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Microfractionation bioactivity-based ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry for the identification of nuclear factor-κB inhibitors and β_2 adrenergic receptor agonists in an alkaloidal extract of the folk herb *Alstonia scholaris*

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ABSTRACT

Traditional Chinese medicines (TCMs) are generally considered complementary or alternative remedies in most Western countries. The constituents of TCMs are hard to define, and their efficacy is difficult to appraise. Thus, the development of suitable methods for evaluating the relationship between bioactivity and the chemical makeup of complex TCM mixtures remains a great challenge. In the present work, the bioactivity-integrated fingerprints of alkaloidal leaf extracts of *Alstonia scholaris*, a folk medicinal herb for chronic respiratory diseases, were established by ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF). This method was coupled with two dual-luciferase reporter assay systems to show nuclear factor-κB (NF-κB) inhibition and β_2 adrenergic receptor (β_2AR) activation. Using UPLC-Q/TOF, 18 potential candidates were identified according to unique mass spectrometric fragmentation. After *in vitro* biological evaluation, several indole alkaloids with anti-inflammatory and anti-asthmatic properties were found, including akuammidine, (E)-alstoscholarine, and (Z)-alstoscholarine. Compared with conventional fingerprints, the microfractionation based bioactivity-integrated fingerprints that contain both chemical and bioactivity details offer a more comprehensive understanding of the chemical makeup of plant materials. This strategy clearly demonstrated that dual bioactivity-integrated fingerprinting is a powerful tool for the improved screening and identification of potential dual-target lead compounds in complex herbal medicines.

1. Introduction

The US Food and Drug Administration reported that drug discovery showed a downward trend in the past decade [1]. One of the reasons was that drug development models focus on searching for disease-related single targets and their corresponding chemical entity [2,3]. This approach has indeed provided many potential drugs that generally interact with diseases caused by a single factor. However, most diseases are multi-factorial, and treating a single target provides only partial treatment. In addition, serious side effects have been reported with some conventional treatments, especially for chronic diseases [4]. Therefore, multi-component drugs are becoming new therapeutic regimens. Single drugs acting on multiple targets have also become a major focus of research [5,6].

Natural products and their metabolites are primary sources of chemical drug entities [7,8]. An ideal drug has efficacy based not only on the regulation of a single target but also on the rebalance of the network of the human body. Focus on multi-target therapeutics from natural products may lead to new insights into drug discovery. Traditional Chinese medicines (TCMs) have played an important role in the treatment of many complex diseases because of their long history of clinical use and reliable therapeutic efficacy. The use of TCMs has also become more popular worldwide in the past decade. TCMs include combinatorial herbs, animal products, and minerals. TCMs are multi-component complex systems interacting with multiple targets and pathways throughout the human body [9]. Therefore, clarifying the mechanism of TCMs is an effective approach to explore multi-component drugs [10].

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TCMs have been proven effective by modern pharmacological studies and clinical trials, but identifying novel bioactive compounds from TCMs remains a challenge. Screening new chemical entities is very important for determining the therapeutic basis of the materials and for identifying lead compounds for drug design. Owing to rapid technical advances and the increasing availability of instrumentation, many techniques have been used to study natural resource medicines. Among them, liquid chromatography (LC)/mass spectrometry (MS) is becoming one of the most essential strategies [11]. Applications of LC/MS in determining chemical entities in TCMs, clarifying fingerprinting, serum pharmacology, and metabolomics have grown rapidly in recent years [12–15]. Unfortunately, LC/MS cannot efficiently evaluate the relationship between bioactivity and chemical characteristics.

Chronic obstructive pulmonary disease (COPD) and allergic asthma are now the fourth leading cause of death. COPD is an airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyper-responsiveness [16]. The inflammatory response is a highly coordinated process involving multiple factors acting in a complex network as stimulators or inhibitors. The synergy of cytokine and chemokine networks amplifies the inflammatory response [17,18]. Considering the complexity of the network, strategies against a single target cannot effectively treat respiratory disease [19,20]. Currently, the combination therapy of an inhaled corticosteroid and a long-acting β_2 adrenergic receptor (β_2AR) agonist is effective for COPD [21], and multi-targeting treatment is receiving more attention [22,23]. These findings inspired us to investigate the anti-inflammatory and anti-asthmatic chemical entities in TCMs.

Leaves of the folk medicinal herb *Alstonia scholaris* (Apocynaceae) have been historically used in ethnopharmacy to treat chronic respiratory diseases in China [24,25]. Traditional research methods use pharmacological models for the detection, isolation, and structural elucidation of biologically active compounds. Some monoterpenoid indole alkaloids have been identified from the *A. scholaris* plant and were shown to possess potent anticancer, antibacterial, antifertility, antitussive, and anti-asthma bioactivity [26–28]. However, due to the limited number of identified rare compounds, biological activity testing was not viable. Our group recently developed a cell-based β_2AR agonist functional evaluation model coupled with high-performance LC (HPLC) to screen potential β_2AR agonist components of an alkaloidal extract of *A. scholaris* leaves. Two new β_2AR agonists with novel skeleton monoterpenoid indole alkaloids were successfully identified, and their activity was confirmed with an *in vivo* relaxant test on guinea pig tracheal muscles [29].

