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Bioactivity-based liquid chromatography-coupled electrospray ionization tandem ion trap/time of flight mass spectrometry for β_2AR agonist identification in alkaloidal extract of *Alstonia scholaris*

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ABSTRACT

Although chromatographic fingerprinting combined with chemometrics, is a rational method for the quality control of traditional Chinese medicine (TCM), chemometrics cannot fully explore the relationship between chemical information and the efficacy of the potential activity. In the present work, a cell-based β_2 adrenergic receptor (β_2AR) agonist functional evaluation model coupled with high-performance liquid chromatography was developed to screen the potential β_2AR agonist components in the alkaloidal extract of *Alstonia scholaris* leaves. Using a liquid chromatography with ion trap time-of-flight mass spectrometry (LCMS-IT-TOF) system, the potential bioactive compounds in the prescription were identified and deduced based on the mass spectrometric fragmentation patterns, tandem mass spectrometry (MS/MS) data, and relevant literature. Several new β_2AR agonists of indole alkaloids were successfully found, and their activities were confirmed through an in vivo relaxant test on guinea pig tracheal muscles. The developed method is rapid and reliable compared with conventional fingerprinting and showed high sensitivity and resolution for the identification of β_2AR agonists in TCM prescriptions. This strategy clearly demonstrates that bioactivity-integrated fingerprinting is a powerful tool not only in screening and identifying potential lead compounds and in determining the therapeutic material basis of Chinese herbal prescriptions, but also in supplying suitable chemical markers for their quality control.

1. Introduction

Traditional Chinese medicine (TCM) has played an important role in the treatment of many complex diseases because of its long history of clinical use and reliable therapeutic efficacy. Nowadays, TCMs have been attracting global attention and are being accepted by an increasing number of people [1]. Although many TCMs have been proven effective by modern pharmacological studies and clinical trials, the identity of the bioactive compounds in most TCMs remain to be elucidated; therefore, their screening and identification are very important not only in determining the therapeutic material basis and identifying the lead compounds, but also in supplying suitable chemical markers for their quality control [2]. The complexity and variability of TCMs present a considerable challenge to their analysis; thus, establishing rapid and reliable analytical methods for the identification of the constituents and the quality control of TCMs is necessary [3]. A more effective strategy is based on a systematic and deeper knowledge of the chemical and biological properties of the TCM components. In recent years, studies on chromatographic fingerprinting, coupled with multivariate analysis tools developed in bioinformatics and chemometrics, was intensified to reveal the mechanisms and quality control of TCMs [4]. The ultimate quality assessment of TCM products has to be based on their pharmacological activities or efficacies [5]. Hence, the quantitative analysis of their active components is the most direct and important method for TCM quality control.

Recently, chromatographic fingerprinting, especially via high-performance liquid chromatography-diode array detection (HPLC-DAD), has become a powerful and widely used technique in the analysis of plant extracts because it can systematically

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profile the composition of samples and focus on the identification and consistency assessment of the sample components [6]. However, HPLC-DAD can only provide very limited structural information. The hyphenated technique of high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) has been shown as a useful analytical tool for the identification and elucidation of compounds in TCM prescriptions or natural products [7,8]. Combined with resolution methods recently developed for chemometrics, as well as database searching and structural elucidation techniques, the qualitative and quantitative analyses of the main compounds detected via chemical fingerprinting became possible [9].

The traditional screening of bioactive compounds using animal models is time-consuming and difficult and cannot be used for the direct screening of bioactive components in TCMs. Organs, tissues, and cellular models have been studied in vivo and in vitro [10,11]. Modern pharmacological studies have revealed that an important indication of drug action is its ability to bind with some receptors, channels, and/or enzymes on cell membranes or inside the cells. High-throughput screening methods using membrane receptors or channels as targets have been extensively developed and employed as promising approaches to the efficient screening of lead compounds as drug candidates from natural resources [12–16]. In addition, the ability to interact with cells is one of the important factors in the biological activities of a drug; thus, biochromatographic techniques, especially those involving immobilized artificial membranes and liposomes, have been developed for the analysis and screening of biologically active compounds in TCMs [17–19]. TCMs are complex mixtures, with the effective components generally found at low levels. Biological fingerprinting analysis is a powerful and efficient method of screening and analyzing bioactive compounds and uses proteins [20,21], DNA [13,22], membranes [23], and even cells [24] as the targets. The identification of biologically active compounds from complex TCM mixtures via biological fingerprinting and obtaining results with high sensitivity and specificity presents a great challenge for many researchers.

