalbumin. Results are presented as means $\pm$ S.D. (n=8). The Dex-group was treated by injection with 1 mg/kg bw of dexamethasone. [#] $p\leq 0.05$ or ^{##} $p\leq 0.01$ compared with model-group; ^{*} $p\leq 0.05$ or ^{**} $p\leq 0.01$ compared with normal control-group. The EEIC-group were treated by gavage with 0.25, 0.5, 1 g/kg bw of EEIC, respectively.

3.3 Effects on the levels of IgE, IL-5, NGF, IFN- γ in BALF and PB of asthmatic guinea pigs

The levels of IgE and IL-5 in the BALF (Fig. 4 A) and PB (Fig. 4 B) of guinea pigs in the normal control group were all lower. Compared with the normal control group, the levels of IgE and IL-5 in the model control group were significantly increased ($p<0.05$). The levels of IgE and IL-5 in BALF and PB of asthmatic guinea pigs were significantly decreased in low, medium and high dose groups. Compared with the model control group, there was a significant difference between the medium and high dose groups ($p<0.05$). In particular, high-dose EEIC showed a significant difference in IL-5 ($p<0.01$). This shows that EEIC can promote the restoration of cytokines IgE, IL-5 to normal levels, which may be one of its anti-asthmatic targets.

In addition, the level of IFN- γ in the serum of the normal control group was higher. The IFN- γ content in the model control group was significantly lower than that in the normal control group ($p<0.05$). After administration, the levels of IFN- γ in BALF and PB were significantly increased. Compared with the model control group, the increase in the medium and high dose groups was significant and there was a significant

difference ($p<0.05$). It shows that EEIC can increase the level of IFN- γ . Compared with the model group, there was no significant difference in IFN- γ in the positive control group (Fig. 4).

Although there was a down-regulation of NGF in BALF and PB in the high, middle and low dose groups and positive control groups, there was no significant difference from the model group ($p>0.05$). It shows that EEIC has less intervention on NGF in BALF and PB of asthmatic guinea pigs. Compared with the model group, there was a significant difference in the level of NGF in the positive control group ($p<0.05$) (Fig. 4) (Supplementary Material A, Tab. S3 and S4).

Fig.4. Effects on the levels of IgE, IL-5, NGF, IFN- γ in BALF and PB of asthmatic guinea pigs induced by ovalbumin. All values are expressed as mean $\pm$ S.D. (n=8). The Dex-group was treated by injection with 1 mg/kg bw of dexamethasone. [#] $p\leq 0.05$ or ^{##} $p\leq 0.01$ compared with model-group; * $p\leq 0.05$ or ** $p\leq 0.01$ compared with normal control-group. The EEIC-group were treated by gavage with 0.25, 0.5, 1 g/kg bw of EEIC, respectively.

3.4 Effects on expression of IL-5 mRNA in lung tissue of the asthmatic guinea pigs

As can be seen from Figure 5, the expression of IL-5 mRNA in the normal control group was low, whereas the expression of IL-5 mRNA in the model control group was significantly increased. With the increase of EEIC dose, the expression of IL-5 mRNA in the asthmatic guinea pigs was gradually lower. Compared with the model control group, there was a significant difference between the medium and high dose groups ($p<0.05$). The results suggest that EEIC can regulate the transcription of IL-5 mRNA from the gene level, which may be an effective mechanism for inhibiting asthma attack (Supplementary Material A, Tab. S5).

Fig.5. Effects on expression of IL-5 mRNA in lung tissue of the asthmatic guinea

pigs induced by ovalbumin. Results are presented as means $\pm$ S.D. (n=8). The Dex-group was treated by injection with 1 mg/kg bw of dexamethasone. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group; ^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group. The EEIC-group were treated by gavage with 0.25, 0.5, 1 g/kg bw of EEIC, respectively.

3.5 Histopathological examination of lung tissue of the asthmatic guinea pigs

No abnormal histopathological changes were observed in the lung tissue sections of normal control guinea pigs. In the model group, there were significant inflammatory cell infiltrations in the bronchi, perivascular and alveolar spaces. The bronchial smooth muscle is thickened. The alveolar space becomes smaller. Compared with the model group, the proliferation of bronchial smooth muscle was decreased, and the inflammatory cells around the bronchus, perivascular area and alveolar cavity were decreased in different treatment groups. The degree of improvement was positively correlated with the dose. The positive control group improved significantly compared with the model group, and a small amount of inflammatory cell infiltration was still seen compared with the normal group. Compared with the model group, the pathological abnormalities in the positive control group were significantly improved, but a small amount of inflammatory cell infiltration was still seen compared with the normal group.

Fig.6. H&E staining of lung tissue sections of guinea pigs (H&E staining, $\times 200$) . A. normal control group (treated with saline); B. model control group (induced with OVA and treated with saline); C. EEIC-0.25 group (induced with OVA and treated with EEIC 0.25 g/kg); D. EEIC-0.5 group (induced with OVA and treated with EEIC 0.5 g/kg); E. EEIC-1 group (induced with OVA and treated with EEIC 1 g/kg); F. positive control group (induced with OVA and i.p. injected with DEX 1 mg/kg). Inflammatory cell infiltrations in the bronchi (green arrow); perivascular (red arrow) and alveolar spaces (blue arrow); Thickened bronchial smooth muscle (yellow arrow)

and smaller alveolar space (violet arrow).

4. Discussion

The respiratory system of guinea pigs is very sensitive to the stimulation of chemical mediators and is an ideal animal model for studying the mechanism of asthma and the development of anti-asthma drugs (Smith and Broadley, 2007; Lim et al., 2015). In this experiment, guinea pigs were sensitized with an intraperitoneal injection of antigen OVA, and 14 days later were stimulated with OVA inhalation to establish a guinea pig asthma model.

EOS acts as an effectors' cell in the development of allergic inflammation in asthma (Li et al., 2009). It participates in mediating the survival, chemotaxis and activation of various inflammatory cells, and is an important cause of allergic inflammation in airways (Johnston et al., 2010). EOS infiltration is a significant physiological feature that distinguishes asthma respiratory inflammation from other inflammatory diseases (Khaitov et al., 2018). Therefore, the key to treating inflammation in asthma is to reduce the amount of EOS in lung tissue (Lugogo et al., 2016). In this experiment, the number of EOS in both BALF and PB of asthmatic guinea pigs was decreased after treatment with EEIC. As a result, the degree of inflammation infiltration of tissue was also decreased. And the results from the latent period of inducing asthma of asthmatic guinea pigs also suggest that treatment with EEIC can significantly relieve the acute attack of asthma.

Although the pathogenesis of bronchial asthma has not yet been fully clarified, it is believed that Th1/Th2 immune response imbalance and Th2 dominant response play a key role in its occurrence and development (Schroder et al., 2010; Tong et al., 2018; Li et al., 2018). Th1 cells mainly secrete IFN- γ cytokines, and IFN- γ is considered to be an anti-asthmatic factor, which inhibits the secretion of IL-5 from Th2 cells, thereby reducing the airway responsiveness (Jin et al., 2011; Xue et al., 2017). Th2 cells secrete inflammatory cytokines such as IL-5. The abnormal increase of Th2 cells leads to the increase of IL-5 and other inflammatory factors, which can stimulate the

proliferation and differentiation of EOS and induce the production of IgE (Zhou et al., 2014). NGF may participate in promoting and amplifying the immune imbalance effect of asthma Th2 cytokines by up-regulating the expression of lymphocyte Th2 cytokine IL-5. NGF can play a role in amplifying airway inflammation by connecting the nervous system and the immune system (Wang et al., 2017). In the PB and BALF of patients involved in the allergen challenge test, the expression level of NGF was found to be significantly higher than that of the normal control group (Gazzinelli-Guimaraes et al., 2016). This experiment examined the expression of the above related cytokines in PB and BALF in OVA-sensitized guinea pigs after administration. Meanwhile, the increase in the number of EOS in allergic diseases is mainly caused by IL-5, so the expression level of IL-5 mRNA was detected experimentally.

Results show that EEIC can regulate the body's immune response by reducing abnormally elevated IgE levels. At the same time, it promotes the secretion of IFN- γ but down-regulates IL-5 levels, thereby regulating the imbalance of Th1/Th2. After asthmatic guinea pigs were treated by EEIC, the regulation of IL-5 mRNA transcription was consistent with the down-regulation of IL-5 in BALF and PB. In addition, the effect of EEIC on NGF did not show a significant difference, whether it is a molecular target for EEIC anti-asthma needs to be further explored.

In this study, EEIC was rich in steroids, polyphenols, and flavonoids. Betulinic acid (polyphenols), oleanolic acid and ursolic acid (steroids), gallic acid (polyphenols), emodin, and flavonoids were identified in EEIC by means of HPLC-MS/MS. Several reports have revealed the role of these phytochemicals in reducing inflammation and improving asthma symptoms. Betulinic acid can interact with the glucocorticoid receptor and simultaneously promote the synthesis of endogenous anti-inflammatory proteins, thus exhibiting anti-inflammatory biological activity (Oosunsanmi et al., 2016). Intraperitoneal injection of gallic acid (GA) has significant anti-inflammatory effects on egg whites or serotonin-induced acute inflammation and formaldehyde-induced chronic inflammation (Tsang et al., 2016). The ester of GA (EGCG) effectively inhibits Th2 type immune responses by inducing expression of Foxp3 and

IL-10, thereby improving airway inflammation and reducing airway remodeling (Kim et al., 2012). Emodin reduces cytokine levels in Th2 subsets to improve asthmatic airway inflammation (Chu et al., 2012). Oleanolic acid (OA) and ursolic acid (UA) are isomeride, and related studies have confirmed that they have inhibitory effects on allergic reactions of type I-IV and various animal models of inflammation (Liu et al., 2016). UA plays an important anti-inflammatory role in bronchial asthma by inhibiting nuclear factor kappa B, increasing antioxidant enzyme activity, and reducing lipid peroxidation (Gnoatto et al., 2008). OA has protective effects on oxidative stress injury of human airway smooth muscle cells cultured in vitro and interferes with airway inflammation and airway remodeling in asthma (Lei et al., 2014; Bueno-Silva et al., 2017). Among flavonoids, quercetin, luteolin, vitexin, apigenin, and chrysin can reduce airway inflammation and airway hyperresponsiveness in asthmatic mice (Yu et al., 2014; Sagun et al., 2017). Moreover, Chrysin can restore the sensitivity of hormone-resistant asthma mice to hormone therapy (Du et al., 2012). The experimental results show that EEIC can reduce the number of EOS in BALF and BA of ovalbumin-induced guinea pig model of asthma, decrease the levels of IgE and IL-5, and increase the levels of IFN- γ . Pathological examination of paraffin section of lung tissue showed that EEIC can reduce the thickening of bronchial smooth muscle and reduce the infiltration damage of tissues by various inflammatory cells. All of these results suggest that steroids, polyphenols and flavonoids in EEIC may be the effective substances for its anti-asthma activity.

In LC-MS/MS analysis of chemical components, it was found that oleanolic acid and ursolic acid can hardly be separated even when using a long gradient, so it can not be discerned whether the signal of 30.66 min in the sample is oleanolic acid or ursolic acid or both. Due to the low response of most compounds in the sample, it is doubted that the signals are the residual components of the system rather than the substances in the sample. And this possibility was then excluded by entering the blank mobile phase after the analysis of the standard test solution and before the sample test solution (Supplementary Material B, Fig. S16).

5. Conclusions

The ethanol extract of IC, was able to reduce the number of EOS in BALF and PB of ovalbumin-induced guinea pig model of asthma. Meanwhile, it also significantly reduced the levels of inflammation-related factors IgE and IL-5, decreased the expression of IL-5 mRNA in lung tissue, and increased the level of IFN- γ factor. All of these may be the important mechanism of IC inhibition of asthmatic airway inflammation. The presence of flavonoids, terpenoids and phenolic compounds in IC might be responsible for these activities.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Financial supports from the National Nature Science Foundation of China (No. 31670354, 31270395) and the Fundamental Research Funds for the Central Universities (2015TS137) are gratefully acknowledged.