Nuclear factor-κB (NF-κB), composed of homo- and heterodimeric complexes of members of the Rel protein family, resides in the cytoplasm. Normally, NF-κB is retained by an association with IκB protein (an endogenous inhibitor). A variety of extracellular stimuli trigger the degradation of IκB by the proteasome pathway [30]. Subsequently, the released NF-κB plays an important role in regulating the expression of several genes involved in immune and inflammatory responses [31]. In the present work, an ultra performance LC (UPLC)–MS method was used to construct advanced chromatographic fingerprints for the recognition of multiple compounds in the alkaloidal extract of *A. scholaris*. Dual-luciferase reporter assay systems for NF-κB inhibitor bioactivity-integrated fingerprinting analysis were then developed. Several indole alkaloid compounds with anti-inflammatory properties were identified and their activities confirmed *via in vitro* inflammatory response inhibition tests. Cell-based β_2AR agonist screening was carried out in parallel, and several multi-target ingredients with anti-inflammatory and anti-asthmatic functions were found.

2. Materials and methods

2.1. Chemicals and materials

HPLC-grade solvent was purchased from Tedia (Fairfield, CA, USA). Deionized water was purified using a Milli-Q system (Millipore Laboratory, Bedford, MA, USA). Purified standard alkaloid samples were isolated from *A. scholaris* [25,27] and used for bioassay and chromatographic analyses. Human tumor necrosis factor-α (TNF-α) was purchased from PeproTech (Rocky Hill, NJ, USA). Dexamethasone (Dex) and salbutamol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). BEAS-2B cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). NF-κB luciferase reporter plasmid pGL4.32 and Renilla luciferase reporter vector pRL-TK plasmid were obtained from Promega (Madison, WI, USA). β_2AR-transfected human embryonic kidney 293 (β_2AR-HEK 293) cells were constructed in our laboratory [32]. All reagents for cell culture were purchased from GibcoBRL Life Technologies (Rockville, MD, USA). Other reagents were analytical grade.

2.2. Sample preparation and UPLC conditions

The alkaloidal extract of dried and powdered leaves of *A. scholaris* was prepared as previously described [29]. The alkaloid fraction was dissolved in CH_3CN and filtered through a 0.22 μm filter prior to UPLC analysis. A Waters Acquity UPLC instrument system (Milford, MA, USA) equipped with a photo DAD was used with the wavelength set at 280 nm. The system was controlled by Waters MassLynx v4.1 software. A Waters Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) was employed for the separations. A gradient elution of water (A) and CH_3CN (B) was performed as follows: 10% B maintained at 0–8 min, 10–40% B at 8–38 min, 40–100% B at 38–48 min, and 100% at 48–55 min. The flow rate was 0.40 mL/min and the column temperature was 30 °C. The injection volume of the test sample was 2 μL.

2.3. Tandem MS (MS/MS) conditions

Accurate mass measurements and MS/MS were performed on a Waters Q/TOF Premier with an ESI system (Waters, Manchester, UK). The ESI–MS spectra were acquired in positive ion mode, and the capillary voltage was set to 3.0 kV. The sample cone voltage was set to 30 V, and high-purity nitrogen (99.9996%) was used as the nebulization and auxiliary gas. The nebulization gas was set to 600 L/h at 350 °C, the cone gas was set to 50 L/h, and the source temperature was 100 °C. The Q/TOF Premier acquisition rate was 0.1 s with a 0.02 s inter-scan delay. Argon was employed as the collision gas at a pressure of 5.3×10^{-5} Torr. The instrument was operated with the first resolving quadrupole in wide-pass mode (50–1000 Da) with the collision cell operating at 30 eV. Leucine enkephalinamide acetate was used as the lock mass ($[M+H]^+ = 555.2931$) at a concentration of 200 μg/L and flow rate of 20 μL/min.

2.4. Measurements of lactate dehydrogenase (LDH)

The release of LDH from the cells was detected to measure the cytotoxicity of drugs. At the end of the incubation period, the supernatants were collected. The activity of LDH was measured according to the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Luciferase reporter assay for NF-κB inhibition and β_2AR activation

UPLC fractions were collected into a 96 deep-well plate every minute and then evaporated to dryness in a vacuum drying oven at 50 °C. The residues were dissolved with 100 μL of cell culture medium for the bioactivity assay. BEAS-2B cells were grown to confluence in 96-well plates using DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. The culture medium was replaced after 24 h, and the cells were co-transfected with 100 ng/well NF-κB luciferase reporter pGL4.32 plasmid and 10 ng/well Renilla luciferase reporter vector pRL-TK plasmid. Transfection was performed for 24 h using Lipofectamine 2000 according to the manufacturer's protocol. The cells were then cultured overnight in serum-free medium before changing to fresh serum-free medium containing drugs or the above-prepared fractions for 1 h before 5 ng/mL TNF-α stimulation for 6 h. Luciferase activity was then measured using a dual-luciferase reporter assay system (Madison, WI, USA), according to the manufacturer's instructions. Luminescence was detected using a Modulus luminometer from Turner Biosystems (Turner Design, Sunnyvale, CA, USA). The ratio of firefly luciferase activity to renilla luciferase activity was used to normalize the differences in transfection efficiency. The luciferase reporter assay for β_2AR activation was carried out as previously described [29].