Alstonia scholaris (Apocynaceae) is widely distributed in the tropical regions of Africa and Asia. As a folk medicinal plant, *A. scholaris* has been historically used in ethnopharmacy to treat chronic respiratory diseases in Yunnan Province, China. The crude aqueous extract of *A. scholaris* leaves has been developed as a TCM consisting of Dengtaiye (DTY) granules based on traditional usage and has been used to treat cough and fever symptoms caused by colds [25,26]. More than 50 monoterpenoid indole alkaloids possessing 18 or 19 carbon atoms on the skeleton have been identified from the *A. scholaris* plant [27] and shown to possess potent anticancer, antibacterial, antifertility, antitussive, anti-asthma, and antitumor bioactivities [28–30]. However, comprehensive anti-asthma compounds and the corresponding mechanisms are yet to be determined.

β_2 adrenergic receptor (β_2AR) agonists are among the most common therapeutic agents for asthma and chronic obstructive pulmonary disease [31]. Previous studies have shown that the extracted agonist bronchodilators from *A. scholaris* leaves may be involved in β_2AR activation. In the present work, a Shimadzu liquid chromatography with ion trap time-of-flight mass spectrometry (LCMS-IT-TOF) system was used to construct specific chromatographic fingerprints for the recognition of multiple compounds in the alkaloidal extract of *A. scholaris*. The constituents of the prescription were identified and deduced according to the MS fragmentation patterns, MS^n data, and relevant literature. Thus, a new strategy for directly purifying and identifying potential β_2AR agonists from the alkaloidal extract of *A. scholaris* using LCMS-IT-TOF-coupled β_2AR agonist functional evaluation method is proposed (Fig. 1). Several new β_2AR agonists of indole alkaloids were found and their activities were confirmed through an in vivo relaxant test on guinea pig tracheal muscles.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Deionized water was purified using the Milli-Q system (Millipore, Bedford, MA, USA). Analytical grade acetic acid was obtained from Tedia (Fairfield, CA, USA). Purified standard alkaloids from *A. scholaris* were isolated [26,29] and stored in a refrigerator for bioassay and chromatographic analyses. β_2AR human embryonic kidney 293 (HEK 293) cells were derived from HEK 293 cells, and the expressed β_2ARs were constructed in our laboratory [32]. All reagents for the cell culture were purchased from GibcoBRL Life Technologies (Rockville, MD, USA). The Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Sample preparation

The dried and powdered leaves of *A. scholaris* were extracted with EtOH under reflux conditions, and the solvent was evaporated in vacuo to afford the ethanolic extract. The other ethanolic extract was dissolved in 1% HCl. The residue was confirmed as a non-alkaloid fraction, and the solution was subsequently basified using ammonia water to pH 9–10. The basic solution was partitioned with EtOAc and afforded the alkaloid fraction (EtOAc layer). The alkaloid fraction was dissolved in CH_3CN and filtered through a 0.2 μm filter prior to HPLC analysis.

2.3. LC–MS/MS conditions

2.3.1. HPLC analysis

A Shimadzu LC-20A series (Shimadzu, Kyoto, Japan) equipped with a binary pump, an autosampler, an ultraviolet detector, and a column compartment was used in the analysis. The detection wavelength was set at 220 nm. A Phenomenex Luna C_{18} column (5 μm, 250 mm × 4.6 mm; Jiangsu, China) was used for the separation. The mobile phase was a gradient elution system of A ($HCOOH:H_2O = 0.1:100$) and B (CH_3CN), and the elution was programmed as follows: 10%–15% B for 0–20 min, 15%–30% B for 20–45 min, 30%–100% B for 45–75 min, and 100% B for 75–80 min. The flow rate was 1 mL min^{-1}, the column temperature was 40 °C, and the injection volume was 10 μL.

2.3.2. LCMS-IT-TOF analysis

The constituent identification in the alkaloidal extract of *A. scholaris* was performed using a Shimadzu LCMS-IT-TOF system (Kyoto, Japan) equipped with an ESI ion source operating in both positive and negative data-dependent MS/MS modes. Data were collected at the scan ranges of 100 Da to 1500 Da for MS and 50 Da to 1500 Da for MS/MS. The source parameters were 2.5 kV electrospray capillary voltage for the positive ionization mode and 3.0 kV for the negative ionization mode; the capillary, CDL, and interface temperatures were set to 200 °C, and the drying gas was set at 90 L h^{-1}. LCMS-IT-TOF was used in the auto-MS^n mode for the determination of indole alkaloids to automatically select the precursor ions. Data were collected at the 50–1000 Da scan range.