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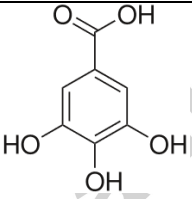
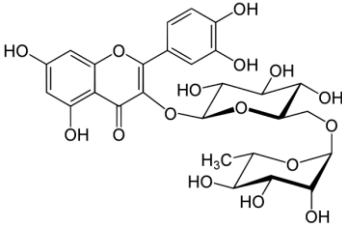
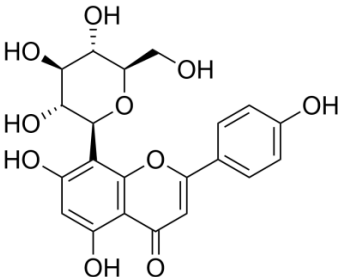
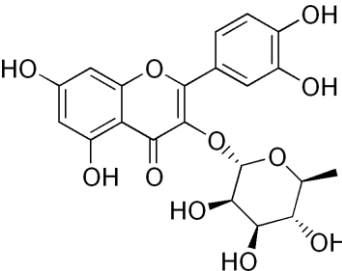
Table 1. The optimum compound dependent parameters for each analysis component.

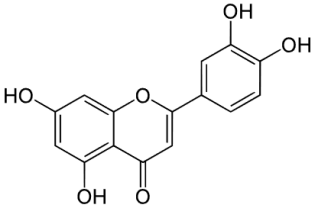
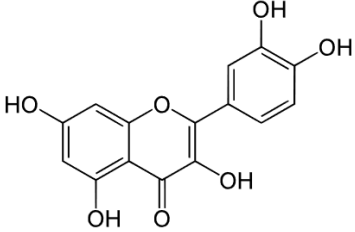
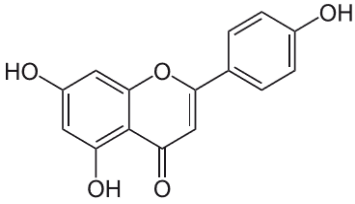
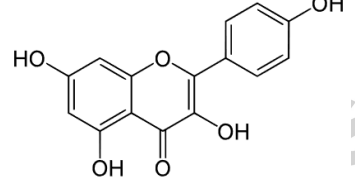
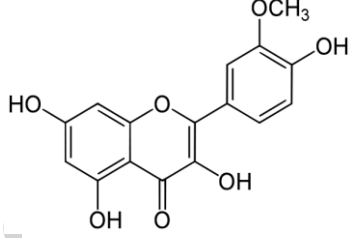
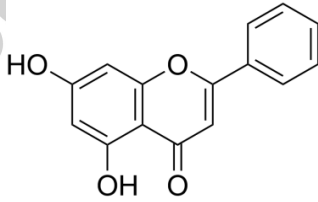
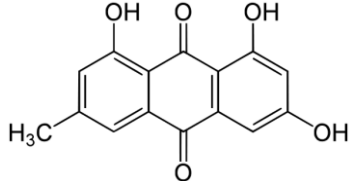
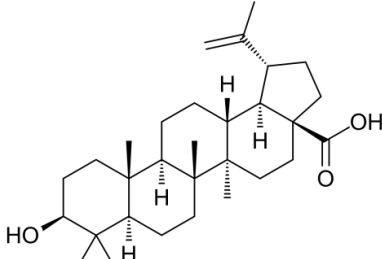
Compounds	Precursor ion (m/z)	Product ion (m/z)	DP (V) ¹	CE (V) ²	EP (V) ³	CXP(V) ⁴
Gallic acid	168.8	125	-65	-22	-10	-7
Rutin	609.3	300	-145	-40	-10	-7
Vitexin	431	311.1	-120	-30	-10	-7
Quercitrin	447.1	300	-105	-34	-10	-7
Luteolin	285	133	-105	-48	-10	-9
Quercetin	301	150.9	-100	-28	-10	-7
Apigenin	268.9	117.1	-105	-46	-10	-7
Kaempferol	284.9	92.8	-115	-48	-10	-5
Isorhamnetin	314.9	300	-100	-30	-10	-7
Chrysin	252.9	63	-115	-50	-10	-3
Emodin	269	224.8	-130	-36	-10	-15

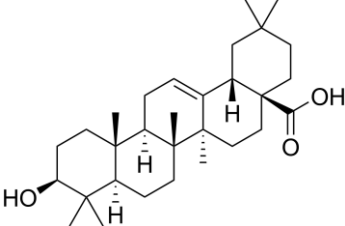
Betulinic acid	455.3	455.3	-150	-5	-10	-15
Oleanolic acid	455.3	455.3	-150	-5	-10	-15
Ursolic acid	455.3	455.3	-150	-5	-10	-15

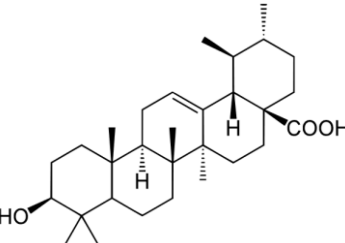
¹ DP is the abbreviation of decluttering potential; ² CE is the abbreviation of collision energy; ³ EP is the abbreviation of entrance potential; ⁴ CXP is the abbreviation of cell exit potential.

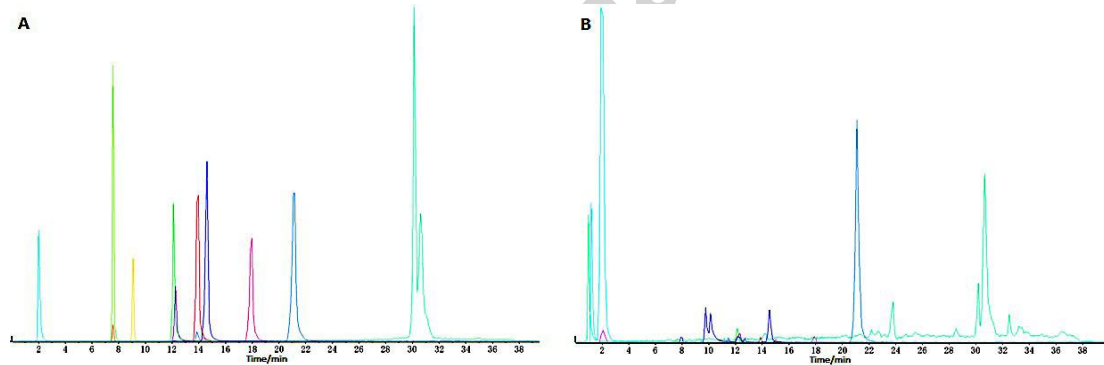
Table 2. Peak retention time for each standard substance and its counterpart in EEIC.

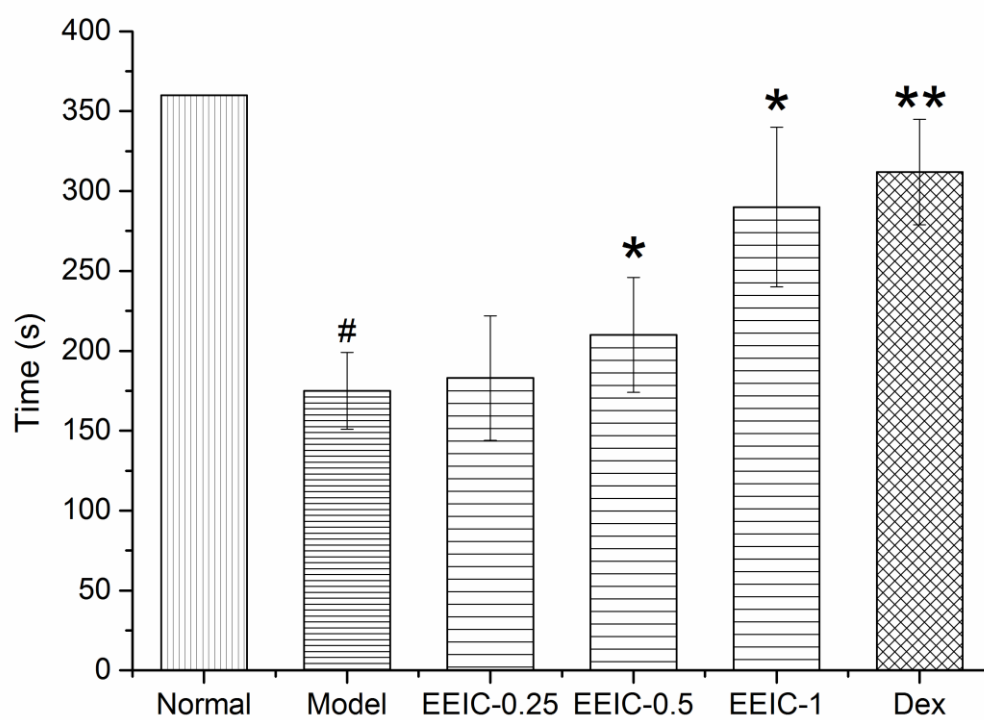
Peak order	Compounds	Molecular formula	Chemical structure	Retention time of reference (min)	Retention time of sample (min)
1	Gallic acid	C ₇ H ₆ O ₅		1.99	2
2	Rutin	C ₂₇ H ₃₀ O ₁₆		7.57	7.53
2	Vitexin	C ₂₁ H ₂₀ O ₁₀		7.57	7.55
4	Quercitrin	C ₂₁ H ₂₀ O ₁₁		9.08	9.04

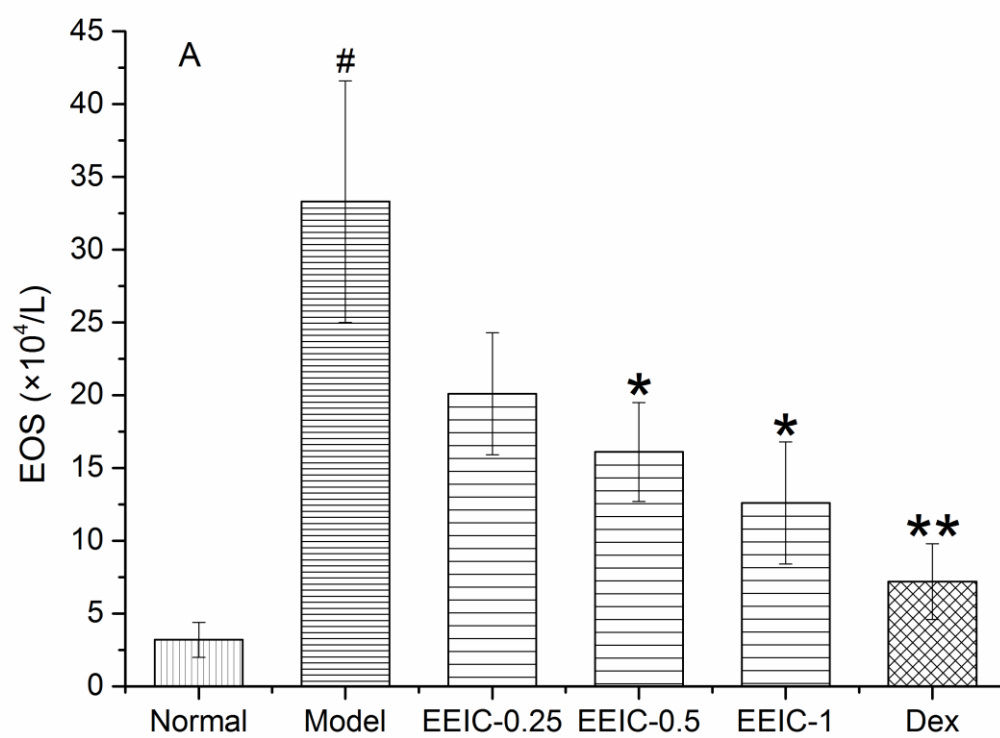
5	Luteolin	$C_{15}H_{10}O_6$		12.11	12.13
6	Quercetin	$C_{15}H_{10}O_7$		12.26	12.29
7	Apigenin	$C_{15}H_{10}O_5$		13.92	13.87
8	Kaempferol	$C_{15}H_{12}O_6$		14.37	14.29
9	Isorhamnetin	$C_{16}H_{12}O_7$		14.61	14.53
10	Chrysin	$C_{15}H_{10}O_4$		17.93	17.89
11	Emodin	$C_{15}H_{10}O_5$		21.13	21.09
12	Betulinic acid	$C_{30}H_{48}O_3$		30.14	30.15

