



Component analysis and antiasthmatic effects of Huashanshen dripping pill

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Abstract

Huashanshen dripping pill (HSS), a commonly used traditional Chinese medicine, has been widely used in China due to its properties of antiasthma, expectorant, and antitussive. However, it was less in-depth understanding of the chemical composition, their absorption in plasma and potential biological mechanism. In this research, ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry was developed to simultaneously identify the chemical constituents and absorption in rat plasma after oral administration of HSS. IgE-sensitized to ovalbumin was induced in BALB/c mice. ELISA and molecular docking analysis were carried out to identify their antiasthmatic mechanisms. As a result, 11 compounds were tentatively characterized by comparing the retention time, ion fragments, and the accurate mass measurement of $[M + H]^+$ ions in the HSS. Two alkaloids including D-anisodamine and L-anisodamine were identified in the rat plasma after oral administration of HSS. The pills alleviated airway inflammation in lung tissues, and inhibited IgE, IL-4, and IL-5 in the serum. Furthermore, there were several amino acid residues involved in anisodamine-targeting receptors of hydrogen bonds and hydrophobic interactions via molecular docking indication. All in all, anisodamine would be the main component in the HSS whose antiasthmatic effects based on the inhibition of IgE, IL-4, and IL-5 production.

Keywords Huashanshen dripping pill · Traditional Chinese medicine · UHPLC-QTOF-MS · Molecular docking · Anisodamine · Asthma

Abbreviations

DM	dexamethasone
ELISA	enzyme-linked immunosorbent assay
HSS	huashanshen dripping pill
IgE	immunoglobulin E
IL	interleukins
MS	mass spectrometry
OVA	ovalbumin
RT	the retention time
UHPLC-	ultra performance liquid chromatography
QTOF-	coupled with quadrupole time-of-flight mass
MS	spectrometry

Introduction

Allergic asthma was considered as one of the important public health issues in the world (Kruse and Vanijcharoenkarn 2018). It was mainly caused by the aggressive reaction to allergens, which was characterized by high airway reactivity, airway inflammation, and high production of allergene-specific immunoglobulin E (IgE). Moreover, interleukin (IL) was considered to be the major cytokine involved in the pathogenesis of allergic responses. IL-4 induced B-cells isotype-switch producing IgE and

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remodeled airway (Steinke and Borish 2001). IL-5 promoted the pathogenesis seen in late phase asthmatic responses by prolonging eosinophil survival and enhancing the inflammation (Hall and Agrawal 2014). Therefore, limiting the production of IgE, IL-4, and IL-5 could inhibit the entire allergic process in allergic asthma (Pelaia et al. 2017).

Currently, the treatment of inflammation was dominated by chemical drugs, including nonsteroidal anti-inflammatory drugs and glucocorticoids, which easily introduced cardiotoxicity, hepatotoxicity, and immunological dysfunction (Wang and Zeng 2019). Thus, in order to compensate for the side effects of chemical medicine, many researchers conducted extensive research on traditional Chinese medicines.

Huashanshen dripping pill (HSS), mainly composed of *Physochlainae Radix* (named Huashanshen in Chinese), was widely used in China because of its antiasthma, expectorant, and antitussive functions. The major active ingredients in HSS contained tropane alkaloids, such as atropine, anisodamine, and scopolamine (Zhou et al. 2017). These compounds were regarded as the traditional anticholinergics, which have been used since 1970s. As previous research, scopolamine, also known as hyoscine, was mainly used to treat nausea, vomiting, motion sickness, as well as smooth muscle spasms (Kohnen-Johannsen and Kayser 2019). Anisodamine could relieve intestinal, microvascular and airway smooth muscle spasms (Xu et al. 2012). Meanwhile, anisodamine significantly suppressed airway hyperreactivity, and eosinophilic inflammation in a murine model of allergic asthma (Xu et al. 2011).

Although HSS was widely used to treat asthma, little was known about its material basis and antiasthma mechanisms. Meanwhile, it was reported that atropine pretreatment may worsen asthma and enhance airway hyperreactivity through the eosinophil-dependent pathway (Verbout et al. 2007, 2009). In order to clarify whether there was similar phenomenon in HSS, systematic analysis of chemical composition of HSS in vivo and in vitro was carried out. Biological activity was elucidated by pharmacological experiments and molecular docking with IgE receptors. It provided an effective approach to the discovery and design of natural products based on highly selective drugs by using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) in combination with virtual computation and biological evaluation.