2.6. Cytokine release measurement

BEAS-2B cells were used in the cytokine release assay. After incubation with TNF-α, the cell culture supernatants of stimulated cells were collected for enzyme-linked immunosorbent assay (ELISA). The releases of IL-6 and IL-8 were measured by commercial ELISA kits (R&D Systems, Hornby, ON, Canada) according to the manufacturer's instructions. Absorbance at 450 nm was detected by an ELISA reader ELX800 (Winooski, VT, USA).

2.7. Statistical analysis

The results are expressed as the standard deviation (SD). A statistical analysis of the data for multiple comparisons was performed using an analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test. For single comparisons, significant differences between the mean were determined using Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Optimization of UPLC-DAD and MS conditions

The chromatographic conditions were evaluated using different Acquity BEH C18 columns (2.1 mm × 100 mm, 1.7 μm; and 2.1 mm × 50 mm, 1.7 μm), and different mobile phases of methanol–water or acetonitrile–water. The 100 mm-long column had a higher peak capacity and better-separated peaks than the shorter column. Acetonitrile showed better baseline results as well as better peak shape and signal response in the retention time of 50 min (Fig. 1A). By comparing the chromatograms at different wavelengths, 280 nm was found to provide the best constituent profile for monoterpenoid indole alkaloids in the extract of *A. scholaris*. The following MS conditions were also investigated: capillary voltage (positive ion mode 2.5, 3.0, and 3.5 kV; negative ion mode 2.5, 3.0, and 3.5 kV), cone gas (45, 50, and 55 L/h), and source temperature (100, 110, and 120 °C). The total peak area was used as the criterion for optimization. The capillary voltage was set to 2.5 kV for the negative mode and 3.0 kV for the positive mode. The cone gas was set to 50 L/h and the source temperature was 100 °C.

3.2. UPLC-Q/TOF–MS/MS analysis of the alkaloidal extract of A. scholaris

The optimal UPLC-Q/TOF conditions were applied in the analysis of the alkaloidal extract of *A. scholaris* (Fig. 1B). The majority of constituents were indole alkaloids, so the positive mode provided higher ion intensities. The $[M+H]^+$ ions provided helpful information on the molecular weight of the constituents. The total ion current (TIC) chromatograms in the positive ESI mode are shown in Fig. 2B. By comparing with the literature data provided the molecular composition, each peak was confirmed using retention time as well as MS and MS/MS fragmentation data from previous studies. In the current study, peak 16 at 20.33 min retention time was chosen to illustrate the identification approach. The base peak in positive ESI mode was *m/z* 353.1481 and confirmed as $[M+H]^+$. The elemental and possible molecular compositions ($C_{20}H_{20}N_2O_4$) were deduced from the exact molecular weight, and these molecular compositions were retrieved from the Chemical Components of Source Plants. The fragmentation patterns (321 $[M-OCH_3]^+$, 293 $[M-COOCH_3]^+$), summarized from the spectra, were compared with other literature findings [33] and peak 16 was then deduced to be nareline. All other constituents were identified using the same approach; about 40 constituents were identified in the alkaloidal extract of *A. scholaris*. The detailed results of 18 potential bioactive compounds and MS/MS information are shown in Table 1. In accordance with the area normalization method, the area of each peak at 280 nm was calculated to assess the relative percent content (RPC) in total alkaloidal extract, as shown in Table 1.

3.3. Identification of bioactive compounds for NF-κB inhibitors and β_2AR agonists in the alkaloidal fraction

To identify the effective components from *A. scholaris* leaves, the alkaloidal extract components were separated by UPLC (Fig. 1A). Each fraction was collected and tested for activity using the dual-luciferase reporter assay system for NF-κB inhibitors and β_2AR agonists. Luciferase activity was expressed as the ratio of firefly to renilla luminescence. As shown in Fig. 1C and D, 17 peaks (fractions 2–18) showed potential activity for NF-κB, and 11 peaks (fractions 3–6, 11–15, 17, and 18) showed potential activity for β_2AR, respectively. Their chromatographic profile, MS/MS fragmentation, and biological activity information are shown in Table 1. According to the RPC of each indole alkaloid, the biological activity contribution of each fraction of NF-κB inhibitor and β_2AR agonist were evaluated, as shown in Table 1. Nine candidate ingredients with intense $[M+H]^+$ molecular ions gave signals at *m/z* 357.1854, 353.1901, 341.1903, 323.1735, 339.1719, 367.1707, 353.1481, 361.1547, and 361.1548. These values corresponded to the indole alkaloids scholaricine, akuammidine, 19Z-vallesamine, strictamine, picrinine, picralinal, nareline, (Z)-alstoscholarine, and (E)-alstoscholarine. The candidate ingredients were obtained after repeated accumulation *via* HPLC. The purity of the above compound was more than 90%, and their structures are shown in Fig. 2.