2.4. Sample preparation and purification for activity assay using HPLC

The components of the alkaloidal extract were separated according to the experimental protocol in Section 2.3.1. HPLC

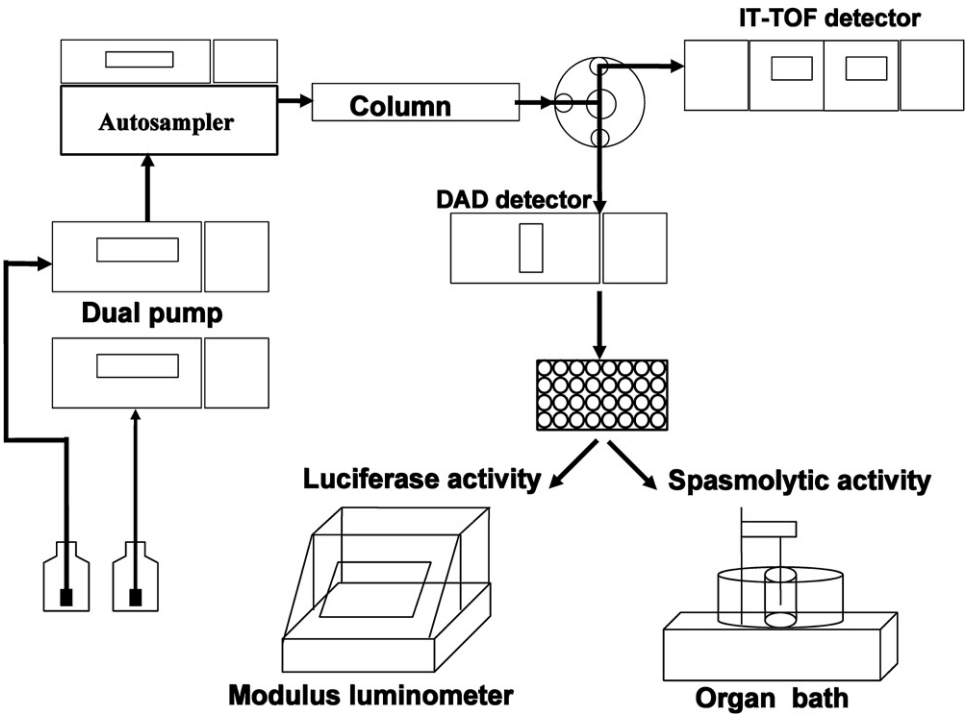

Fig. 1. Schematic representation for the dual-luciferase reporter assay-guided LC–MS method for the identification of β_2AR agonists in the alkaloidal extract of Alstonia scholaris.

fractions were collected into a 96 deep-well plate (2.2 mL) every 1 min and evaporated to dryness in a vacuum drying oven at 60 °C. The residues were dissolved in dimethyl sulfoxide (50 μL) and diluted with Dulbecco's Modified Eagle's Medium (DMEM) for the luciferase reporter activity assay. Each active peak, which was confirmed via both LCMS-IT-TOF and luciferase reporter assay, was repeatedly ($n = 3$) accumulated and purified via HPLC for use in the subsequent cytological and in vitro spasmolytic activity tests.

2.5. Luciferase reporter assay for β_2AR activation

The β_2AR HEK 293 cells were plated in 96-well microplates at a density of 4×10^4 cells/well and grown in DMEM containing 10% fetal bovine serum, 100 mg/L Zeocin, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin. The culture was maintained at 37 °C under 5% CO_2. The culture medium was replaced after 24 h, and the cells were co-transfected with pCRE-Luc reporter and pRL-TK plasmids at 51 and 1.1 ng/well, respectively. Transfection was performed overnight using Lipofectamine 2000 according to the manufacturer's instructions. The cells were then washed with phosphate-buffered saline (PBS), and a fresh medium was added 24 h prior to the 5 h treatment with drugs or the prepared fractions. To stop the reaction, the cells were washed with PBS before the addition of 20 μL/well passive lysis buffer. Luciferase activity was then measured using a dual-luciferase reporter assay system (Promega WI, USA). Luminescence was detected using a Modulus luminometer from Turner Biosystems (Turner Designs, Sunnyvale, CA, USA). The ratio of firefly luciferase activity to renilla luciferase activity was used to normalize the differences in the transfection efficiency.

2.6. Relaxant effect of the indole alkaloids on guinea pig tracheal muscles

In vitro spasmolytic activity tests on isolated tracheas were conducted in a previous study [33]. The tracheal strips were vertically mounted in a 20 mL water-jacketed organ bath filled with Krebs-bicarbonate buffer under 95% O_2 and 5% CO_2 at 37 °C. In all experiments, 200 μL of each drug was added into the organ bath. All drug concentrations were expressed as the final concentration in the organ bath. The test samples were added 10 min before the addition of acetylcholine, and propranolol was added 10 min before the addition of the test samples. The peak contractile response was recorded as 100%. The half maximal effective concentration (EC_{50}) values of the tensions, expressed as 50% of the peak contractile response, were used for activity evaluation.