Materials and method

Materials

HSS was obtained from Zhongxin Pharmaceuticals (Tianjin, China). LC-MS grade acetonitrile, methanol, and formic

acid were purchased from Merck Company (Darmstadt, Germany). Water was purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). The reference standards including atropine, D/L-anisodamine, and scopolamine were provided from Shanghai Standard Technology Co. Ltd (Shanghai, China).

Sample preparation for UHPLC-QTOF-MS analysis

Preparation of samples

HSS powder (2 mg, batch number: 20160331) was completely dissolved in 1 mL of methanol under ultrasonic extraction for 30 min. The methanol solution was centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.22 µm filter and stored at 4 °C until analysis.

Preparation of plasma samples

After an overnight fast, Sprague–Dawley rats were orally administered 200 mg/kg (scaled from ten times of human dose) of HSS once a day for 3 consecutive days. Blood sample was collected into heparinized tubes from each rat by the puncture of the retro-orbital sinus at 0, 15, 30, and 120 min after the last administration of HSS. Blood was immediately processed for plasma by centrifugating at 3500 rpm for 15 min at 4 °C. The supernatants were obtained. Then acetonitrile (100 µL) and methanol (100 µL) were added to the plasma (100 µL) and vortexed for 5 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was dried with nitrogen gas. The residue was dissolved by 200 µL of 50% methanol, and then vortexed and centrifuged at 12,000 rpm for 15 min. The supernatant was injected into UHPLC system for analysis.

UHPLC-QTOF-MS analysis

A Dionex UltiMate 3000 UHPLC System (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a photodiode array detector was used in the range of 190–800 nm. The system was controlled by Chromeleon Xpress software (Thermo Fisher Scientific, Waltham, MA, USA). A Kinetex XB-C18 column (50 mm × 2.1 mm, 1.3 µm; Phenomenex, Torrance, California USA) was used for the separation, at a temperature of 30 °C. The mobile phase was a gradient of A (acetonitrile) and B (0.1% formic acid/H₂O): 2–7% A for 0–2 min, 7% A for 2–4 min, 7–30% A from 4 to 10 min, 30–50% A for 10–12 min, 50–95% for 12–16 min, and 95% A from 16 to 20 min. The flow rate was set at 0.3 mL/min. The injection volume of the test sample was 1 µL.

The mass analysis was performed using a QTOF-MS (Bruker Daltonik GmbH, Germany) equipped with electrospray ionization in positive ionization mode. The

operating parameters were set as follows: end plate offset 500 V, capillary voltage 4.5 kV, nebulizer 2.0 bar, drying gas temperature 200 °C, drying gas flow 6.0 L/min. To ensure mass accuracy and reproducibility, the mass spectrometer was calibrated over a range of 50–1500 Da before analysis. The operations were conducted by the Hystar software (Bruker Daltonik GmbH, Germany).

In vivo evaluation of antiasthmatic activity

Allergic asthma with ovalbumin (OVA)

BALB/c mice were randomly divided into five groups (Fig. 1): (1) control group (IgE-sensitized with saline), (2) model group, (3) oral administration of 54 mg/kg HSS (HSS-L group, scaled from three times of human dose), (4) oral administration of 108 mg/kg HSS (HSS-H group, scaled from six times of human dose), and (5) oral administration of 2 mg/kg dexamethasone (DM group). Model, HSS, and DM groups were actively IgE-sensitized to ovalbumin (OVA) as described previously (Hirose et al. 2017). In short, 200 µL of allergen solution containing 100 µg OVA (grade II, Sigma-Aldrich) and 2 mg Al(OH)₃ (Sigma-Aldrich) in saline was injected intraperitoneally on the 1st and 8th days. After 7 days of the second sensitization, challenged with 5% OVA was performed by the inhalation of aerosolized solutions for 20 min once a day within 5 days in mice. Then the challenges continued for 2 weeks once

2 days. The treatment groups were oral administration 1 h after OVA challenge from the 15th to 34th days. The control group and the model group were treated with saline instead. All the mice were killed 24 h after the last challenge and treatment with HSS and DM. Subsequently, blood samples were collected into tubes by the puncture of the retro-orbital sinus. Bloods were processed for serum by centrifugating at 4000 rpm for 10 min at 4 °C. The supernatants were obtained and stored at −80 °C until analysis.