3.4. Cytotoxicity assays of anti-inflammatory compounds from A. scholaris leaves

As shown in Fig. 3, anti-inflammatory compounds (10 or 100 μmol/L) from *A. scholaris* leaves did not show a significant increment in the level of LDH activities in the serum of cells compared to the control, which indicated that the compounds (10 or 100 μmol/L) did not showed damage in the cells.

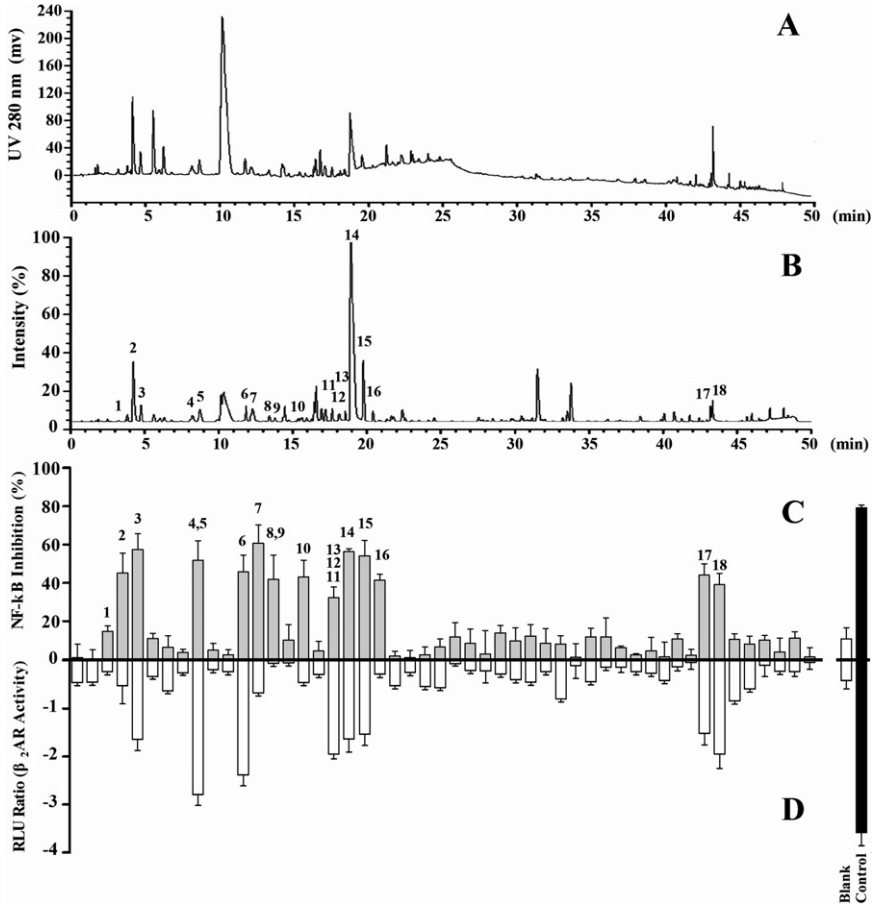

Fig. 1. (A) UPLC-UV chromatograms at 280 nm of *A. scholaris* alkaloidal extract; (B) Q/TOF TIC chromatograms in the positive ESI mode; (C) Bioactivity chromatograms obtained *via* the dual-luciferase reporter assay system for NF-κB inhibitors; (D) β_2AR agonist activation. The peak numbers are consistent with those in Table 1.

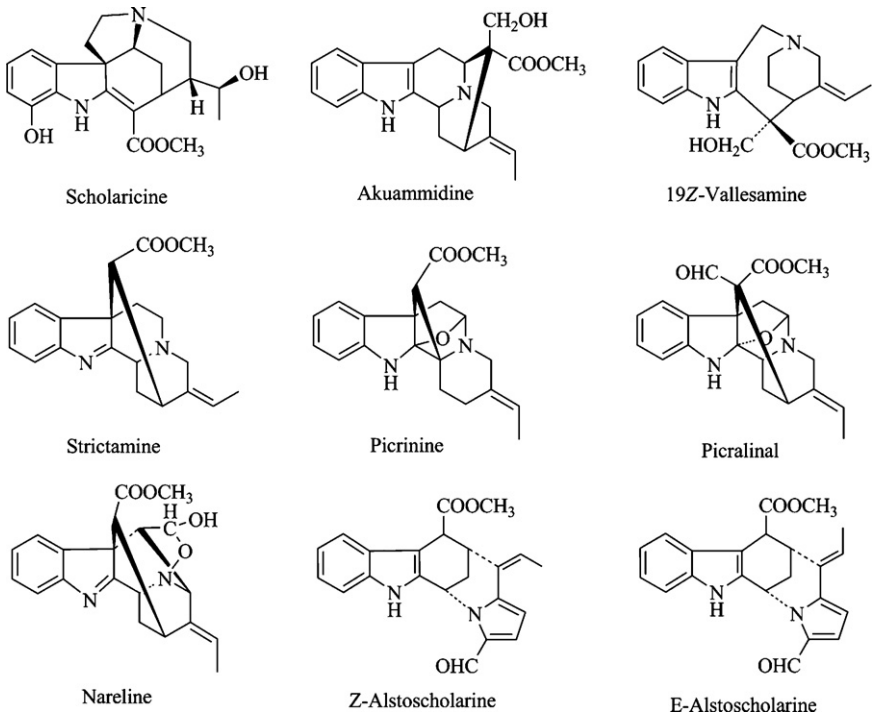

Fig. 2. Chemical structures of compounds identified as NF-κB inhibitors and β_2AR agonists in a functional evaluation model for *A. scholaris* alkaloidal extract.