2.7. Statistical analysis

Results are expressed as the standard error of the mean (SEM). Statistical analysis of the data for multiple comparisons was performed using ANOVA, followed by the Bonferroni post hoc test. For single comparisons, significant differences between the means were determined using the Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Optimization of LC and MS conditions

HPLC and MS conditions were optimized to obtain better detections. The effects of the acid concentration, column temperature, CDL temperature, capillary temperature, capillary voltage, and drying gas flow rate were investigated. The experiments were arranged as follows: acid concentration (0.05% and 0.1% acetic acid, 0.05% and 0.1% formic acid); column temperature (20, 30, and 40 °C); capillary temperature (180, 200, and 220 °C); CDL temperature (180, 200, and 220 °C); capillary voltage (positive ion mode: 2.5, 3.0, and 3.5 kV; negative ion mode: 2.5, 3.0, and 3.5 kV); and drying gas flow rate (1, 1.25, and 1.5 L/min). The total peak area was used as the criterion for optimization. The optimum conditions obtained were 0.1% formic acid, 40 °C column temperature, 200 °C capillary

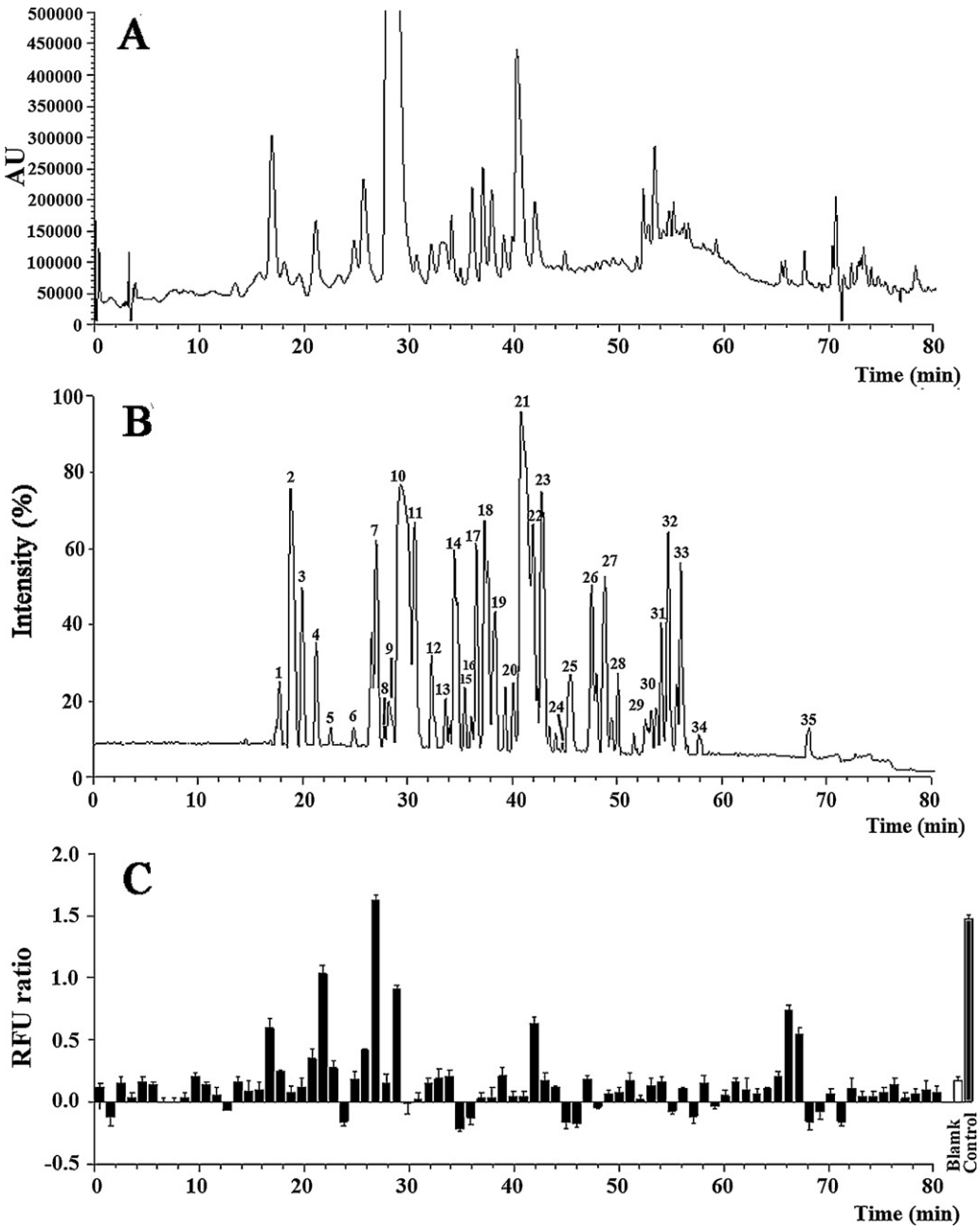

Fig. 2. (A) HPLC-UV chromatograms of *A. scholaris* alkaloidal extract; (B) LCMS-IT-TOF TIC chromatograms in the positive ESI mode; and (C) bioactivity chromatograms obtained via the dual-luciferase reporter assay system for β_2AR agonist activation. The peak numbers are consistent with those in Table 1.

temperature, 200 °C CDL temperature, 2.5 kV capillary voltage for the positive ion mode, 3.0 kV for the negative ion mode, and 1.5 L/min drying gas flow rate.