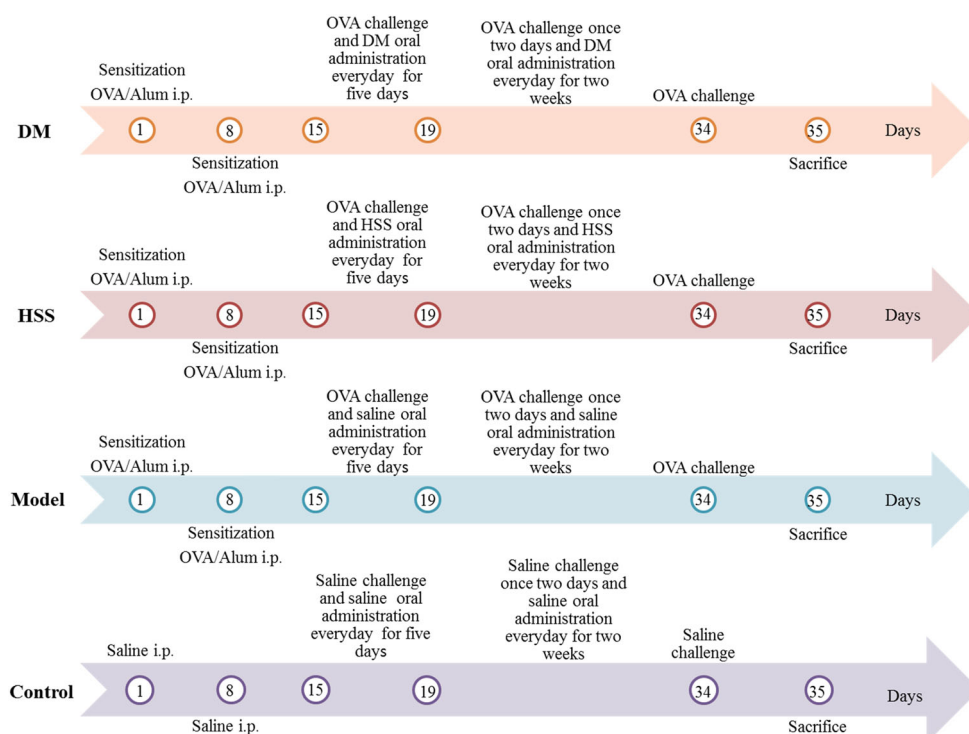
Histological analysis

For the histopathological examination, the pulmonary tissues were fixed in 10% formalin processed for paraffin embedding according to standard histological procedures. Five-micrometer-thick sections were prepared and stained with hematoxylin and eosin. It was randomly cut into six histological sections. Histopathology examination was completed using Leica DM5000 biological microscope and performed by a pathologist who was unaware of whether tissues were treated.

Determination of IgE

Measurement of serum IgE levels was performed by the enzyme-linked immunosorbent assay (ELISA) for mice, according to the manufacturer's instructions (Nanjing Sen-BeiJia Biological Technology Co. Ltd).