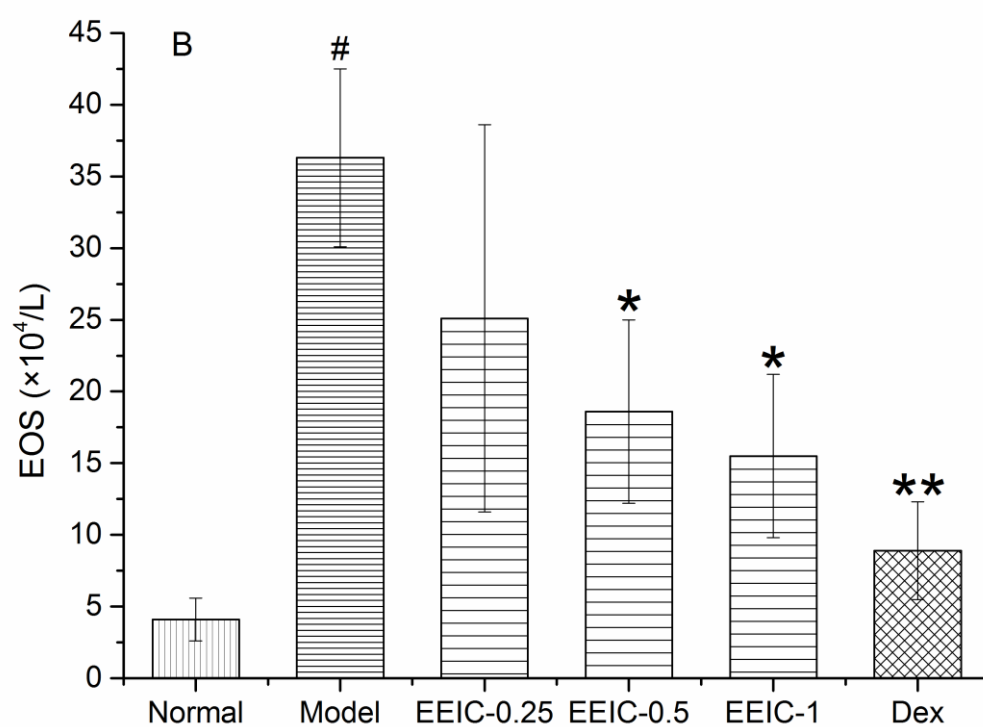
13	Oleanolic acid	$C_{30}H_{48}O_3$		30.63	30.66
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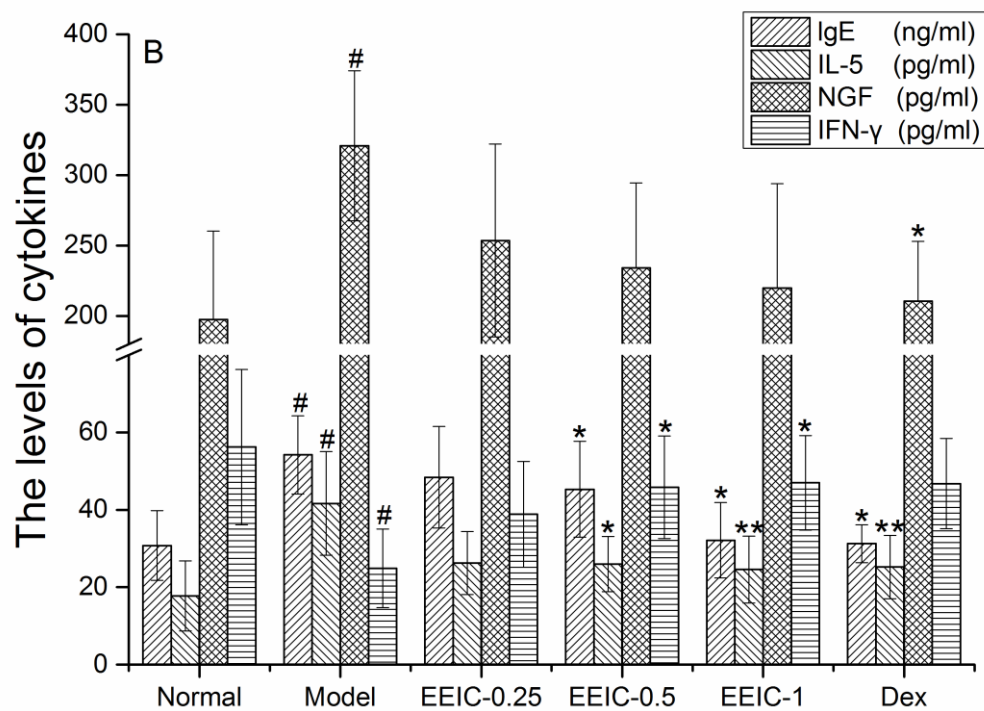
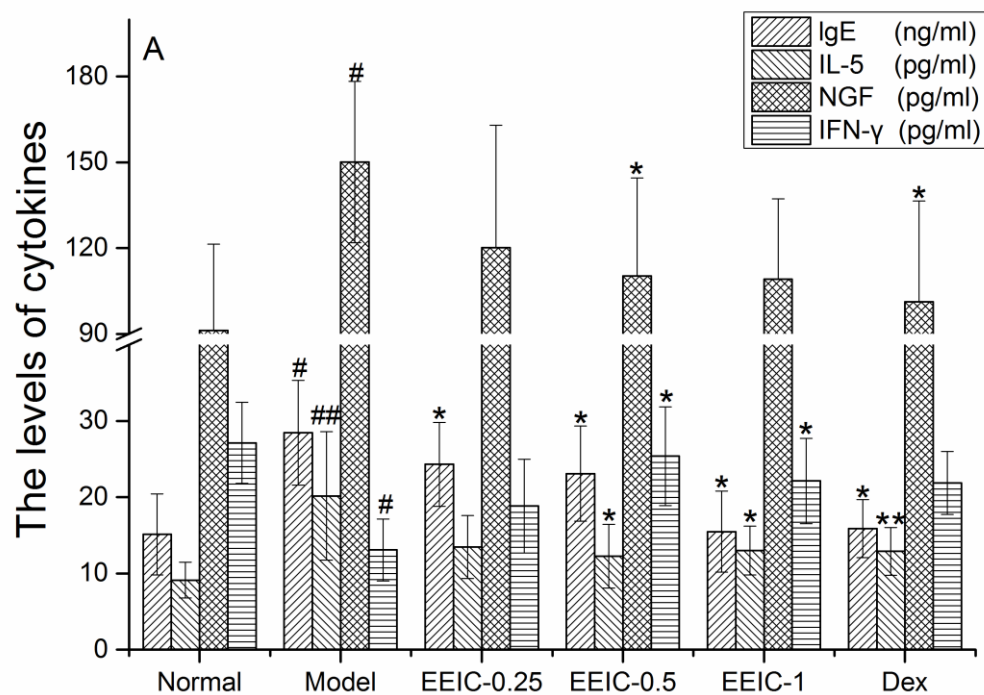
13	Ursolic acid	$C_{30}H_{48}O_3$		30.63	30.66
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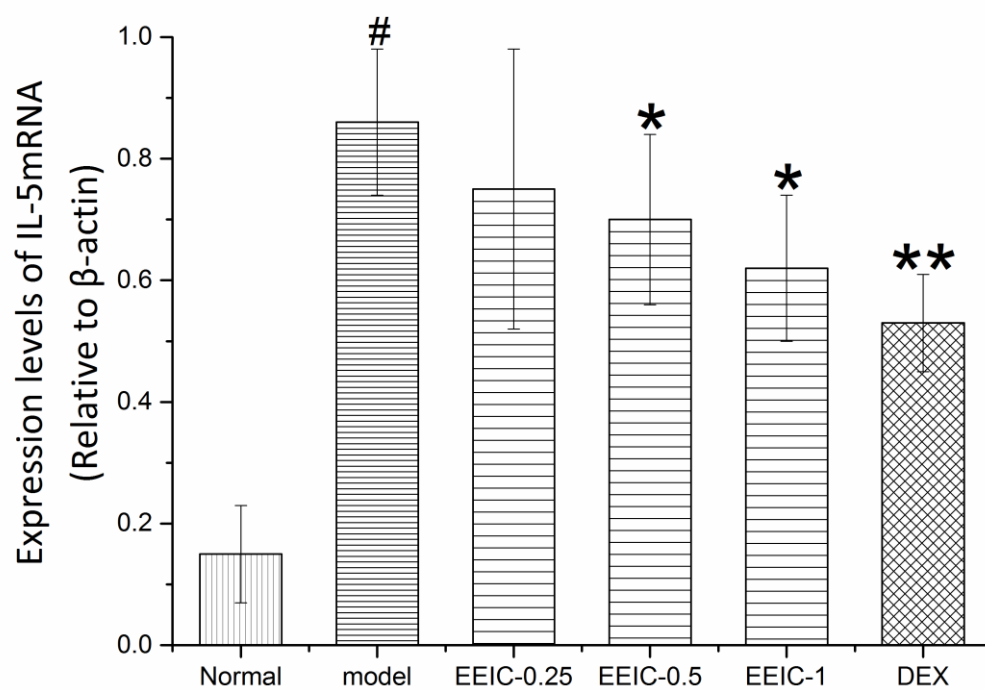