Table 1
Chromatographic profile, MS/MS data of (+) ESI–MS, and the identification results of the bioactive compounds in an alkaloidal extract of *A. scholaris*.

No.	RT	UV (λ_{max})	Identification	*m/z* ([M+H]$^+$)	Formula	MS/MS	g/mol	RPC (%)	NF-κB (BAC)	β_2AR (BAC)
1	3.12	281	12-Hydroxy-echitamidine N^b-oxide	373.1774	$C_{20}H_{24}N_2O_5$	373 $[M+H]^+$, 355 $[M-OH]^+$, 341 $[M-OCH_3]^+$	372.17	0.16	–	–
2	4.12	233, 336	Scholaricine	357.1854	$C_{20}H_{24}N_2O_4$	357 $[M+H]^+$, 325 $[M-OCH_3]^+$, 279 $[M-H_2O-OCH_3-CO]^+$	356.17	5.83	+	–
3	4.63	238, 334	11-Methoxygelsemamide	357.1799	$C_{20}H_{24}N_2O_4$	357 $[M+H]^+$, 339 $[M-H_2O]^+$, 215 $[M-H_2O-OCH_3-C_7H_8]^+$	356.17	1.41	+	+
4	8.12	242, 281	Alstomaline	339.1667	$C_{20}H_{22}N_2O_3$	339 $[M+H]^+$, 309 $[M-CH_2O]^+$, 282 $[M-CHO-CO]^+$	338.16	1.07	++	++
5	8.60	226, 269	Akuammidine	353.1901	$C_{21}H_{24}N_2O_3$	353 $[M+H]^+$, 323 $[M-OCH_3]^+$, 306 $[M-OCH_3-OH]^+$, 293 $[M-COOCH_3]^+$	352.18	1.29		
6	11.73	239, 329	19Z-Vallesamine	341.1903	$C_{20}H_{24}N_2O_3$	341 $[M+H]^+$, 309 $[M-OCH_3]^+$, 282 $[M-COOCH_3]^+$	340.18	1.1	++	++
7	12.18	240, 283	*N*(4)-Demethylalstophyllal oxindole	369.1793	$C_{21}H_{24}N_2O_4$	369 $[M+H]^+$, 351 $[M-OH]^+$, 339 $[M-CHO]^+$	368.17	0.86	+	–
8	13.34	272	Rhazimanine	355.1994	$C_{21}H_{26}N_2O_3$	355 $[M+H]^+$, 323 $[M-CH_2OH]^+$	354.19	0.57	+	–
9	13.70	242, 282	Affinisine	309.1579	$C_{20}H_{24}N_2O$	309 $[M+H]^+$, 291 $[M-OH]^+$, 281 $[M-CO]^+$	308.19	0.18		
10	15.28	272, 334	Strictamine	323.1735	$C_{20}H_{22}N_2O_2$	323 $[M+H]^+$, 291 $[M-OCH_3]^+$, 263 $[M-OCH3-CO]^+$	322.17	0.20	++	–
11	16.81	234, 274	Alloyohimbine	355.1988	$C_{21}H_{26}N_2O_3$	355 $[M+H]^+$, 327 $[M+H-CO]^+$	354.19	1.54	+	+
12	17.14	269	19E-Vallesamine	341.1839	$C_{20}H_{24}N_2O_3$	341 $[M+H]^+$, 309 $[M-OCH_3]^+$, 282 $[M-COOCH_3]^+$	340.18	0.92		
13	17.57	234, 272	Pseudoyohimbine	355.1983	$C_{21}H_{26}N_2O_3$	355 $[M+H]^+$, 327 [M+H-CO]	354.19	0.54		
14	18.82	235, 285	Picrinine	339.1719	$C_{20}H_{22}N_2O_3$	339 $[M+H]^+$, 307 $[M-OCH_3]^+$, 279 $[M-OCH_3-CO]^+$	338.17	8.21	+	+
15	19.64	240, 289	Picralinal	367.1707	$C_{21}H_{22}N_2O_4$	367 $[M+H]^+$, 335 $[M-OCH_3]^+$, 307 $[M-CO-OCH_3]^+$	366.17	0.99	+	+
16	20.33	266	Nareline	353.1481	$C_{20}H_{20}N_2O_4$	353 $[M+H]^+$, 321 $[M-OCH_3]^+$, 293 $[M-COOCH_3]^+$	352.14	0.12	++	–
17	43.14	275	(Z)-Alstoscholarine	361.1547	$C_{22}H_{20}N_2O_3$	361 $[M+H]^+$, 301 $[M-COOCH_3]^+$	360.15	0.16	++	++
18	43.28	275	(E)-Alstoscholarine	361.1548	$C_{22}H_{20}N_2O_3$	361 $[M+H]^+$, 301 $[M-COOCH_3]^+$	360.15	0.46	++	++