Fig. 1 Treatment protocols of allergic asthma with ovalbumin



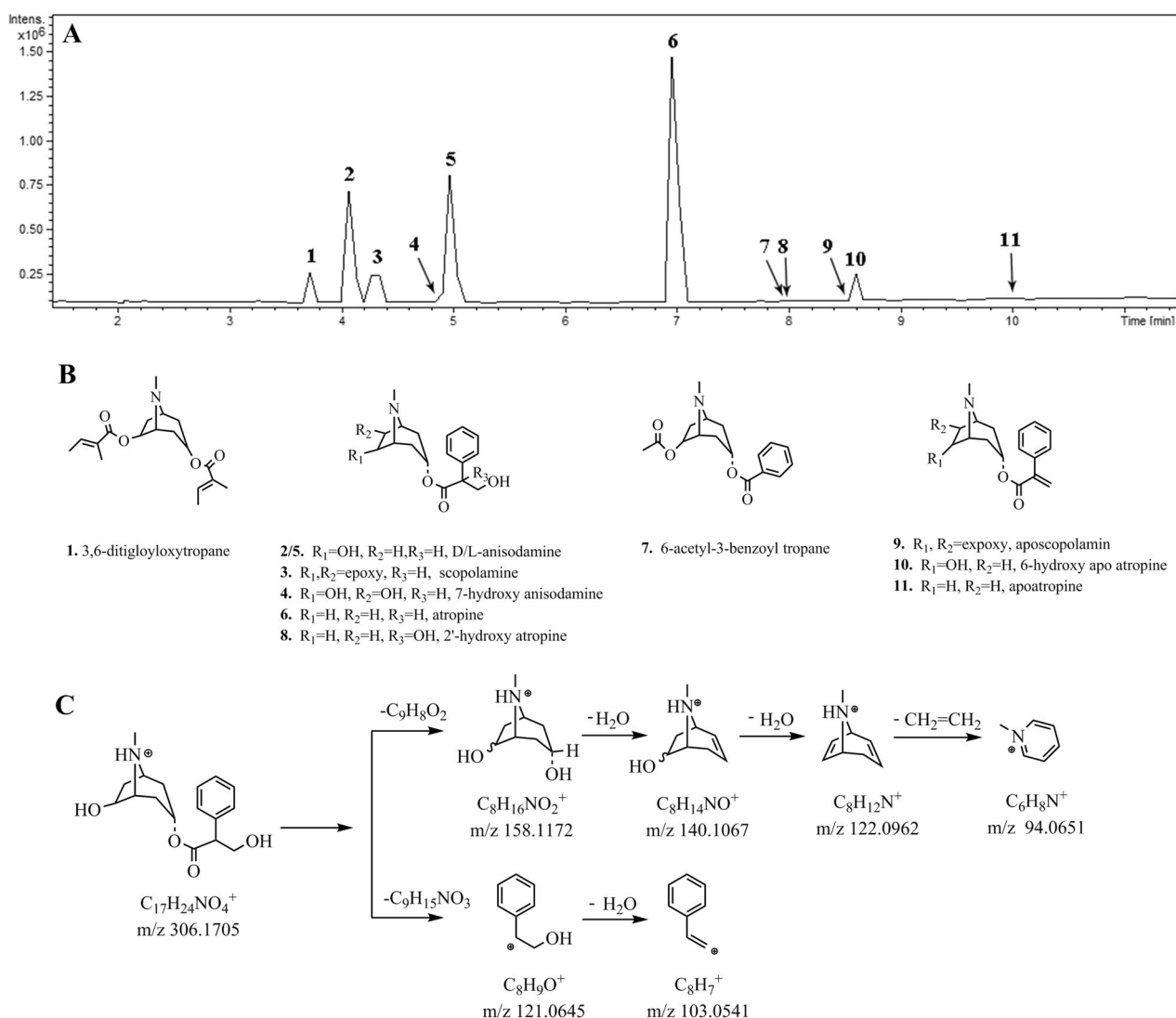


Fig. 2 **a** UHPLC-QTOF-MS base peak intensity chromatogram of HSS in positive mode. **b** Chemical structures of compounds identified in HSS. The numbers indicated each alkaloid in Table 1. **c** Proposed fragmentation pathways and characteristic ions of anisodamine

Cytokines quantification

The IL-4 and IL-5 levels in serum of mice were measured by ELISA, in accordance to the manufacturer's instructions (Nanjing SenBeiJia Biological Technology Co. Ltd).

Molecular docking

Docking simulations were carried out with Accelrys Discover Studio 2.5, Ligplus and Pymol 1.8. Three-dimensional structures of anisodamine were downloaded from PubChem Compound (<http://pubchem.ncbi.nlm.nih.gov>). Ligand was prepared to pdbqt format. The crystal structures of IgE receptors (FcεRI and CD23) were obtained

from the Protein Data Bank (<http://www.rcsb.org/pdb>, PDB ID: 2WQR and 4EZM) (Holdom et al. 2011; Dhaliwal et al. 2012). Docking was performed sequentially in three steps. Initially, the ligand was minimized using Accelrys Discover Studio 2.5. Then, the second step involved localized docking with a smaller ball (radius 9) centered at the potential binding site of interest via Accelrys Discover Studio 2.5. Finally, three-dimensional pictures of the ligand binding to the IgE receptors were obtained from Pymol 1.8.