3.2. LCMS-IT-TOF analysis

The optimal LCMS-IT-TOF conditions were applied in the analysis of the alkaloidal extract of *A. scholaris* (Fig. 2). The total ion current chromatograms in the positive ESI mode are shown in Fig. 2B, indicating the high precision of the developed method. Majority of the DTY constituents were alkaloids; thus, the positive mode provided higher ion intensities, and the $[M+H]^+$ ions provided helpful information on the molecular weight and structure of the constituents. The identities of all constituents were initially deduced from several aspects, as follows: the elemental composition and possible molecular composition were based on the exact molecular weight, and comparison with literature data provided the molecular composition of the sample. The MS/MS fragments and the retention time verified the results. Some peaks had the same protonated molecules in the MS spectra and similar fragment ions in the MS/MS spectra; however, their retention behaviors were different, which is helpful in the identification of the sample components. In the current study, peak 9 at the 29.311 min retention time was chosen to illustrate the identification approach. The base peak in the positive ESI mode was *m/z* 341.1903 and confirmed as $[M+H]^+$. The elemental and possible molecular compositions were deduced from the exact molecular weight. These molecular compositions were retrieved from the Chemical Components of Source Plants in Traditional Chinese Medicine and showed that 3-hydroxy quinine ($C_{20}H_{24}N_2O_3$) is most probably the

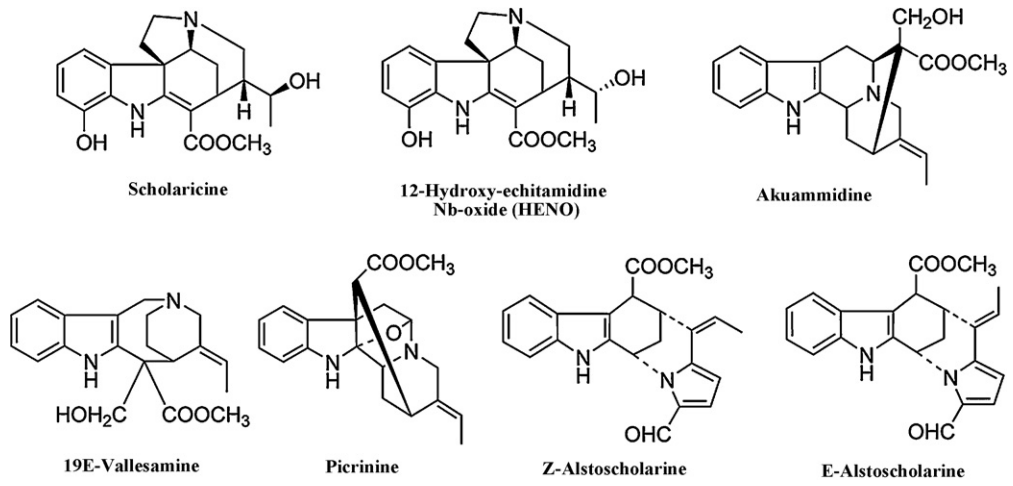

Fig. 3. Chemical structures of compounds identified by β_2AR agonist functional evaluation model for *A. scholaris* alkaloidal extract.

Table 1
MS/MS data of (+) ESI-MS and the identification results of the bioactive compounds in alkaloidal extract of *A. scholaris*.

Peak no.	t_R (min)	Identification	M/Z ([M+H]$^+$)	MS^2	*m/z*	Composition
17	17.810	Scholaricine	357.1854	357 [M+H]$^+$, 325 [M−OCH_3]$^+$, 279 [M−H_2O—OCH_3—CO]$^+$	356.1776	$C_{20}H_{24}N_2O_4$
22	21.298	12-Hydroxy-echitamidine-Nb-oxide	373.1774	373 [M+H]$^+$, 355 [M−OH]$^+$, 341 [M−OCH_3]$^+$	372.1696	$C_{20}H_{24}N_2O_5$
27	28.157	Akuammidine	353.1901	353 [M+H]$^+$, 323 [M−OCH_3]$^+$, 306 [M−OCH_3—OH]$^+$, 293 [M−$COOCH_3$]$^+$	352.1823	$C_{21}H_{24}N_2O_3$
29	29.311	19,20-(*E*)-Vallesamine	341.1903	341 [M+H]$^+$, 309 [M−OCH_3]$^+$, 282 [M−$COOCH_3$]$^+$	340.1825	$C_{20}H_{24}N_2O_3$
42	40.711	Picrinine	339.1719	339 [M+H]$^+$, 307 [M−OCH_3]$^+$, 279 [M−OCH_3—CO]$^+$	338.1641	$C_{20}H_{22}N_2O_3$
67	67.867	19,20-(*Z*)-Alstoscholarine	361.1547	361 [M+H]$^+$, 301 [M−$COOCH_3$]$^+$	360.1469	$C_{22}H_{20}N_2O_3$
68	68.137	*E*-Alstoscholarine	361.1548	361 [M+H]$^+$, 301 [M−$COOCH_3$]$^+$	360.1470	$C_{22}H_{20}N_2O_3$