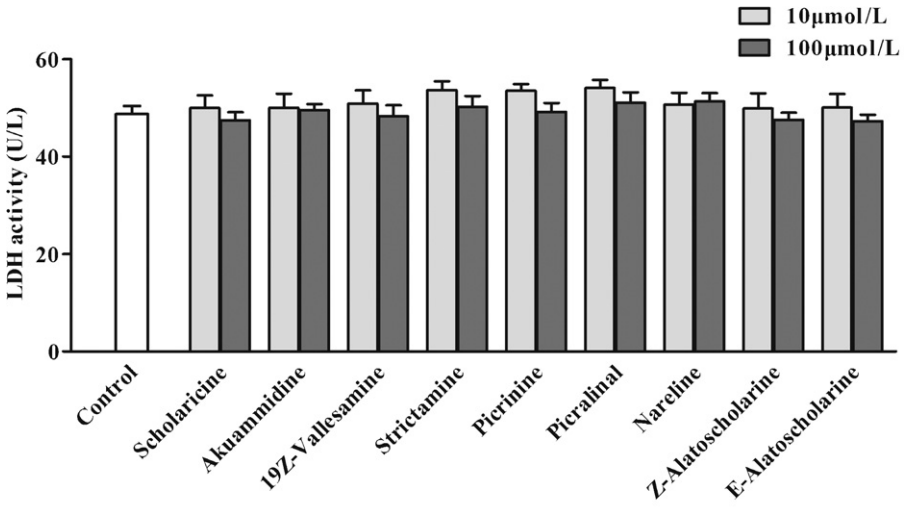

Fig. 3. Effect of anti-inflammatory compounds (10 or 100 μmol/L) from *A. scholaris* leaves on LDH activities in the serum of cells ($n = 5$).

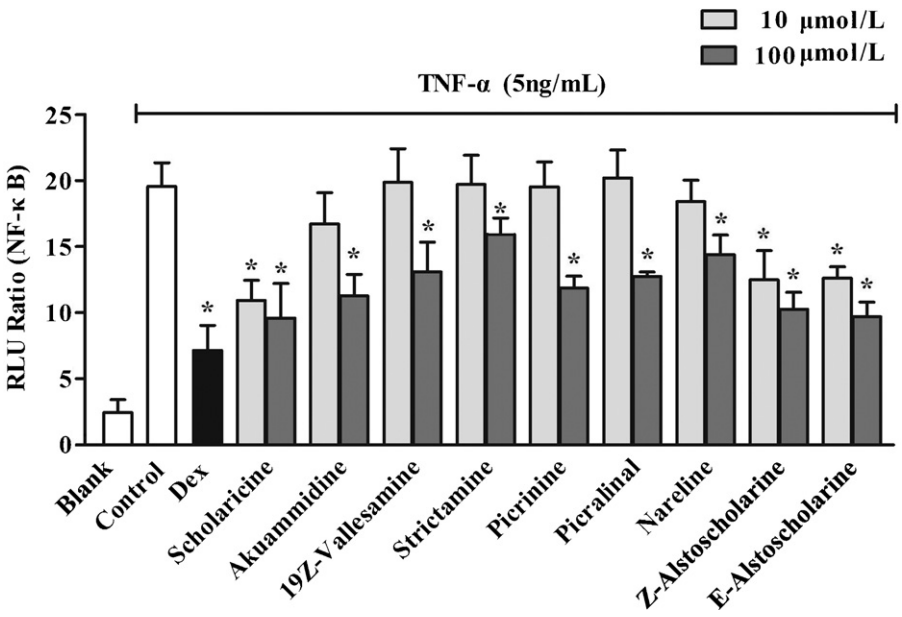

Fig. 4. Verification of NF-κB inhibitor activity using the dual-luciferase reporter assay system. Each bar represents the mean ± SD ($n = 5$), $^{*}p < 0.01$ *vs.* control.

3.5. Confirmation of anti-inflammatory compounds from A. scholaris leaves

The NF-κB inhibitors of the purified indole alkaloids (10 and 100 μmol/L) and the positive control drug Dex (10^{-5} mol/L) were determined by the luciferase reporter assay. The effects on the NF-κB level in TNF-α-induced BEAS-2B cells are shown in Fig. 4. Compared with the control, the Dex groups significantly inhibited TNF-α-induced NF-κB production ($p < 0.01$). All samples displayed good NF-κB inhibitor activation in high concentrations; however, only scholaricine, (Z)-alstoscholarine, and (E)-alstoscholarine showed significant effects at low concentrations ($p < 0.01$). A cytokine release test was performed to confirm the synergistic effects of the aforementioned alkaloids at the cellular level. The same samples (100 μmol/L) were added to TNF-α-stimulated BEAS-2B cells to determine efficacy in inhibiting the release of IL-6 and IL-8. As shown in Fig. 5, the expression of IL-6 decreased ($p < 0.05$) in cells treated with akuammidine, strictamine, picrinine, picralinal, (Z)-alstoscholarine, and (E)-alstoscholarine. For IL-8, all samples showed obvious suppression ($p < 0.05$). The positive control Dex (10 μmol/L) inhibited both IL-6 and IL-8 ($p < 0.01$).