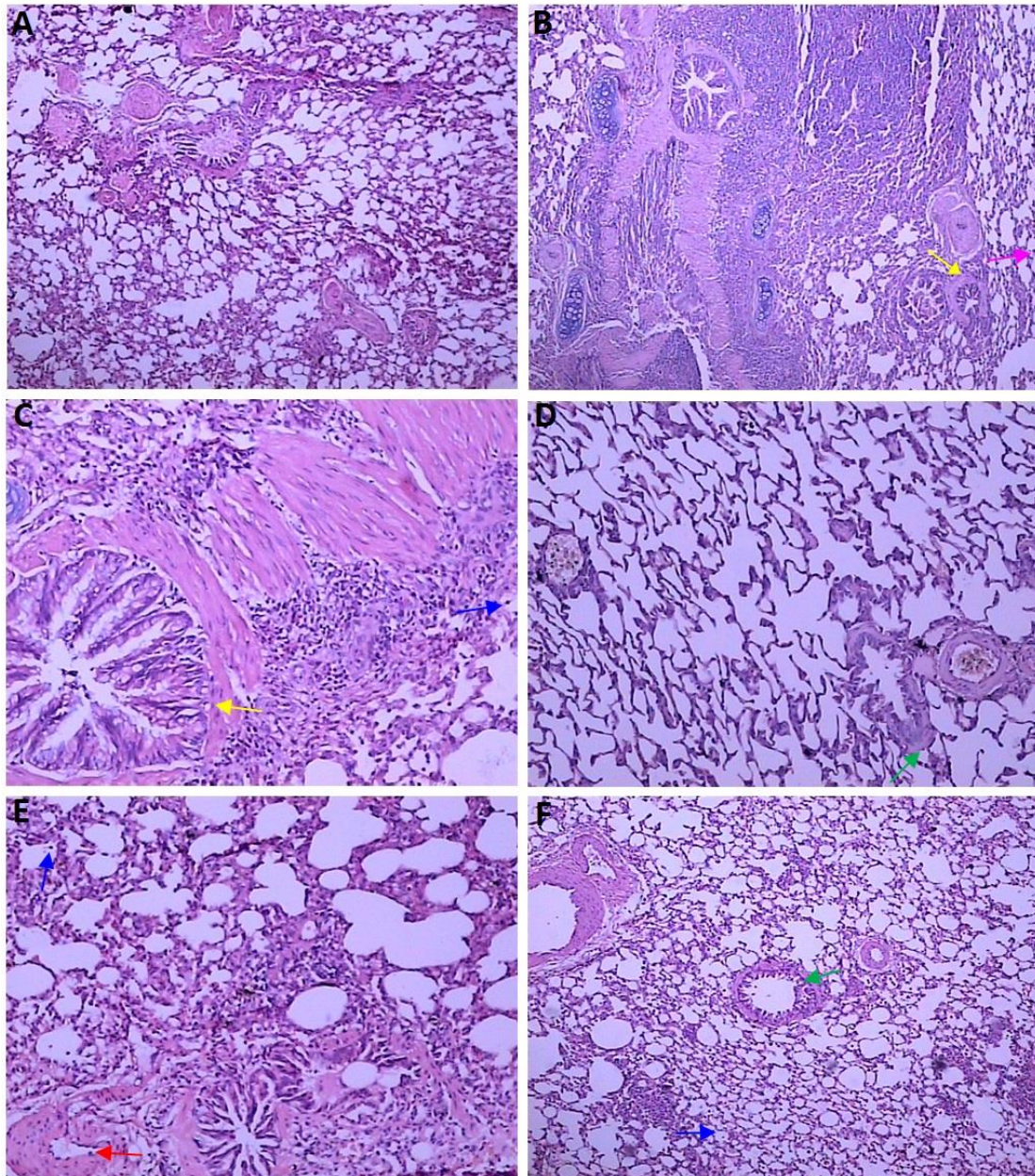












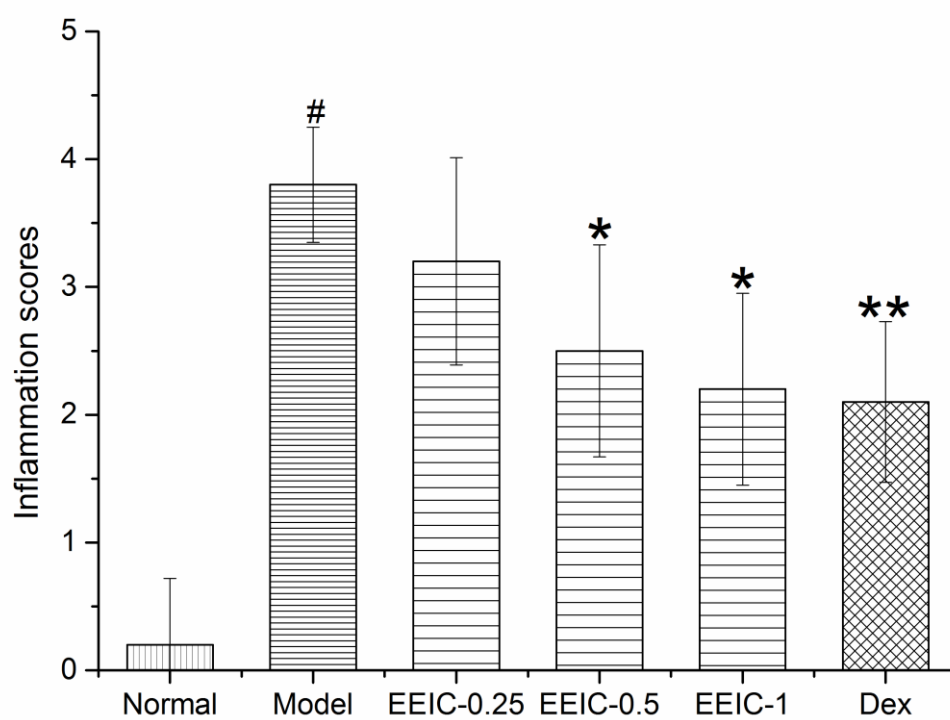


Table S1. Effects on the latent period of inducing asthma of asthmatic guinea pigs.

Group	Time (s)
Normal	360±0
model	175±24 [#]
EEIC-0.25	183±39
EEIC-0.5	210±36 [*]
EEIC-1	290±50 [*]
DEX	312±33 ^{**}

Values are mean ± S.E.M; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group; ^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

Table S2. Effects on the number of EOS in BALF and PB of asthmatic guinea pigs

Group	the number of EOS in BALF	the number of EOS in PB
Normal	3.2±1.2	4.1±1.5
model	33.3±8.3 [#]	36.3±6.2 [#]
EEIC-0.25	20.1±4.2	25.1±13.5
EEIC-0.5	16.1±3.4 [*]	18.6±6.4 [*]
EEIC-1	12.6±4.2 [*]	15.5±5.7 [*]
DEX	7.2±2.6 ^{**}	8.9±3.4 ^{**}

Values are mean ± S.E.M, $\times 10^4/\text{mL}$; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group; ^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

Table S3. Effects on the levels of IgE, IL-5, NGF, IFN- γ in BALF of asthmatic guinea pigs

cytokines	group					
	Normal	model	EEIC-0.25	EEIC-0.5	EEIC-1	DEX
IgE(ng/ml)	15.13±5.32	28.45±6.87 [#]	24.32±5.49 [*]	23.10±6.23 [*]	15.48±5.32 [*]	15.89±3.81 [*]
IL-5(pg/ml)	9.13±2.36	20.18±8.43 ^{##}	13.47±4.12	12.27±4.18 [*]	13.01±3.21 [*]	12.90±3.15 ^{**}
NGF(pg/ml)	91.25±30.12	150.13±28.17 [#]	120.14±42.87	110.32±34.28 [*]	109.12±28.19	101.25±35.24 [*]
IFN- γ (pg/ml)	27.14±5.32	13.08±4.07 [#]	18.84±6.14	25.39±6.48 [*]	22.14±5.59 [*]	21.89±4.13

Values are mean ± S.E.M; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group; ^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

Table S4. Effects on the levels of IgE, IL-5, NGF, IFN- γ in PB of asthmatic guinea pigs

cytokines	group					
	Normal	model	EEIC-0.25	EEIC-0.5	EEIC-1	DEX
IgE(ng/ml)	30.76 $\pm$ 9.02	54.18 $\pm$ 10.12 [#]	48.41 $\pm$ 13.09	45.29 $\pm$ 12.37 [*]	32.13 $\pm$ 9.75 [*]	31.24 $\pm$ 4.87 [*]
IL-5(pg/ml)	17.71 $\pm$ 9.05	41.64 $\pm$ 13.42 [#]	26.22 $\pm$ 8.13	26.01 $\pm$ 7.12 [*]	24.59 $\pm$ 8.63 ^{**}	25.20 $\pm$ 8.17 ^{**}
NGF(pg/ml)	197.45 $\pm$ 62.71	320.79 $\pm$ 53.17 [#]	253.47 $\pm$ 68.45	234.10 $\pm$ 60.37	219.78 $\pm$ 74.19	210.47 $\pm$ 42.34 [*]
IFN- γ (pg/ml)	56.18 $\pm$ 20.09	24.90 $\pm$ 10.16 [#]	38.82 $\pm$ 13.70	45.79 $\pm$ 13.25 [*]	46.98 $\pm$ 12.19 [*]	46.75 $\pm$ 11.63

Values are mean $\pm$ S.E.M; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group;
^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

Table S5. Effects on expression of IL-5 mRNA in lung tissue of the asthmatic guinea pigs

Group	IL-5/ β -actin(mRNA)
Normal	0.15 $\pm$ 0.08
model	0.86 $\pm$ 0.12 [#]
EEIC-0.25	0.75 $\pm$ 0.23
EEIC-0.5	0.70 $\pm$ 0.14 [*]
EEIC-1	0.62 $\pm$ 0.12 [*]
DEX	0.53 $\pm$ 0.08 ^{**}

Values are mean $\pm$ S.E.M; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group;
^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

Table S6. Effects on inflammation scores of inducing asthma of asthmatic guinea pigs.

Group	Inflammation scores
Normal	0.21 $\pm$ 0.52
model	3.84 $\pm$ 0.45 [#]
EEIC-0.25	3.22 $\pm$ 0.81
EEIC-0.5	2.51 $\pm$ 0.83 [*]
EEIC-1	2.23 $\pm$ 0.75 [*]
DEX	2.11 $\pm$ 0.63 ^{**}

Values are mean $\pm$ S.E.M; $n = 8$ in each group. [#] $p \leq 0.05$ or ^{##} $p \leq 0.01$ compared with model-group;
^{*} $p \leq 0.05$ or ^{**} $p \leq 0.01$ compared with normal control-group.

1. Chromatograms of each standard and the corresponding substance extracted from the sample chromatogram are as follows.

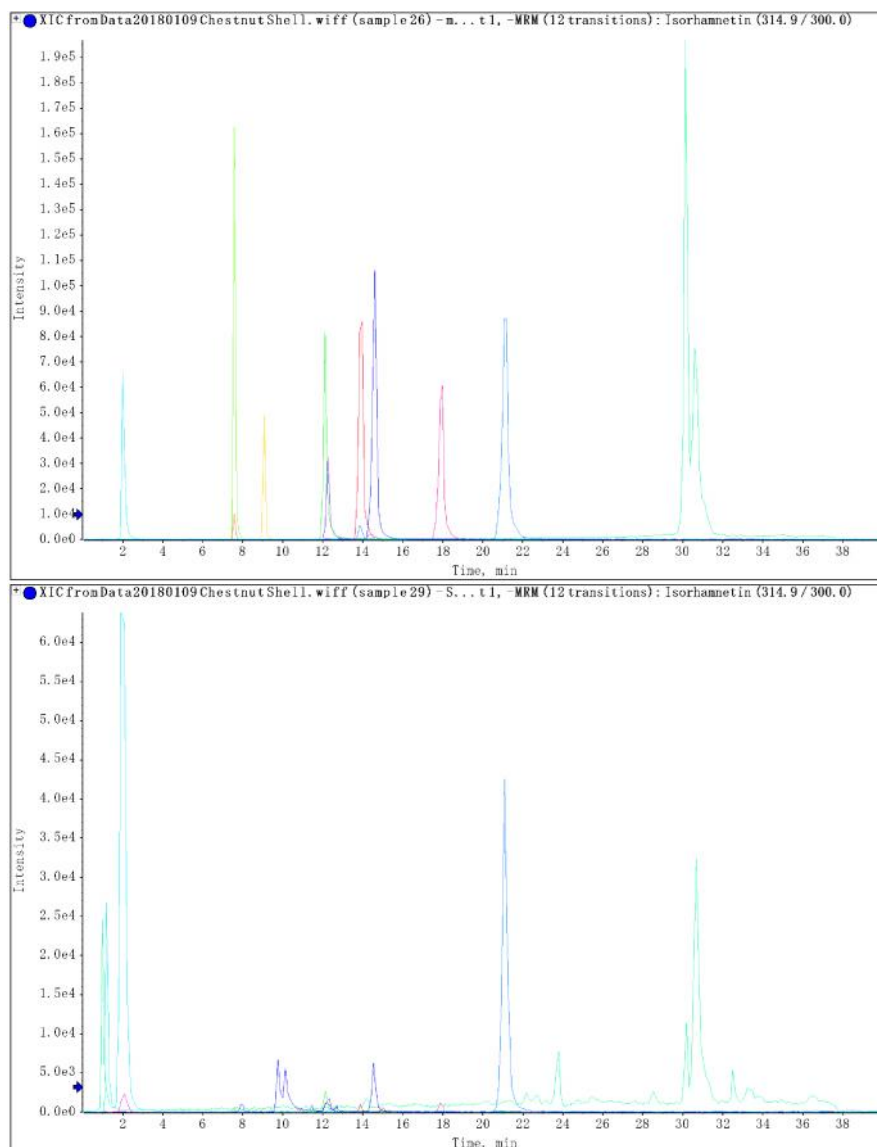


Fig.S1 Chromatograms of standard substances (upper) and the sample (lower).