Data analysis

All the data were statistically analyzed using statistical software IBM SPSS 21 (Armonk, New York, USA) and expressed as means $\pm$ SEM. Statistical analyses were

conducted using the one-way ANOVA by Tukey's multiple comparison tests.

Result and discussion

Identification of the compounds in HSS

In order to obtain the global view of the compounds in HSS, it's analyzed by the base peak intensity chromatogram of HSS in ESI (+)-MS data (Fig. 2). According to the retention time of the reference, the ion fragments information and the accurate mass measurement of $[M + H]^+$ ions, 11 compounds were identified in HSS (Table 1). To identify the structures of these peaks in detail, the elemental composition of software Compass DataAnalysis (Bruker Daltonik GmbH, Germany) comparing with previous LC-MS data (Zhou et al. 2017) was used to confirm molecular weight and calculate molecular formula. For example, a series of ion fragment of peak 2 (Fig. 2) were given in positive ion ESI modes. According to its molecular ion 306.1705 at $[M + H]^+$. Compass DataAnalysis deduced some possible molecular compositions including $C_{17}H_{24}NO_4$, $C_{13}H_{20}N_7O_2$, and $C_{18}H_{20}N_5$. Meanwhile, the major product ions including m/z 158.1172 $[M + H - C_9H_8O_2]^+$, 140.1067 $[M + H - C_9H_8O_2 - H_2O]^+$, 122.0962 $[M + H - C_9H_8O_2 - 2H_2O]^+$, 121.0645 $[M + H - C_9H_{15}NO_3]^+$ and 103.0541 $[M + H - C_9H_{15}NO_3 - H_2O]^+$ indicated that its molecular composition should be $C_{17}H_{24}NO_4$. According to the above analysis, the structural analysis of alkaloids in HSS was constructed in Fig. 2b.

Identification of HSS absorption in rat plasma

Based on the composition analysis of HSS, two alkaloids after 120 min oral administration of HSS in rat plasma, including D-anisodamine and L-anisodamine were separated and identified through the comparison of their retention time and mass spectra with the characteristics of HSS analyzed above (Table 2). However, no alkaloid was identified in 0, 15, and 30 min plasma. As previous reported, anisodamine could significantly inhibit airway hyperreactivity and eosinophilic inflammation in allergic asthma (Xu et al. 2011). Therefore, anisodamine should be the main antiasthma compounds in HSS.

HSS alleviated allergic asthma

HSS as one of the most common traditional Chinese medicines was used to prevent the recruitment of inflammatory cells into the lung tissue. To re-evaluate the antiasthma effect of HSS, a model of chronic allergic lung inflammation was set up through repeated sensitization with OVA