The alkaloid fractions of *A. scholaris* leaf extract exhibit remarkable anti-inflammatory bioactivity [34]. Further investigation revealed that some purified alkaloids (100 μmol/L) inhibited the pro-inflammatory enzymes COX-1, COX-2, or 5-LOX *in vitro*. The three main alkaloids picrinine, vallesamine, and scholaricine also produced anti-inflammatory effects in animal models [25]. The promoter region of the human COX-2 gene contains specific motifs with sequence similarity to the consensus-binding site for NF-κB. Importantly, NF-κB has a role in TNF-α-induced COX-2

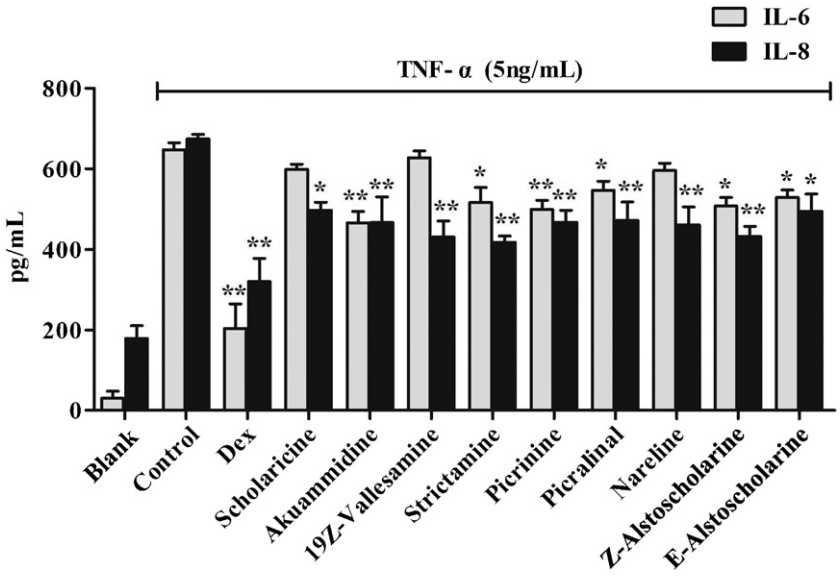

Fig. 5. Effect of purified indole alkaloids on anti-inflammatory activity by cytokine release tests. The response of alkaloids to the inhibition of the release of IL-6 (white column) and IL-8 (black column) are represented as mean ± SD for triplicate samples. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the saline control group.

expression [35,36]. The current findings using UPLC-Q/TOF coupled with a dual-luciferase reporter assay for NF-κB inhibitor screening was in accord with previous reports [25]. Combined with chromatographic fingerprint analysis, several low content (RPC, 0.12–0.2%) but highly bioactive NF-κB inhibitors, namely, strictamine, nareline, and (Z)-alstoscholarine were found.

3.6. Dual-target candidate analysis of A. scholaris leaves

The microfractionation bioactivity-integrated UPLC-Q/TOF functional evaluation system had a clear technological advantage in screening and identifying the dual-target lead compounds in a complex herbal medicine. Integrated chemical information, NF-κB inhibitor detection, and β_2AR agonist screening [29] revealed that three potential monoterpenoid indole alkaloids, namely, akuammidine, (Z)-alstoscholarine, and (E)-alstoscholarine, had both anti-inflammatory and anti-asthmatic activities. Thus, these dual-functional lead compounds have potential use in chronic airway inflammatory diseases therapy. Interestingly, total synthesis approaches for (E)- and (Z)-alstoscholarine were reported recently [37], indicating that the dual-target candidate (Z)-alstoscholarine may be worthy of further study.

4. Conclusions

In the present work, a method using UPLC-Q/TOF and two microfractionation based dual-luciferase reporter assays for guided screening and identification was established for NF-κB inhibitors and β_2AR agonists. Consequently, 18 monoterpenoid indole alkaloids were identified from the *A. scholaris* extract. After *in vitro* evaluation, three dual-functional lead compounds, akuammidine, (Z)-alstoscholarine, and (E)-alstoscholarine, were found. Among them, (Z)-alstoscholarine is worthy of further attention in chronic airway inflammatory diseases therapy. Compared with conventional fingerprints, this strategy clearly demonstrates that dual bioactivity-integrated fingerprinting is a powerful tool for improving the screening and identification of potential dual-target lead compounds from complex herbal medicines.

