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Short communication

Quantification of huperzine A in *Huperzia serrata* by HPLC-UV and identification of the major constituents in its alkaloid extracts by HPLC-DAD-MS-MS

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Abstract

A rapid, simple and reliable high performance liquid chromatography (HPLC) method has been established for the analysis of the major alkaloids in *Huperzia serrata*, a traditional Chinese medicine (TCM) herb. After chromatographic separation on a reversed-phase C_{18} column with methanol-ammonium acetate (pH 6.0; 80 mM, 30/70, v/v) as the mobile phase, nine alkaloid compounds in the alkaloid extracts of *H. serrata* were identified by online diode array detection–MS and by comparing with data from literature and standard samples. One compound in the extract, huperzine A, is a drug for treating Alzheimer's disease. Its content was quantified by HPLC coupled with UV-vis. The method was the validated. The recovery rates were 96.8–97.7% with R.S.D <2.44%. The intra- and inter-day precisions, expressed as R.S.D., ranged from 0.53% to 1.51%. Good linear regression was observed in the concentration range of 5–100 μg/ml ($r = 0.9997$). The results demonstrate that this method is simple, selective, and suitable for the quality control of this TCM herb.
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Keywords: Huperzine A; *Huperzia serrata*; HPLC/DAD/MS

1. Introduction

Huperzia serrata, a member of the *Lycopodium* species, has been used in traditional Chinese medicine for thousands of years as a diuretic, hemostyptic, antispasmodic, and analgesic agent [1]. In recent years, huperzine A, one of the chemicals in *H. serrata*, has been studied extensively for its potential in treating dementias such as Alzheimer's disease.

Alzheimer's disease is one of the major diseases affecting the elderly population throughout the world. The disease is a neurodegenerative disorder associated with neuritic plaques that affect the cerebral cortex, amygdala and hippocampus. There is also neurotransmission damage in the brain [2]. One of the major functional deficits in Alzheimer's disease is a hypofunction of cholinergic neurons. This leads to the cholinergic hypothesis of Alzheimer's disease and the rationale for strategies to increase acetylcholine in the brains of Alzheimer's disease patients [3]. Two FDA-approved drugs for the treatment of Alzheimer's disease, tacrine and donepezil, are acetylcholinesterase inhibitors. Huperzine A is also an acetylcholinesterase inhibitor and has been found to increase acetylcholine levels in the rat brain following its administration. It also increases norepinephrine and dopamine, but not serotonin levels [4]. Synthesis of huperzine A and its numerous analogues has been reported [5,6]. Due to the high cost, however, extraction from plants is still the major source of huperzine A. Natural huperzine A is a chiral molecule also called L-huperzine A or (−)-huperzine A. Synthetic huperzine A is a racemic mixture called (±)-huperzine A. The synthetic racemic mixture (±)-huperzine-A was three times less potent than (−)-huperzine A in vitro [7]. As the natural source of huperzine A, the whole herb also contains a large amount of various alkaloids, such as the selagine, lycodine, fawcettidine, obscurinine and lycopodine type alkaloids [8–13]. In recent studies, some alkaloids with similar structures to huperzine A in *H. serrata*, such as huperzine B, 6 β-hydroxyhuperzne A and *N*-methyl-huperzine B have been proved to exert AChE inhibiting effects [14–16].

In this study a HPLC method for the determination of huperzine A was developed, and based on the chromatographic conditions, the major constituents in alkaloid extracts of this

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plant were identified by high performance liquid chromatography (HPLC)–diode array detection (DAD)–tandem mass spectrometry (MS–MS). The technique takes advantage of chromatography as a separation method and both DAD and MS as an identification method. DAD and MS can provide on-line UV and MS information for each individual peak in a chromatogram, and the protonated molecular ions were further analyzed by MS/MS. The further structural information can be acquired by collision-induced dissociation (CID) of pseudomolecular ions. So in most cases, these peaks can be identifical directly by comparison with literature data or with standard compounds.