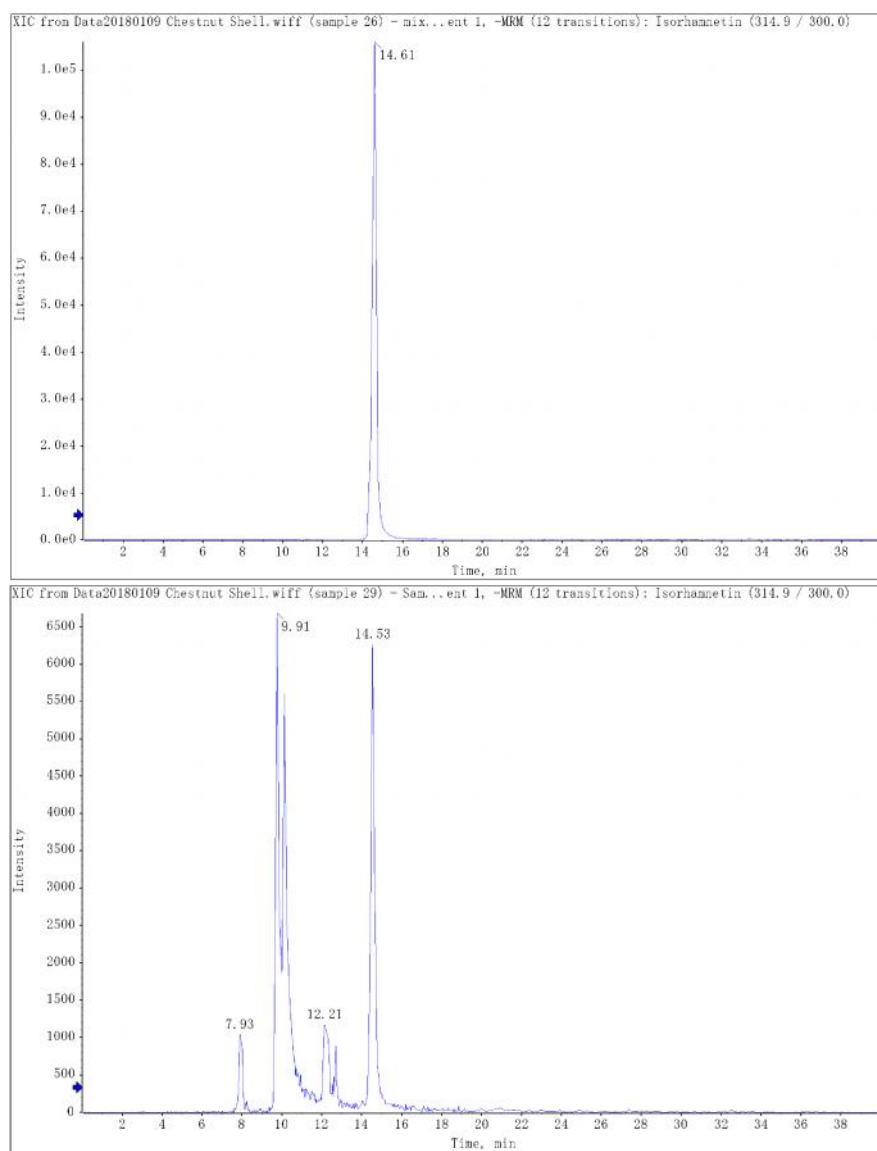


Fig.S2 Chromatograms of Isorhamnetin in the standard substance (upper) and the sample (lower).

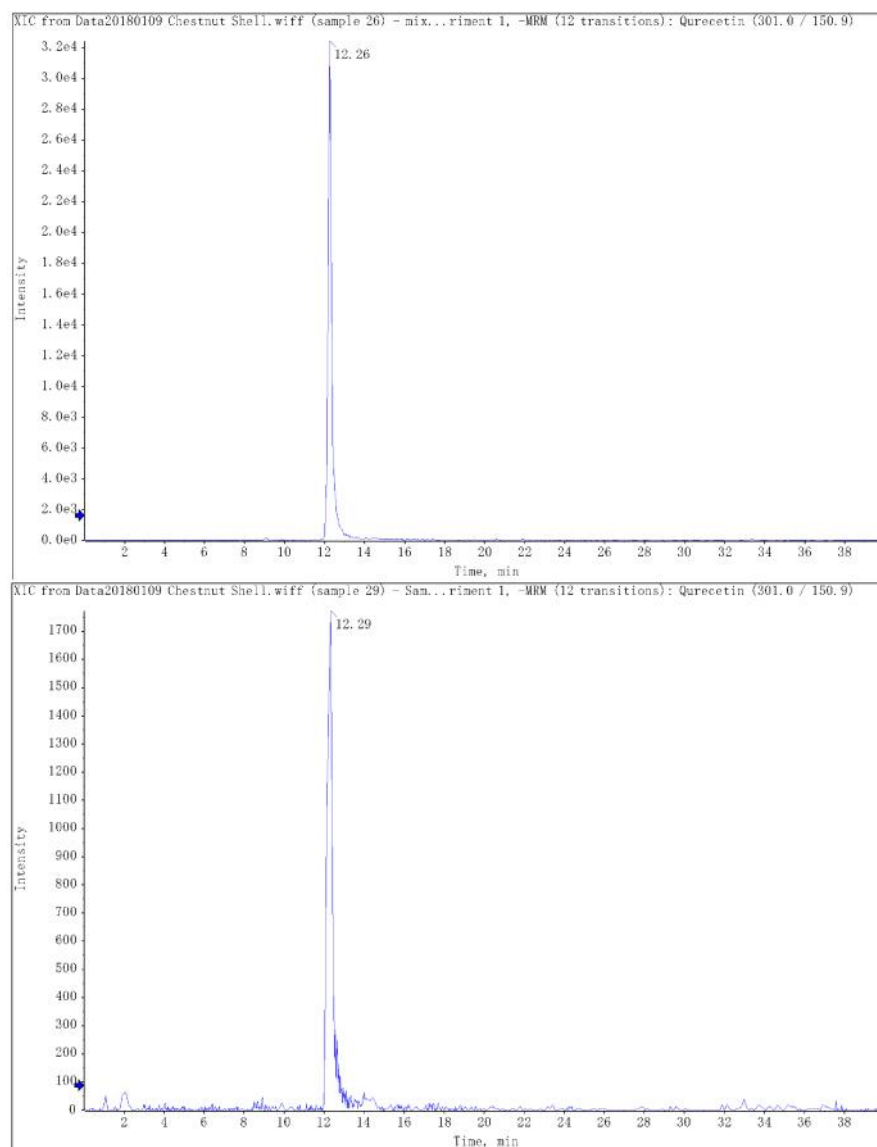


Fig.S3 Chromatograms of Quercetin in the standard substance (upper) and the sample (lower).

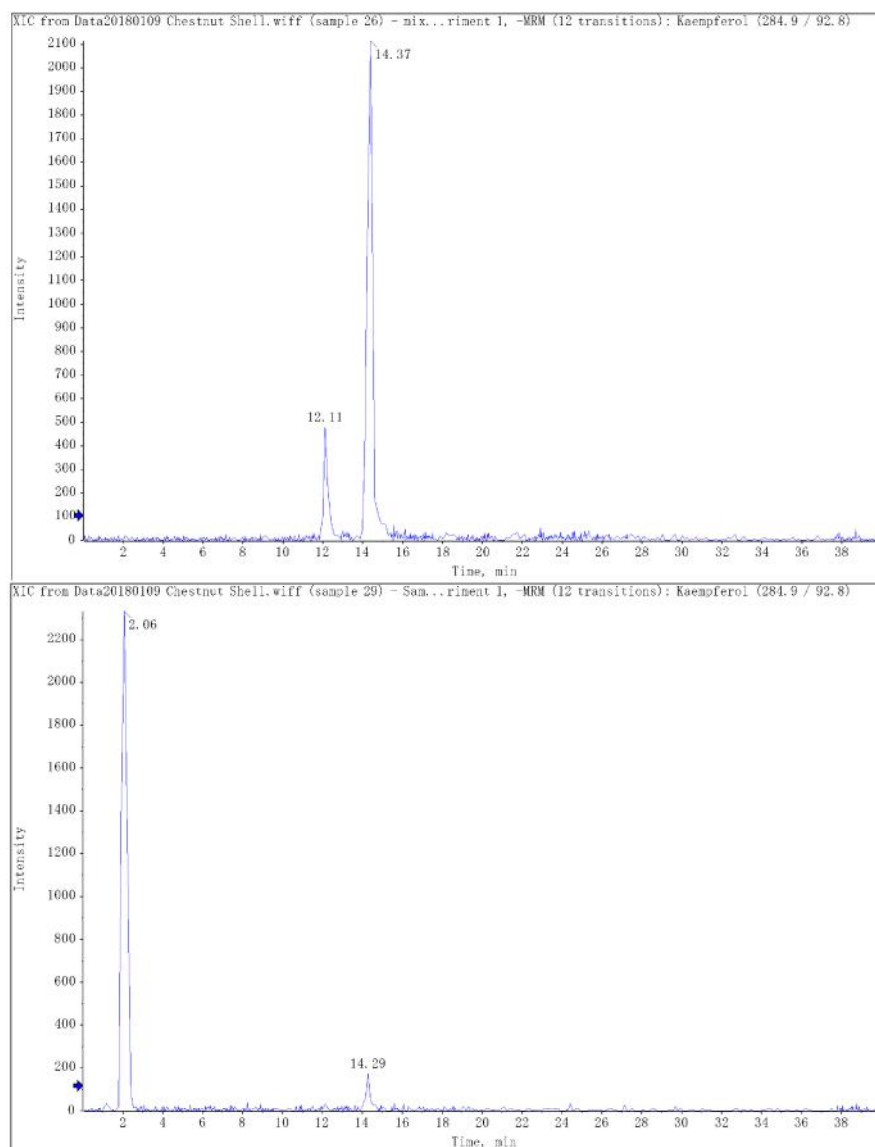


Fig.S4 Chromatograms of Kaempferol in the standard substance (upper) and the sample (lower).