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References

[1] A. Mullard, Nat. Rev. Drug Discov. 11 (2012) 91–94.
[2] R.D. Clark, Curr. Top. Med. Chem. 9 (2009) 791–810.
[3] B. Hughes, Nat. Rev. Drug Discov. 8 (2009) 93–96.
[4] C.T. Keith, A.A. Borisy, B.R. Stockwell, Nat. Rev. Drug Discov. 4 (2005) 71–78.
[5] P. Csermely, V. Agoston, S. Pongor, Trends Pharmacol. Sci. 26 (2005) 178–182.
[6] A. Petrelli, S. Giordano, Curr. Med. Chem. 15 (2008) 422–432.
[7] D.J. Newman, G.M. Cragg, K.M. Snader, Nat. Prod. Rep. 17 (2000) 215–234.
[8] N.M. Njuguna, C. Masimirembwa, K. Chibale, J. Nat. Prod. 75 (2012) 507–513.
[9] M. Wang, R.J. Lamers, H.A. Korthout, J.H. van Nesselrooij, R.F. Witkamp, R. van der Heijden, P.J. Voshol, L.M. Havekes, R. Verpoorte, J. van der Greef, Phytother. Res. 19 (2005) 173–182.
[10] P. Tian, Nature 480 (2011) S84–S86.
[11] T. Guan, Y. Liang, C. Li, L. Xie, G. Wang, L. Sheng, Chin. J. Nat. Med. 9 (2011) 385–400.
[12] J.L. Zhou, L.W. Qi, P. Li, J. Chromatogr. A 1216 (2009) 7582–7594.
[13] M. Yang, J. Sun, Z. Lu, G. Chen, S. Guan, X. Liu, B. Jiang, M. Ye, D.A. Guo, J. Chromatogr. A 1216 (2009) 2045–2062.
[14] D. Steinmann, M. Ganzera, J. Pharm. Biomed. Anal. 55 (2011) 744–757.
[15] M.J. Gray, D. Chang, Y. Zhang, J. Liu, A. Bensoussan, Biomed. Chromatogr. 24 (2010) 91–103.
[16] A.B. Kay, J. Allergy Clin. Immunol. 87 (1991) 893–910.
[17] M. Gouwy, S. Struyf, P. Proost, J. van Damme, Cytokine Growth Factor Rev. 16 (2005) 561–580.
[18] A.M. Vignola, S. la Grutta, G. Chiappara, A. Benkeder, V. Bellia, G. Bonsiqnore, Paediatr. Respir. Rev. 3 (2002) 41–46.
[19] P.J. Barnes, Br. J. Pharmacol. 147 (2006) S297–S303.
[20] A. Viola, A.D. Luster, Annu. Rev. Pharmacol. Toxicol. 48 (2008) 171–197.
[21] D.W. Mapel, J.S. Hurley, A.A. Dalal, C.M. Blanchette, Prim. Care Respir. J. 19 (2010) 93–103.
[22] L.A. O'Neill, Nat. Rev. Drug Discov. 5 (2006) 549–563.
[23] C.M. Hai, Curr. Med. Chem. 14 (2007) 67–76.
[24] J.H. Shang, X.H. Cai, Y.L. Zhao, T. Feng, X.D. Luo, J. Ethnopharmacol. 129 (2010) 293–298.
[25] J.H. Shang, X.H. Cai, T. Feng, Y.L. Zhao, J.K. Wang, L.Y. Zhang, M. Yan, X.D. Luo, J. Ethnopharmacol. 129 (2010) 174–181.
[26] M.R. Khan, A.D. Omoloso, M. Kihara, Fitoterapia 74 (2003) 736–740.
[27] X.H. Cai, Z.Z. Du, X.D. Luo, Org. Lett. 9 (2007) 1817–1820.
[28] P. Kamarajan, N. Sekar, V. Mathuram, S. Govindasamy, Biochem. Int. 25 (1991) 491–498.
[29] Y. Hou, X. Cao, L. Dong, L. Wang, B. Cheng, Q. Shi, X. Luo, G. Bai, J. Chromatogr. A 1227 (2012) 203–209.
[30] P.J. Barnes, M. Karin, N. Engl. J. Med. 336 (1997) 1066–1071.
[31] S. Ghosh, M.J. Ma, E.B. Kopp, Annu. Rev. Immunol. 16 (1998) 225–260.
[32] G. Bai, Y. Yang, Q. Shi, Z. Liu, Q. Zhang, Y.Y. Zhu, Acta Pharmacol. Sin. 29 (2008) 1187–1194.
[33] L. Jain, M.B. Pandey, S. Singh, A.K. Singh, V.B. Pandey, Nat. Prod. Res. 23 (2009) 1599–1602.
[34] S. Arulmozhi, P.M. Mazumder, P. Ashok, B. Hulkoti, L.S. Narayanan, Pharmacogn. Mag. 3 (2007) 106–111.
[35] J. Martel-Pelletier, J.P. Pelletier, H. Fahmi, Semin. Arthritis Rheum. 33 (2003) 155–167.
[36] J. Ke, X. Long, Y. Liu, Y.F. Zhang, J. Li, W. Fang, Q.G. Meng, J. Dent. Res. 86 (2007) 363–367.
[37] T. Gerfaud, C. Xie, L. Neuville, J. Zhu, Angew. Chem. Int. Ed. Engl. 50 (2011) 3954–3957.