Table 1 UPLC-QTOF-MS analyses of tropane alkaloids in HSS

No.	RT (min)	Identification	Formula	$[M + H]^+$	UHPLC-ESI-MS/MS	Measure m/z $[M + H]^+$	Err [ppm]	mSigma
1	3.7	3,6-Ditigloyloxytropane	$C_{17}H_{24}NO_5$		322.1652, 174.119, 156.1016, 138.0910, 121.0645, 110.0963, 103.0544, 79.0534, 74.0598	322.1654	-1.5	9.6
2	4.1	D-anisodamine	$C_{17}H_{24}NO_4$		306.1705, 158.1172, 140.1067, 122.0961, 103.0541, 91.0540, 79.0540	306.1705	-1.8	3.7
3	4.4	Scopolamine	$C_{17}H_{22}NO_4$		304.1553, 156.1017, 138.0913, 121.0646, 110.0963, 103.0542, 79.0540	304.1552	2.7	4.6
4	4.7	7-Hydroxyanisodamine	$C_{17}H_{24}NO_5$		322.1645, 156.1019, 140.1060, 122.0961, 121.0643, 103.0535, 94.0651, 79.0539	322.1660	-4.0	5.4
5	4.9	L-anisodamine	$C_{17}H_{24}NO_4$		306.1706, 158.1174, 140.1067, 122.0962, 103.0541, 91.0541, 79.0539	306.1706	-1.9	0.9
6	7.0	Atropine	$C_{17}H_{24}NO_3$		290.1759, 142.1229, 124.1119, 103.0540, 93.0698, 91.0543, 77.0384, 67.0541	290.1751	-2.6	1.8
7	7.8	6-Acetyl-3-benzoyl tropane	$C_{17}H_{22}NO_3$		304.1557, 156.1020, 140.1072, 138.0914, 124.1120, 110.0965, 103.0542, 93.0699, 79.0545, 67.0552	304.1556	-4.2	85.0
8	7.9	2'-Hydroxy atropine	$C_{17}H_{24}NO_4$		306.1736, 156, 140.1069, 133.0314, 124.1123, 121.0649, 103.0543, 93.0701, 91.0544, 79.0551, 67.0524	306.1736	-11.9	28.8
9	8.5	Aposcopolamin	$C_{17}H_{20}NO_4$		286.1454, 140.1070, 122.0974, 103.0541, 91.0289, 79.0545	286.1448	-3.4	23.9
10	8.6	6-Hydroxy apoatropine	$C_{17}H_{22}NO_3$		288.1605, 140.1071, 122.0965, 103.0542, 91.0544, 79.0539	288.1604	-3.4	7.6
11	10.0	Apoatropine	$C_{17}H_{22}NO_2$		272.1657, 140.1078, 124.1120, 103.0545, 93.0698, 91.0543, 77.0386, 67.0539	272.1654	-3.3	5.1

RT retention time

Table 2 UPLC-QTOF-MS analyses of tropane alkaloids absorbed in rat plasma

No.	RT (min)	Identification	Formula [M + H] ⁺	UHPLC-ESI-MS/MS	Measure <i>m/z</i> [M + H] ⁺	Err [ppm]	mSigma
1	4.1	D-anisodamine	C ₁₇ H ₂₄ NO ₄	306.1727, 158.1171, 140.1076, 122.0967, 103.0558, 91.0528, 79.0555	306.1727	−8.8	34.0
2	5.0	L-anisodamine	C ₁₇ H ₂₄ NO ₄	306.1700, 158.1138, 140.1067, 122.0979, 103.0505, 91.0577, 79.0559	306.1700	0.1	n.a.