compound obtained from DTY. The fragmentation patterns summarized from the spectra are consistent with other literature findings [34]; thus, peak 9 was deduced as 19,20-(*E*)-vallesamine. All other constituents were identified using the same approach. The detailed results of the identified compounds and the MS and MS/MS information at the positive mode are shown in Table 1. The major constituents of DTY were indole alkaloids. These results provide helpful chemical information for future activity studies and can be used in the constituent identification of other similar Chinese herbal medicines.

3.3. Identification of hit compounds for β_2AR agonists

To isolate the effective components from *A. scholaris* leaves, the alkaloidal extract components were further separated using HPLC (Fig. 2A). Eighty fractions were isolated and collected, and each fraction was tested for activity using the dual-luciferase reporter assay system for β_2AR agonists. The luciferase activity was expressed as the ratio of firefly to renilla luminescence. As shown in Fig. 2C, seven peaks (fractions 17, 22, 27, 29, 42, 67, and 68) showed potential activity; their MS/MS fragmentation behaviors are shown in Table 1. Their intense [M+H]$^+$ molecular ions gave signals at *m/z* 357.1854, 373.1774, 353.1901, 341.1903, 339.1719, 361.1547, and 361.1548, respectively, which correspond to the indole alkaloids scholaricine, 12-hydroxy-echitamidine Nb-oxide (HENO), akuammidine, 19,20-(*E*)-vallesamine, picrinine, Z-alstoscholarine, and E-alstoscholarine; the structures are shown in Fig. 3. Although studies on natural β_2AR agonist screening of Chinese medicines and natural products have been reported [33,35–37], the LCMS-IT-TOF-coupled β_2AR agonist functional evaluation method described above provides a clear technological advantage. Purified HPLC fractions were directly used for the β_2AR activity analysis, and the identification using MS was faster and more reliable than conventional methods. This strategy clearly demonstrates that bioactivity-integrated fingerprinting is a powerful and meaningful tool not only in screening and identifying potential lead compounds and in determining the therapeutic material basis of Chinese herbal medicines, but also in supplying suitable chemical markers for quality control.

3.4. Confirmation of β_2AR agonists from A. scholaris leaves

The bioactive peak fractions were obtained after repeated accumulation via HPLC. The purified compounds showed good separation. In accordance with the area normalization method, the purity of the seven compounds was 90.1% to 95.2%. The β_2AR

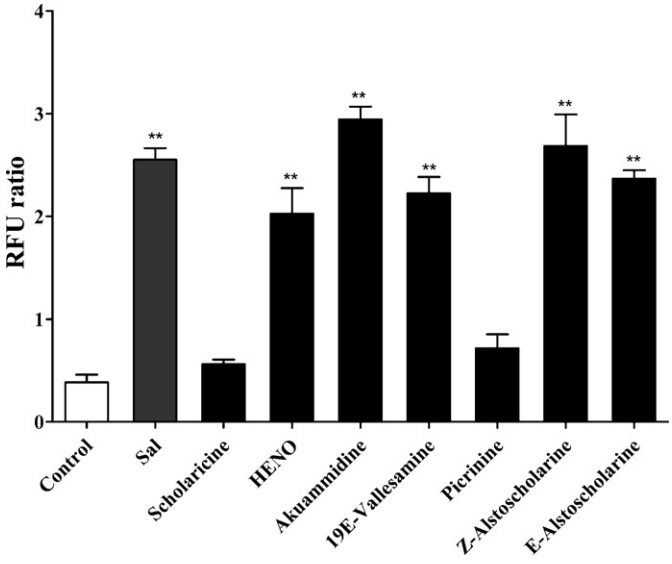

Fig. 4. Verification of β_2AR agonist activity using the dual-luciferase reporter assay system. Each bar represents the mean ± SEM. $n = 5$, $^*P < 0.01$ vs. control.