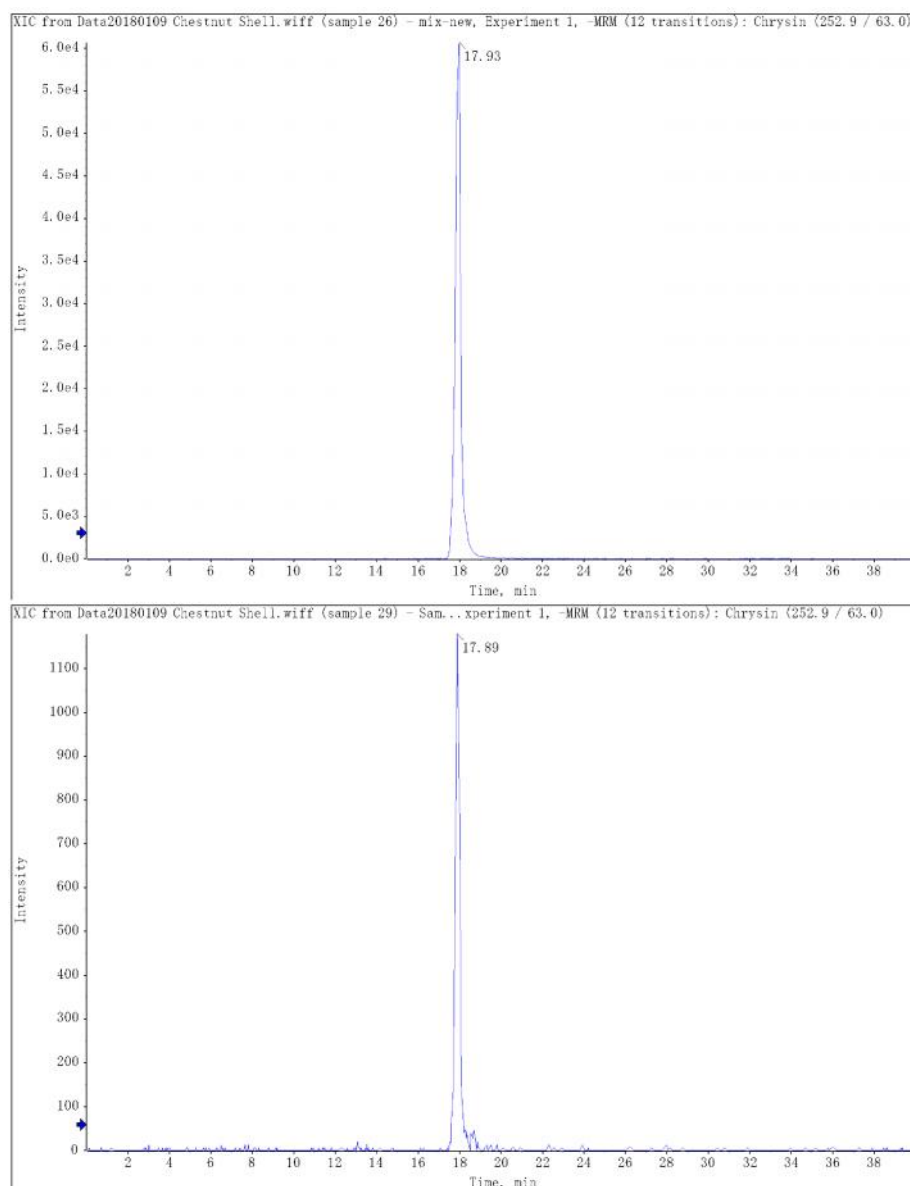


Fig.S5 Chromatograms of Chrysin in the standard substance (upper) and the sample (lower).

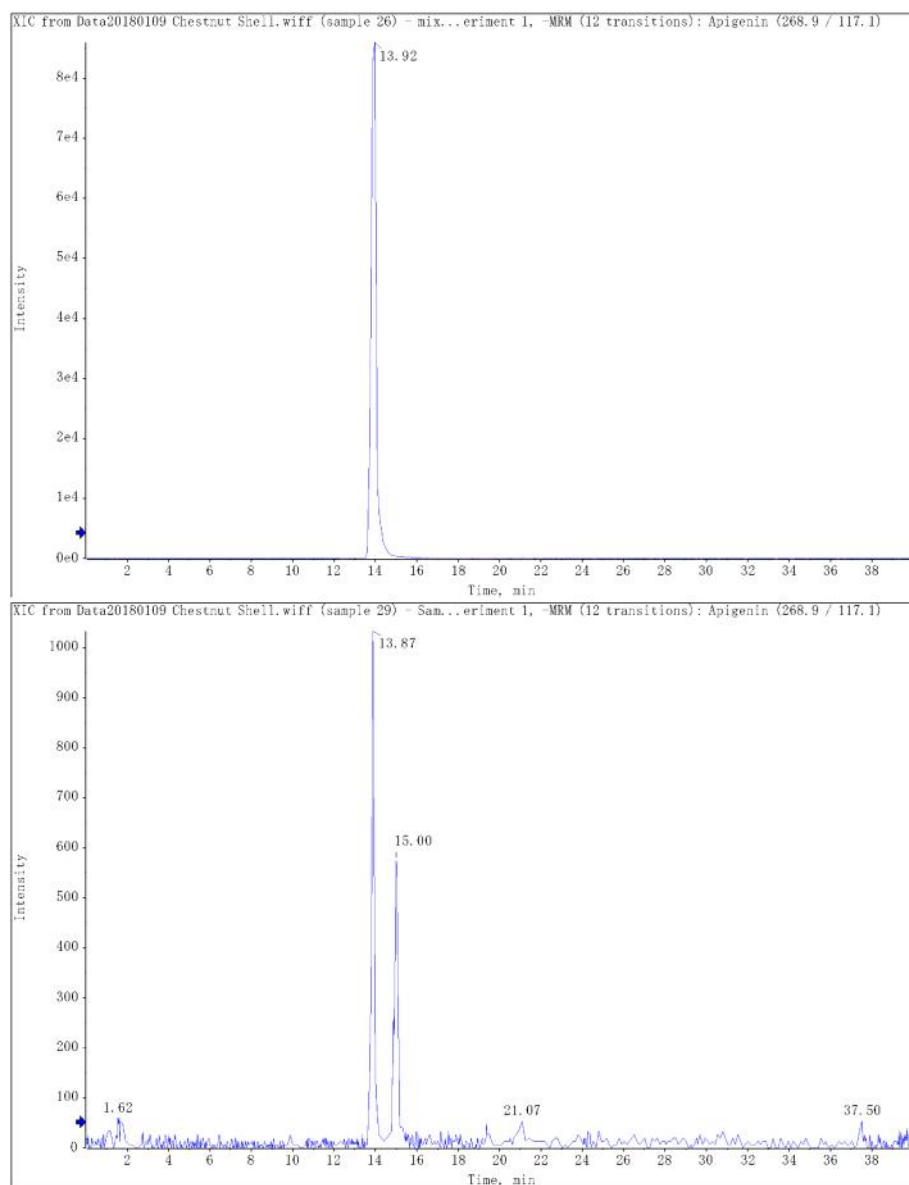


Fig.S6 Chromatograms of Apigenin in the standard substance (upper) and the sample (lower).

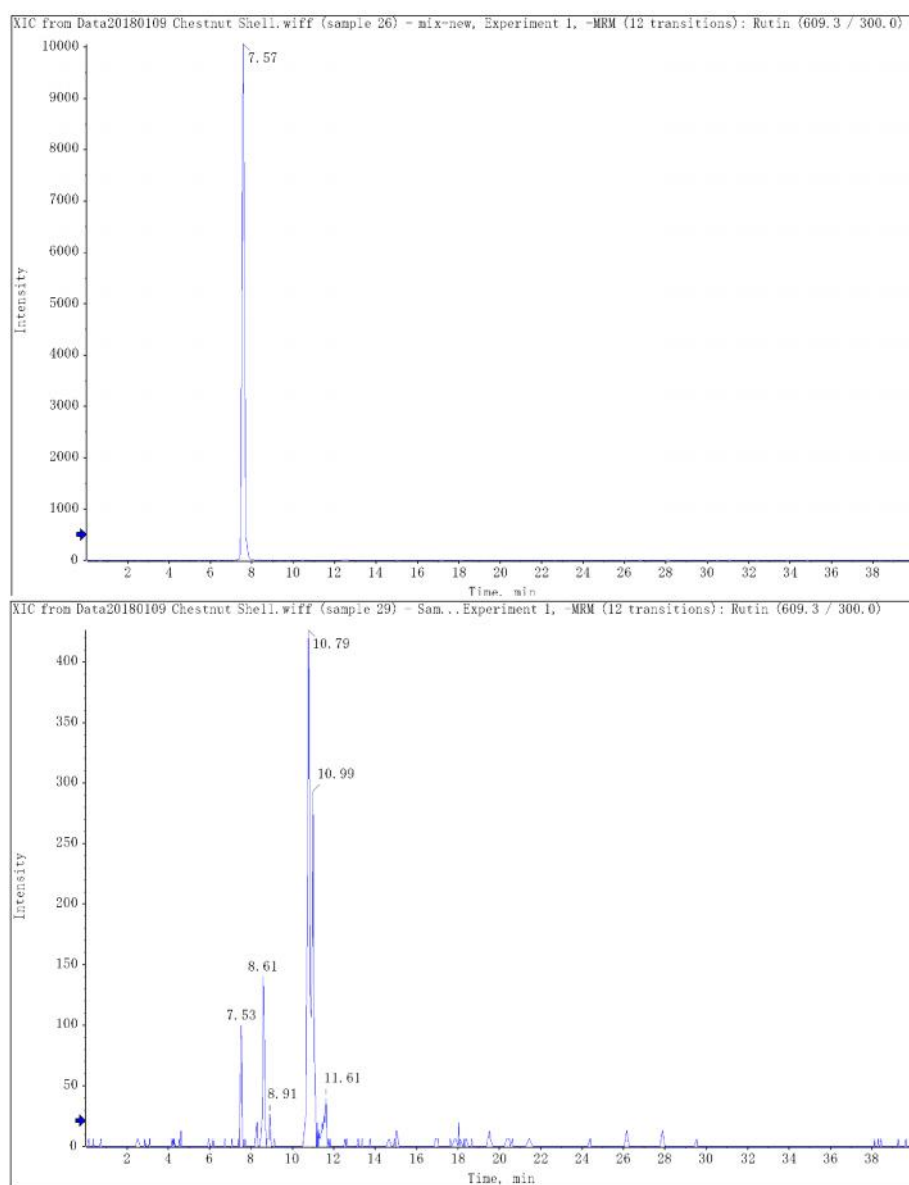


Fig.S7 Chromatograms of Rutin in the standard substance (upper) and the sample (lower).

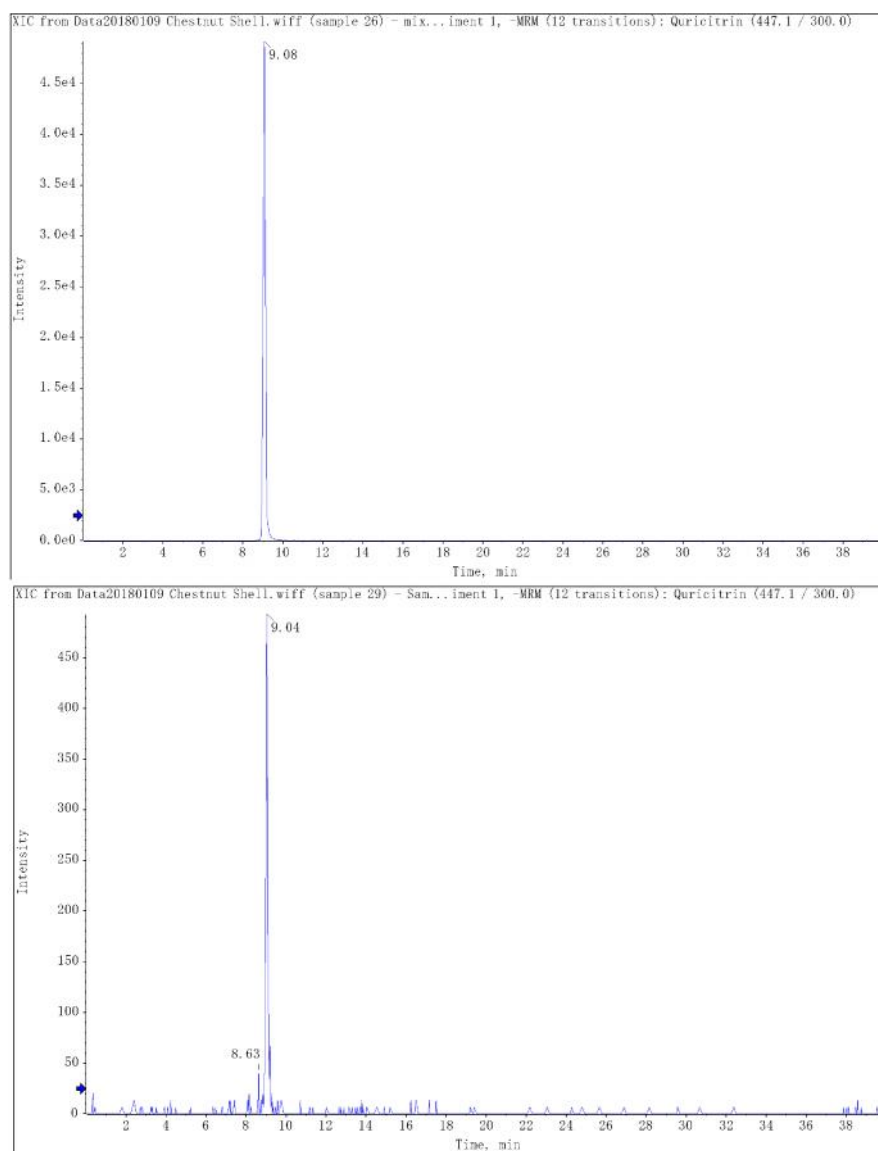


Fig.S8 Chromatograms of Quercitrin in the standard substance (upper) and the sample (lower).