2. Experimental

2.1. Regents and chemicals

Acetic acid, ammonium acetate and chloroform were of analytical grade from Shanghai Company of Chemical Reagent (Shanghai, China). Ammonium solution was of analytical grade from Shanghai China Reagent Company (Shanghai, China). Hydrochloric acid was obtained from Shanghai Zhenxin no. 2 Chemical Reagent Factory (Shanghai, China). Water for HPLC analysis was purified by Kemflo HY-50 system (Kemflo, USA). Double distilled water was used for the extraction and preparation of samples. Methanol for HPLC analysis was of HPLC grade from Tedia (Fairfield, OH, USA). The standard samples of (−)-huperzine A were purchased from NingBo Traditional Chinese Pharmaceutical Factory (Zhejiang, China).

Table 1
Experimental values obtained in the recovery test of huperzine A

Amount of standard (μg/ml)			Recovery[a] (%)	R.S.D. (%)
	Added	Recovered		
R_1	5	4.84	96.8	2.44
R_2	10	9.77	97.7	1.77
R_3	20	19.42	97.1	2.13

[a] Mean of three replicate analyses.

2.2. Plant material and sample preparation

H. serrata was grown in the university gardens. Plants were collected (October 2003), gently cleaned, sorted (whole herbs, roots, stems, leaves), and dried at 60 °C until constant weight.

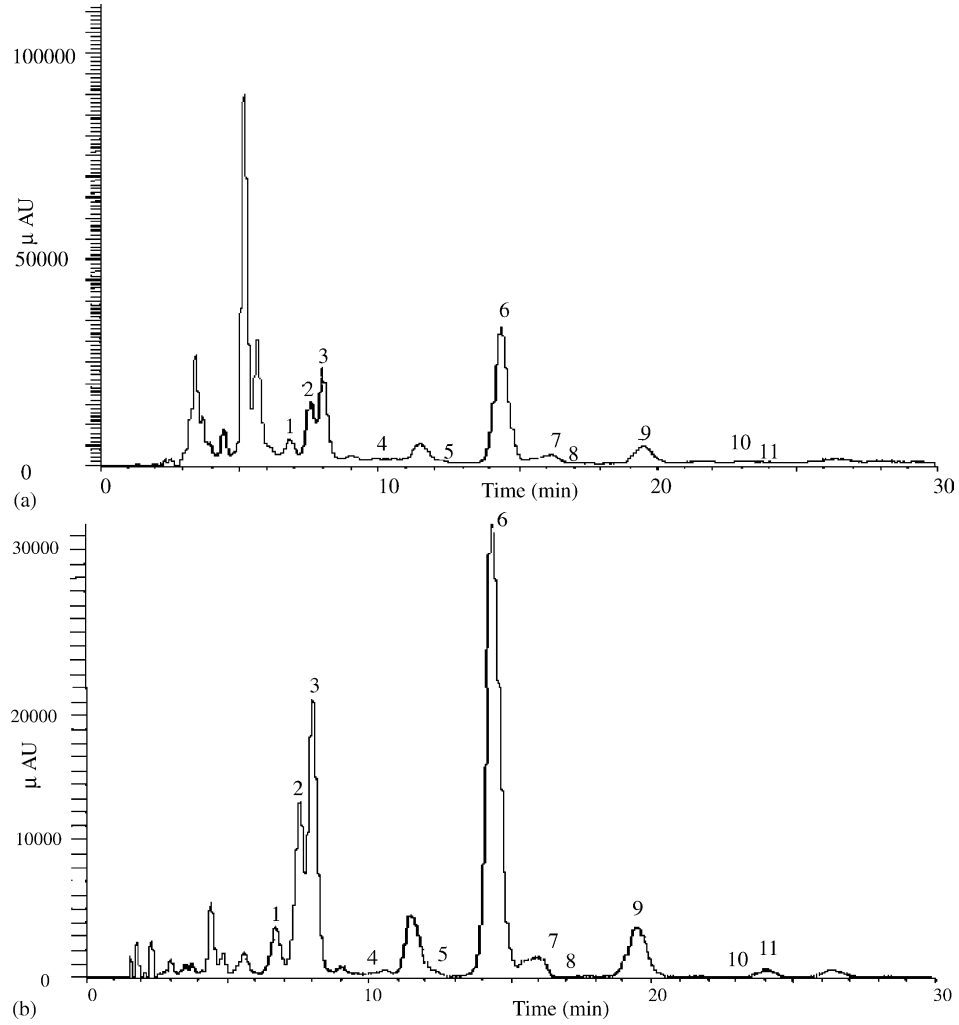

Fig. 1. HPLC chromatograms of the alkaloid extract of *Huperzia serrata* at (a) 230 nm and (b) 310 nm. Compound names and retention times see Table 3.