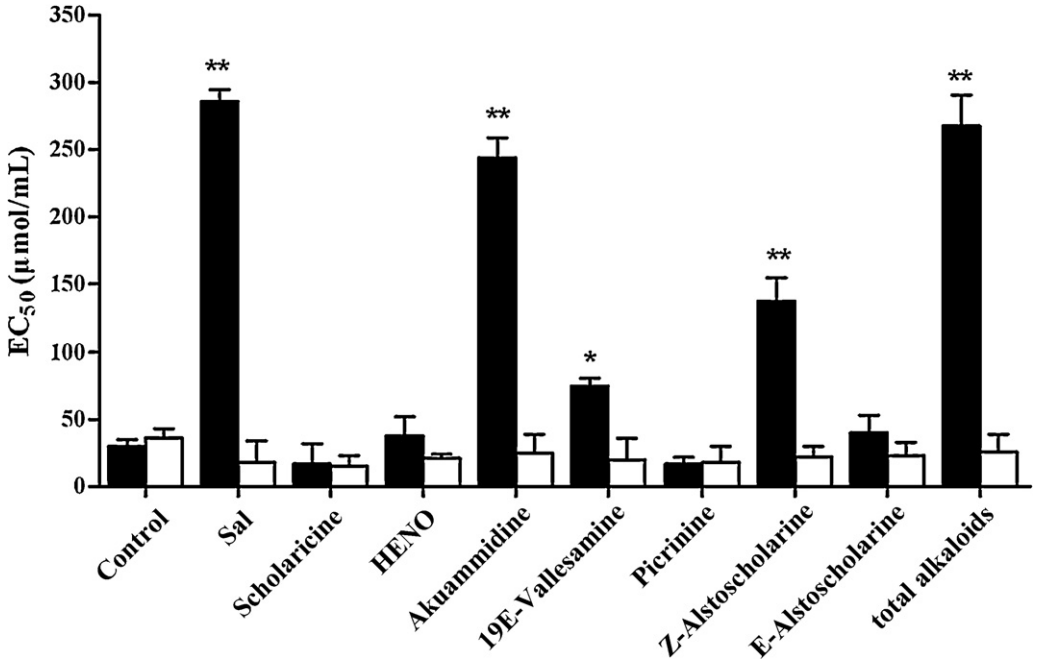

Fig. 5. The effect of purified indole alkaloids on spasmolytic activity tests by isolated guinea pig trachea. Tensions are expressed as EC_{50} values, the half concentration of peak contractile response (black column). Propranolol (1 μmol/L) significantly inhibited the effect of alkaloids on tracheal contractions (white column). Data represent means ± SEM for triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the saline control group.