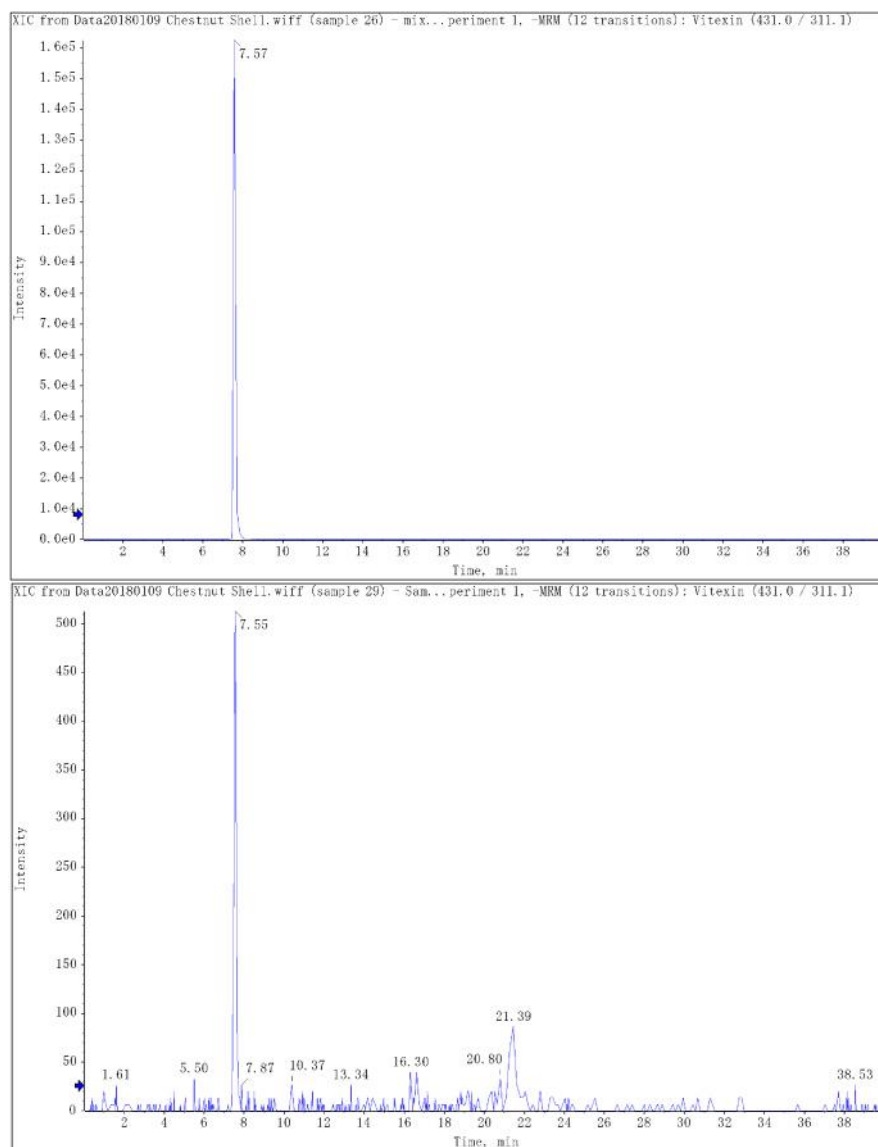


Fig.S9 Chromatograms of Vitexin in the standard substance (upper) and the sample (lower).

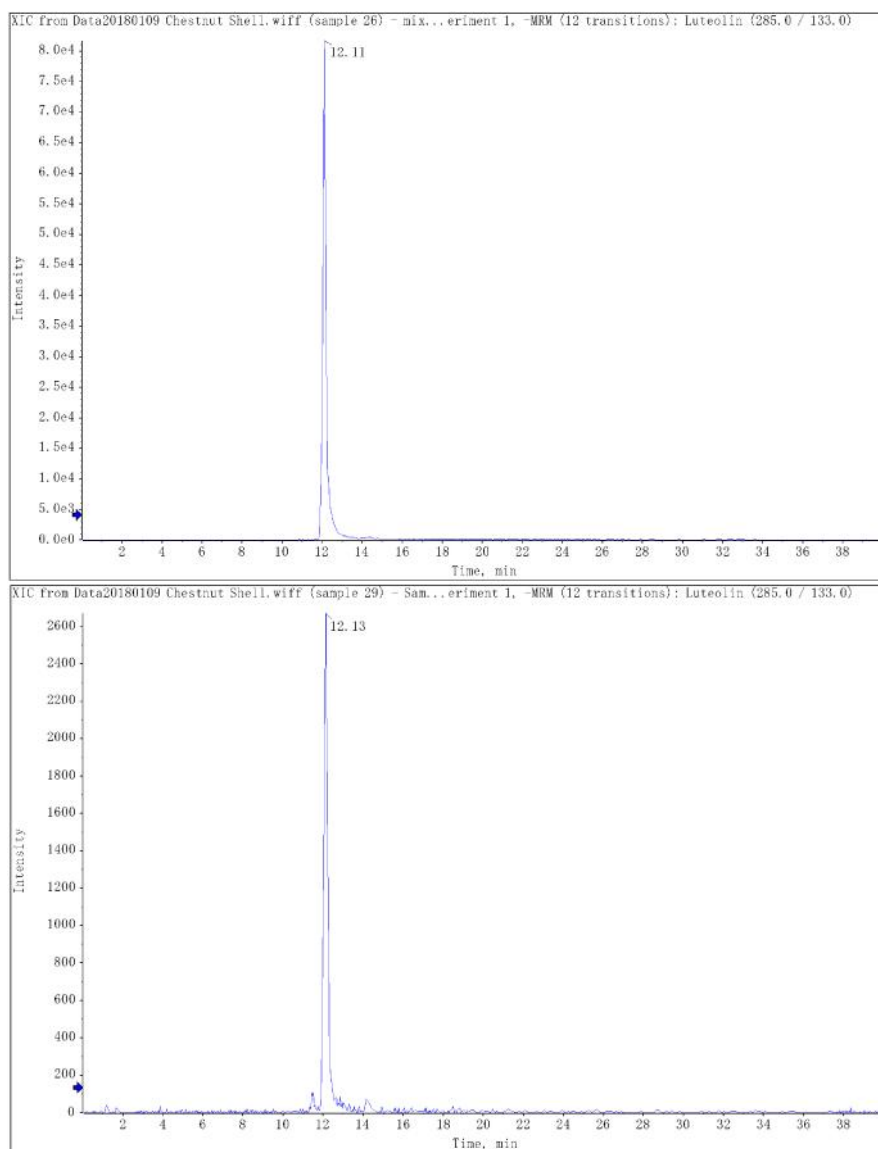


Fig.S10 Chromatograms of Luteolin in the standard substance (upper) and the sample (lower).

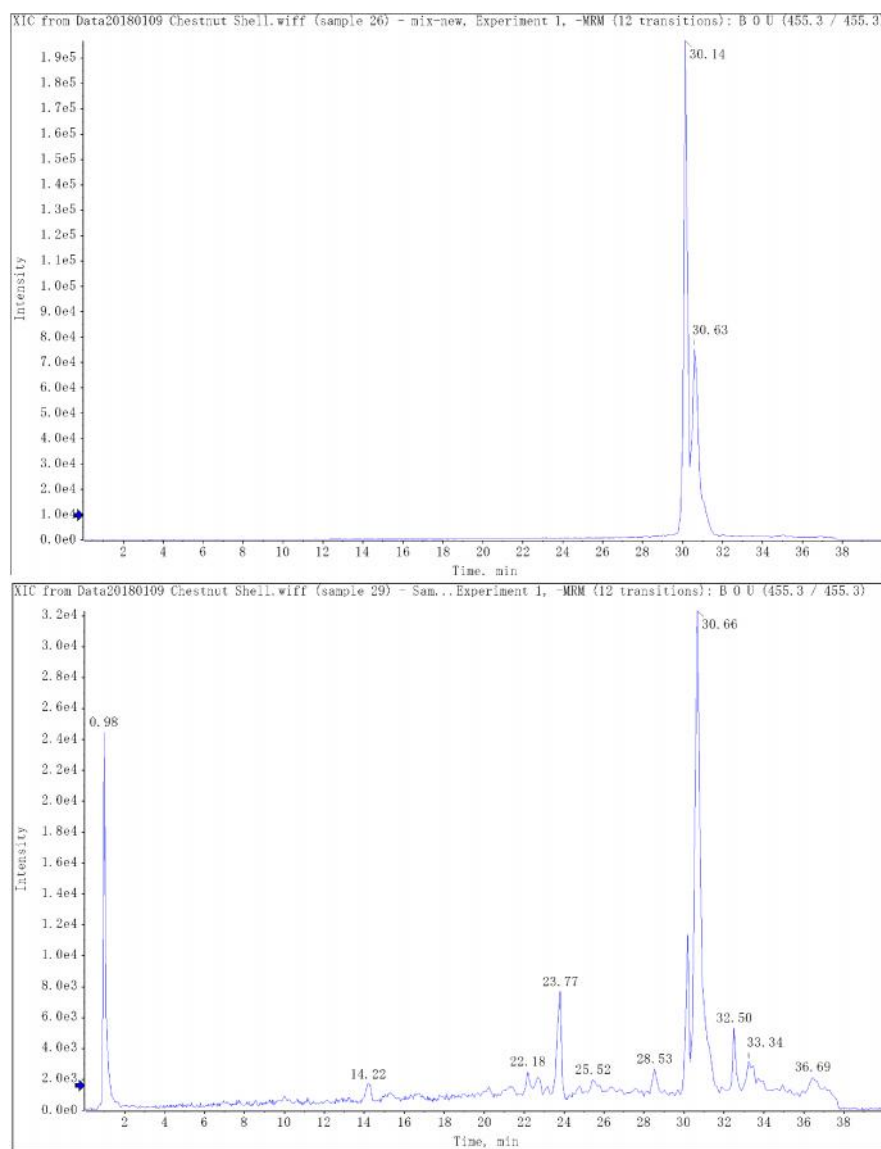


Fig.S11 Chromatograms of Betulinic acid, Oleanolic acid and Ursolic acid in the standard substance (upper) and the sample (lower).