RT retention time

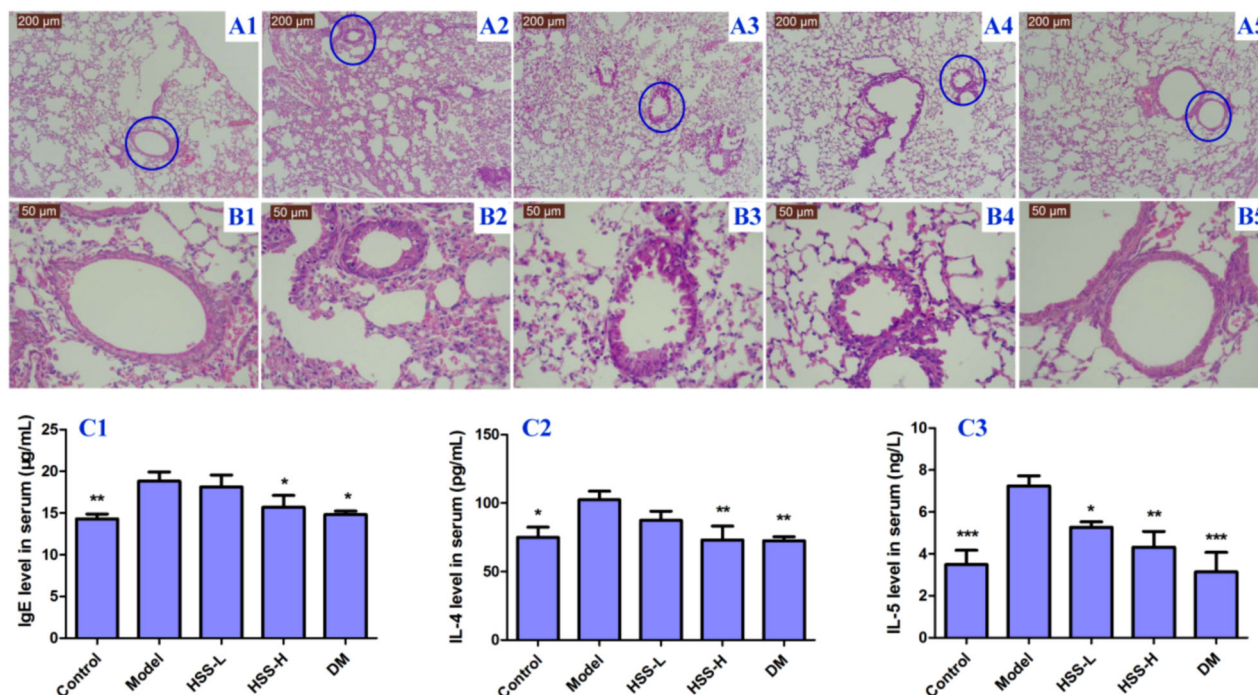


Fig. 3 HSS suppressed inflammatory cell infiltration in the lung tissue of OVA-induced asthma models. Effect of daily oral HSS for 23 days on histopathological examination in lung tissue (×100) (a), (×400) (b). (1) Control group, (2) model group, (3) HSS-L group, (4) HSS-H

group, and (5) DM group. C1 IgE level in serum. C2 IL-4 level in serum. C3 IL-5 level in serum. Values were expressed as means ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with model groups

nebulization to the mice (Delescluse et al. 2012). As a result, OVA nebulization induced the thickening of basement membrane, and peribronchial infiltration of inflammatory cells in the lungs of OVA-challenged mice (Fig. 3a, b) compared with saline-challenged control mice (Kistemaker et al. 2016). One hundred and eight milligrams per kilogram of HSS and DM significantly inhibited airway inflammation in response to OVA compared with model mice.

As previous report, IgE was involved early in the inflammatory cascade and regarded as a cause of allergic asthma, while eosinophilia as a consequence of the whole process (Matucci et al. 2018). Meanwhile, OVA induced Th2 cell-mediated allergic airway inflammation, producing IgE when differentiated B-cells underwent isotype switching (Hirose et al. 2017; Hall and Agrawal 2014). Compared with the control group, the model group sensitized and challenged with OVA increased IgE level in serum.

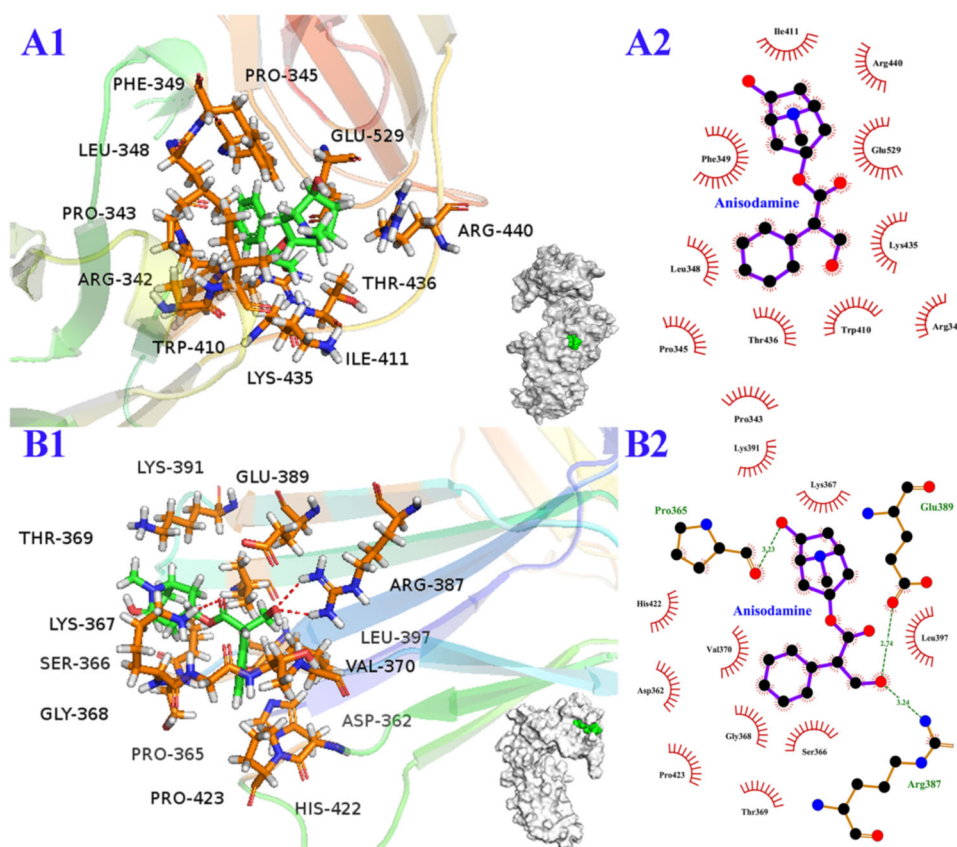
However, the treatment of sensitized mice with HSS or DM significantly reduced serum level of IgE (Fig. 3C1). These results supported the conclusion that HSS inhibited the release of IgE into serum.