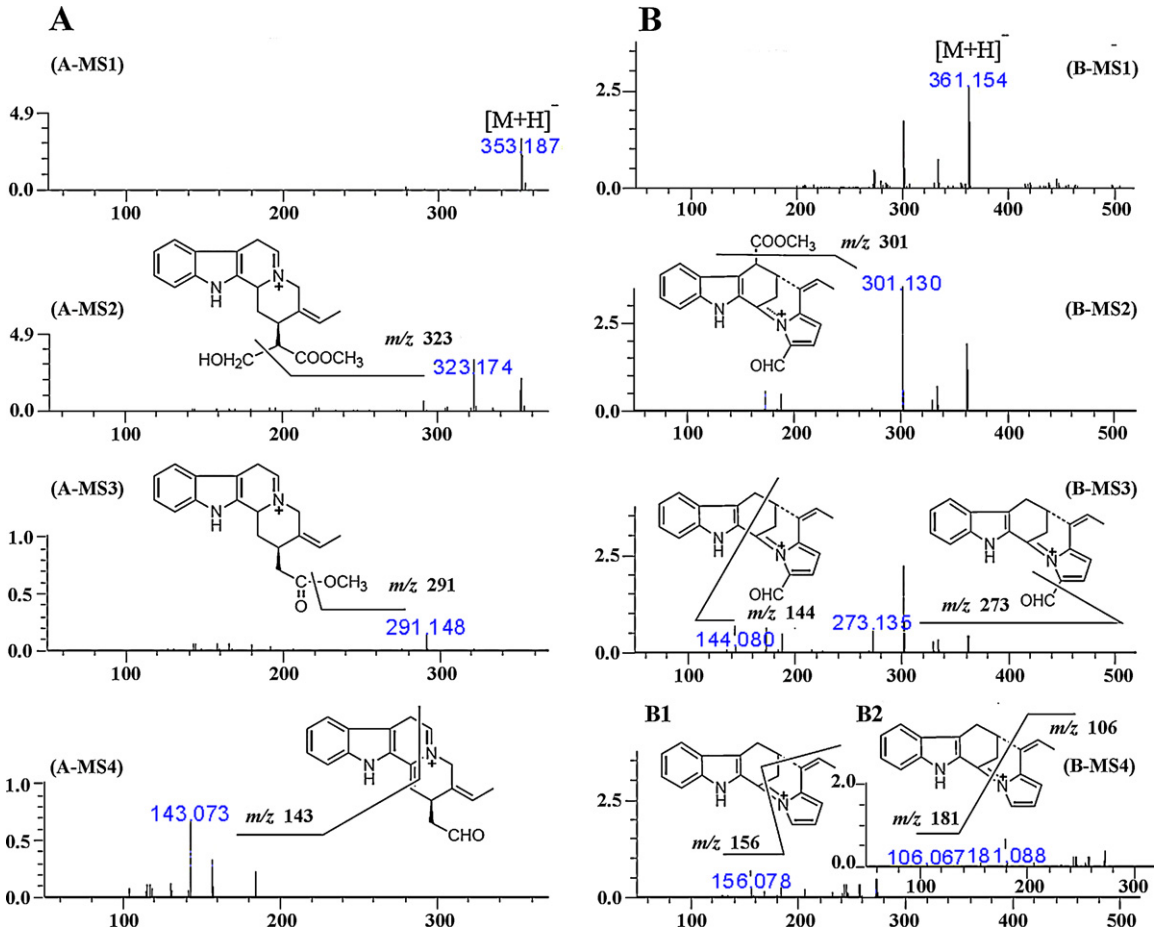

Fig. 6. ESI-MS^n spectra of akuammidine (A), Z-alstoscholarine and E-alstoscholarine in positive ion modes (B).

activities of the purified indole alkaloids (0.5 μg/mL) and the positive drug salbutamol (Sal, 0.01 μmol/L) were determined using luciferase reporter assay. As shown in Fig. 4, the β_2AR agonist of the classic phenylethylamine-type Sal presented the most promising results ($p < 0.01$). Compared with the control, HENO, akuammidine, 19,20-(*E*)-vallesamine, Z-alstoscholarine, and E-alstoscholarine also showed significant effects ($p < 0.01$), but scholaricine and picrinine displayed much poorer β_2AR activation.

Spasmolytic activity tests using isolated guinea pig trachea were performed to confirm the synergistic effects of the aforementioned alkaloids in an in vitro model. The EC_{50} values for the spasmolytic activity of the total alkaloidal extract (500 μg/mL) and the purified alkaloids (5 μg/mL) are shown in Fig. 5. The order of the EC_{50} values is as follows: Sal (285.7 μmol/L), akuammidine (243.9 μmol/L), Z-alstoscholarine (137.5 μmol/L), 19,20-(*E*)-vallesamine (74.8 μmol/L), and total alkaloidal extract (267.5 μmol/L). The significant increases observed in akuammidine and Z-alstoscholarine are similar to that observed in 0.1 μmol/L Sal ($p < 0.01$), and the effects were completely blocked with 1 μmol/L of the β/AR blocker, propranolol. Therefore, akuammidine and Z-alstoscholarine were considered as new indole alkaloid-type β_2AR agonists.

3.5. Characterization of indole alkaloid β_2AR activity

Vellosimine-type alkaloids are bioactive components in the *A. scholaris* extract whose $[M+H]^+$ ions are the base peak in the positive ESI mode. The MS^n data on these ions mainly gave fragment ions corresponding to α-bond cracking in an N-close position and to McLafferty rearrangements; most of the fragments were β-carboline derivatives (*m/z* 168). This result is coincidental with the known vellosimine-type alkaloid fragmentation patterns [38]. For example, peak 7 at the 28.157 min retention time (*m/z* 352) gave the fragment ions $[M+2H-CH_2OH]^+$ at *m/z* 323, $[M+H-CH_2OH-OCH_3]^+$ at *m/z* 291, and $[M+2H-C_{11}H_7N_2-C_2H_4]^+$ at *m/z* 143, which correspond to akuammidine. The MS^n spectrum of akuammidine is shown in Fig. 6A. The chemical formula of peaks 35 and 36 correspond to the exact molecular weight (*m/z* 360) and fragmentation pathways of $[M-COOCH_3]^+$ at *m/z* 301 and $[M+H-COOCH_3-CHO]^+$ at *m/z* 273, respectively. However, because of the different spatial locations on the MS^4 spectrum, the methyl indole fragment $[M-COOCH_3-CHO-C_8H_6N]^+$ of Z-alstoscholarine at *m/z* 156 (Fig. 6B1) and the components of E-alstoscholarine correspond to the methyl carbazole debris $[M-COOCH_3-CHO-C_6H_5N]^+$ at *m/z* 181 (Fig. 6B2).

4. Conclusions

In the present work, a dual-luciferase reporter assay guided LCMS-IT-TOF system for β_2AR agonist biological fingerprinting analysis was developed. The Chinese folk herbal prescription alkaloidal extract of *A. scholaris* was divided into fractions using a conventional chromatographic separation, and the fractions were screened for β_2AR activity. An LCMS-IT-TOF system was then used for the characterization of the multiple compounds in these fractions. After experimental validation of the pharmacological effects of the purified samples, two new indole alkaloid-type β_2AR agonists, akuammidine and Z-alstoscholarine, were characterized. The results indicate that the developed system has advantages over conventional methods in the investigation of potential bioactive components in TCMs. This strategy is rapid, reliable, and shows high sensitivity and resolution in identifying the bioactive constituents in complex chemical systems such as TCM prescriptions.

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