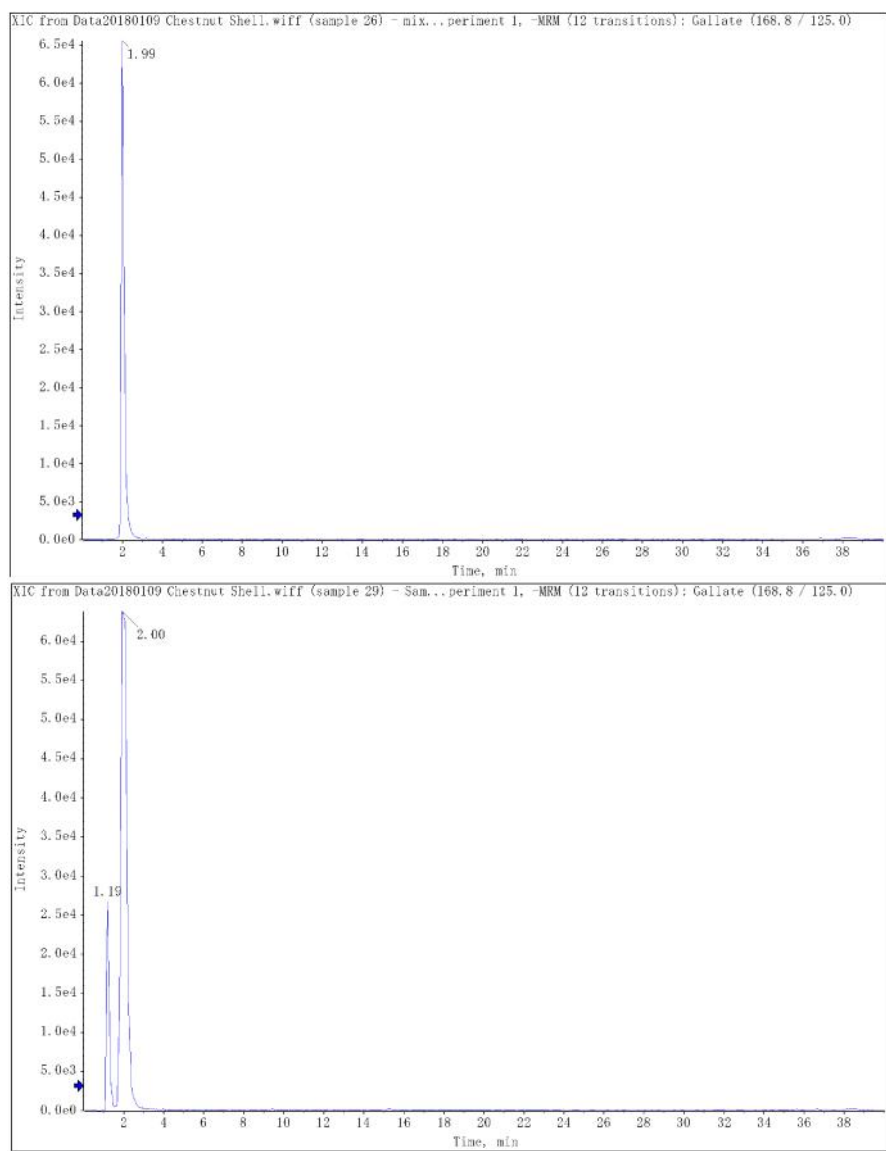


Fig.S12 Chromatograms of Gallic acid in the standard substance (upper) and the sample (lower).

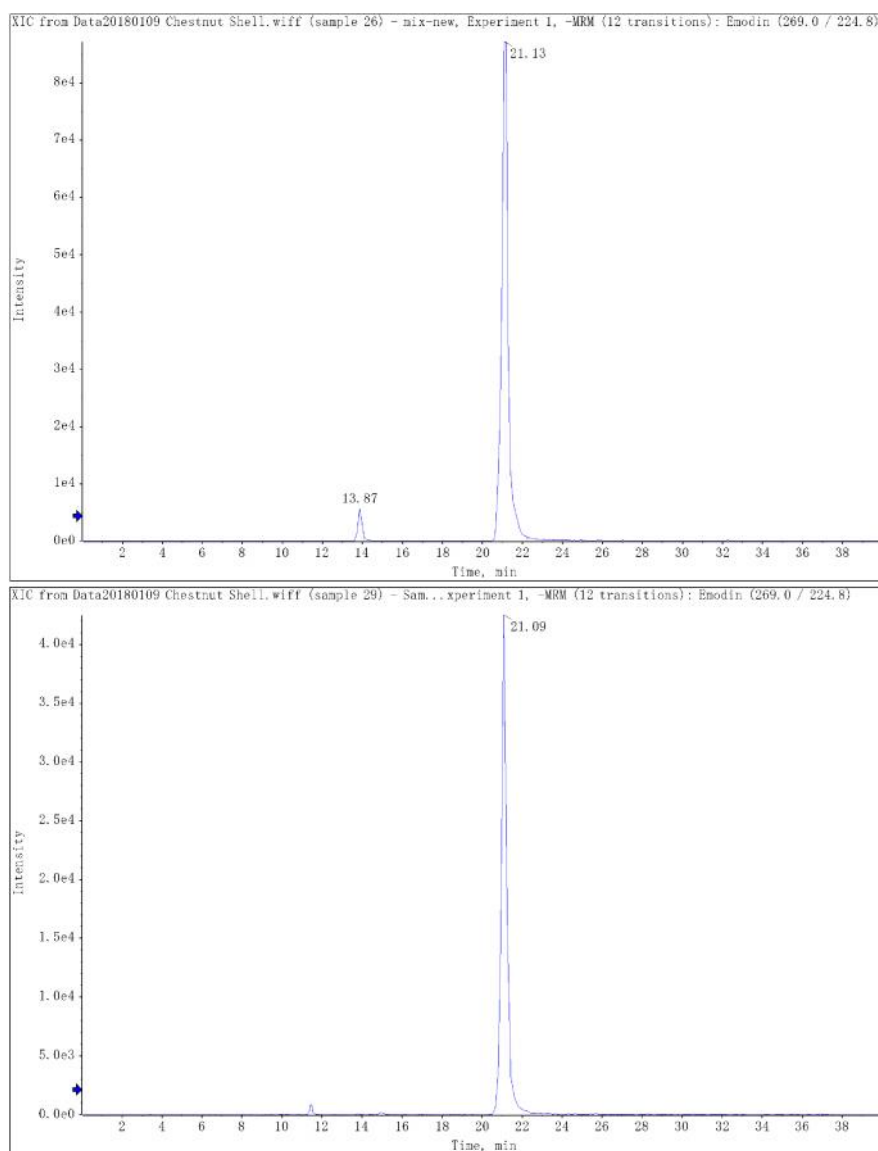


Fig.S13 Chromatogram of Emodin in the standard substance (upper) and the sample (lower).

2. Difficult to separate oleanolic acid and ursolic acid

During the experiment, it was found that even with a long gradient, oleanolic acid and ursolic acid are still difficult to separate (Figures 14 and 15). Therefore, it cannot be determined whether the signal with a retention time of 30.66 min in the sample is oleanolic acid or ursolic acid or both.

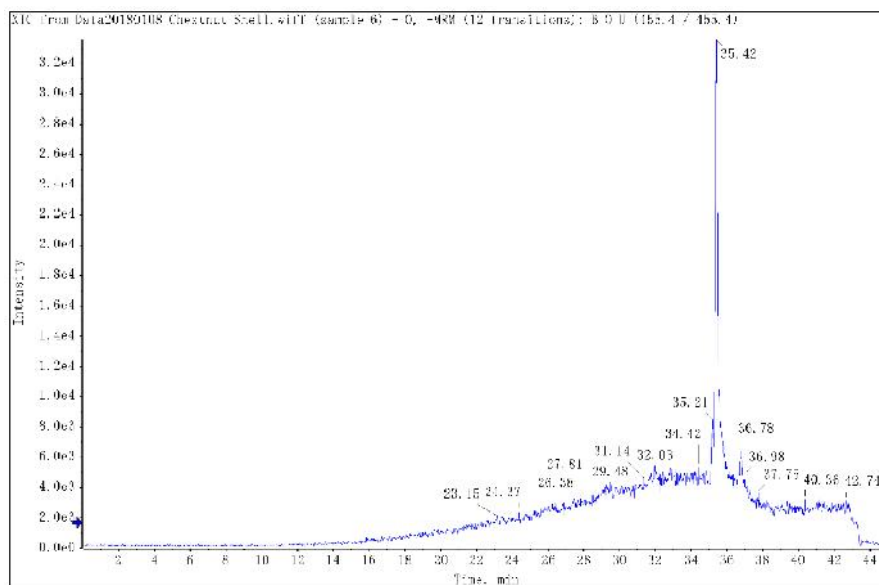


Fig.14 The chromatogram of Oleanolic acid

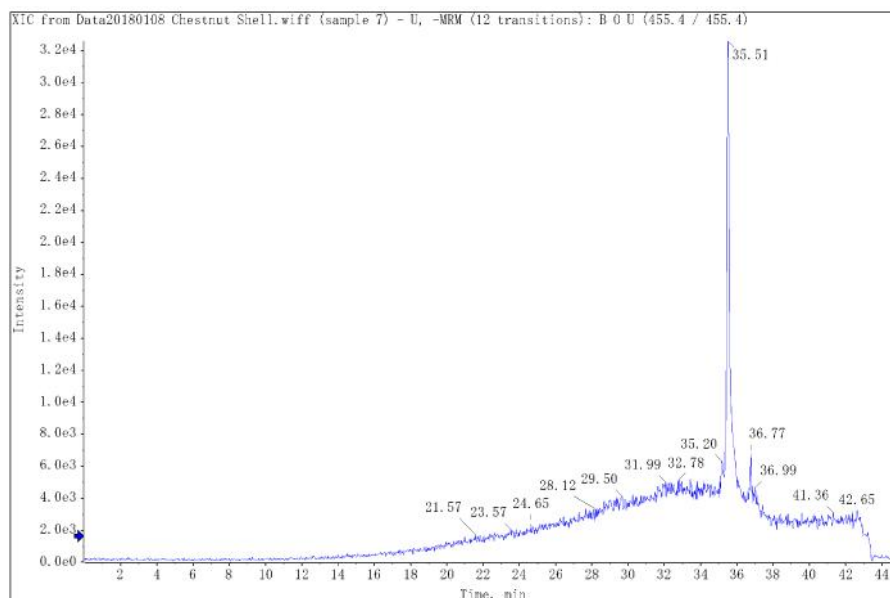


Fig.S15 The chromatogram of Ursolic acid

3. Discussion on residues

Due to the low response of most compounds in the sample, it is doubted whether it is possible to remain in the system rather than the substance in the sample. This is then excluded by the use of blank mobile phase before the sample test solution after analysis of the standard test solution.

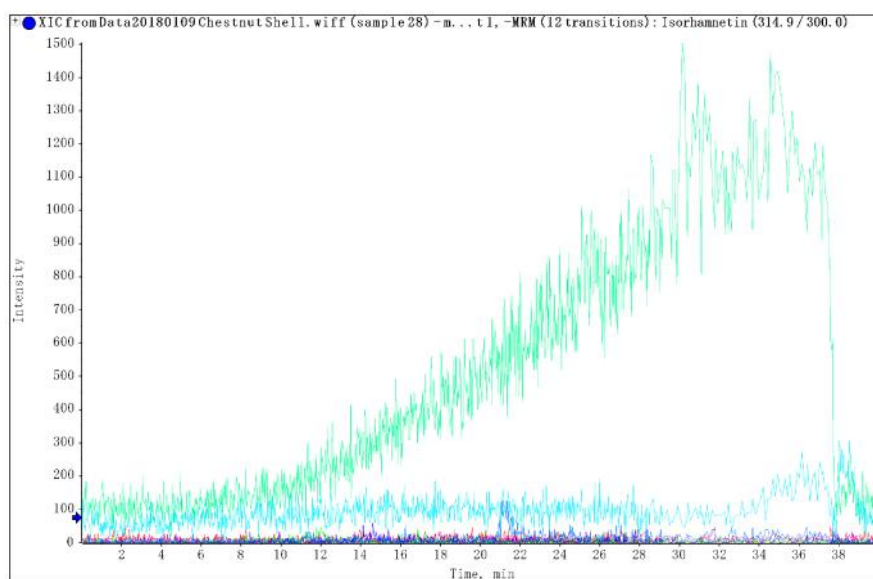


Fig. S16 The chromatogram of the mobile phase.