Meanwhile, the model group with OVA nebulization increased IL-4 and IL-5 levels in serum compared with the control group. However, treatment with HSS similar to DM significantly reduced the release of IL-4 and IL-5 (Fig. 3C2, C3), which was involved in the production of IgE and the pathogenesis of several aspects of bronchial inflammation in asthma (Foster et al. 2017).

Anisodamine presented high affinity with IgE receptors

As previous reported, IgE antibodies were the core of most allergic reactions, binding to high-affinity receptor (FcεRI) sensitizing mast cells to respond to allergens. (Pennington

Fig. 4 3D and 2D intermolecular interactions of anisodamine with **a** FcεRI (PDB: 2WQR) and **b** CD23 (PDB: 4EZM). The binding pockets of the compound and important amino acids are depicted in the stick model, while proteins are shown in the ribbon model (A1 and B1). The intermolecular interaction pattern is consisted in hydrophobic contacts and hydrogen bonds (A2 and B2). The figure was generated using PyMol and Ligplus



et al. 2016). Moreover, IgE bounding to low-affinity receptor (CD23) regulated IgE-sensitized antigen presentation and mediated IgE antibody production. (Cooper et al. 2012). To investigate the interaction between anisodamine and IgE receptors, molecular docking experiments were analyzed by Accelrys Discover Studio 2.5 program. Therefore, FcεRI and CD23 were applied for the following experiments.

The main interactions between anisodamine and the active binding sites of FcεRI and CD23 were hydrophobic interactions and hydrogen bonds. As shown in Fig. 4, anisodamine was adjacent to eleven amino acid residues ARG-342, PRO-343, PRO-345, LEU-348, PHE-349, TRP-410, ILE-411, LYS-435, THR-436, ARG-440, and GLU-529 of FcεRI via hydrophobic interactions. Meanwhile, anisodamine was adjacent to thirteen amino acid residues ASP-362, SER-366, GLU-368, THR-369, VAL-370, LYS-367, LYS-391, LEU-397, HIS-422, and PRO-423 of CD23 via hydrophobic interactions. Furthermore, three hydrogen bonds were found between the hydroxyl groups of anisodamine and the residues PRO365, Arg387, and Glu389 of CD23.

Docking study indicated that anisodamine presented high affinity via hydrophobic contacts and hydrogen bonds with the above IgE receptors containing FcεRI and CD23.

Therefore, it was speculated that anisodamine might inhibit the ability of IgE to bind to its receptors on effector cells.

Conclusion

This study provided useful information to identify the structures of alkaloids in HSS and proposed the fragmentation pathways and characteristic ions of typical alkaloids by UHPLC-QTOF-MS analysis. In addition, two alkaloids after oral administration of HSS in rat plasma, including D-anisodamine and L-anisodamine were separated and identified. The antiasthmatic effect of HSS was proved by lung tissue histological analysis, immunoglobulin, and inflammatory factors comparison. Through molecular docking, it was found that anisodamine had affinity to IgE receptors. It was speculated that anisodamine played a major role in HSS antiasthmatic effect through the inhibition of IgE. All in all, this research would be helpful to understand its mechanism of action and promote its recognition by the international community.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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