Approximately 15 g of the dried material were pulverized, accurately weighed, and then placed in a Soxhlet extraction apparatus. The material was refluxed with 300 ml of 95% ethanol for 24 h. The ethanol extracts were pooled, evaporated to dryness, and were dissolved in 100 ml of 0.5 M HCl with stirring for 2 h at room temperature. The filtered solution was then extracted three times with $CHCl_3$. After alkalization with NH_4OH to pH 9, the aqueous phase was extracted for another five times with $CHCl_3$. After evaporation, the obtained residues were reconstituted into 50 ml of methanol and filtered through a syringe filter (0.45 μm). Aliquots (10 ml) of the resultant extracts were subjected to HPLC analysis.

The reference of Huperzine A was weighed accurately then dissolved in methanol and diluted to 0.1 mg/ml. Standard solution was stored in the dark under refrigeration at 4 °C and was found to be stable for several months. A series of working standard solutions were prepared by the appropriate dilution of the above-mentioned standard solution with methanol to create huperzine A concentrations of 5.0, 10, 20, 40, 70 and 100 μg/ml.

2.3. HPLC-UV analysis

HPLC analyses were carried out on a Waters (Milford, MA, USA) 600-MS pump system, connected to a tunable UV–vis Waters 486 detector. A Diamonsil (Beijing, China) C_{18} column (5 μm, 200 mm × 4.6 mm I.D.) was used. The column was eluted with methanol-ammonium acetate (pH 6.0; 80 mM) (40/60, v/v) at a flow rate of 1 ml/min. Samples were filtered through a syringe filter (0.45 μm) prior to each injection.

2.4. HPLC-DAD-MS–MS analysis

The HPLC conditions for HPLC-DAD-MS-MS analysis were the same as those used for HPLC-UV analysis, except that the mobile phase was methanol-ammonium acetate (pH 6.0; 80 mM) (30/70, v/v). A Finnigan LCQ Advantage HPLC/MS^n (Thermo Finnigan, USA) system with an ESI interface was coupled to a Surveyor PDA Detector set at 230 and 310 nm. The ESI–MS spectra were acquired in positive ion mode. ESI values were as follows: source voltage 4.5 kV, sheath gas flow-rate 35 p.s.i., auxiliary gas flow 10 p.s.i., capillary voltage 3.14 V, capillary temperature 275 °C and scan range 100–500 u.

3. Results and discussion

3.1. HPLC determination of huperzine A in Huperzia serrata

Optimized chromatographic conditions were achieved after several trials with methanol, water, and ammonium acetate buffer in various proportions. It was found that the presence of ammonium acetate in the mobile phase had a significant effect on the retention behavior of alkaloids of *H. serrata*. When ammonium acetate was absent in the methanol–water (40:60, v/v) system, there was no peck within 30 min. Once 80 mM ammonium acetate buffer (pH 6.0) was used instead of water, the t_R of alkaloids shortened dramatically. In the system an increase in the percentage of methanol decreases the retention of the alkaloid compounds. Subsequently, the optimal mobile phase consisting of methanol- ammonium acetate (pH 6.0; 80 mM) (40/60, v/v) was employed, which leads to well-separated resolution, satisfactory peak shape as well as relatively short time for analysis.

Table 2
Sample analysis result

Sample origin	Average content of huperzine A (%)	R.S.D. (%)
The whole herb	0.0118	2.11
Root of *Huperzia serrata*	0.0019	2.86
Stem of *Huperzia serrata*	0.0071	1.72
Leaf of *Huperzia serrata*	0.0175	1.57

We used the external standard method, because it is simple**,** fast and accurate for sample preparation. The retention time repeatability during the precision studies was found to be excellent for all the solutions. The calibration curves for huperzine A were constructed by plotting concentration versus peak area and showed good linearity in the 5.0–100.0 μg/ml range. The linear equation was $y = 44695x + 7149.8$ ($r = 0.9997$). A series of sample analyses were performed to validate the performance of the method. The measurements of intra- and inter-day precisions were utilized to assess the repeatability and reproducibility of the developed assay. In the concentration of 15, 30, and 60 μg/ml the percent R.S.D. on the basis of peak area ratios for five replicate

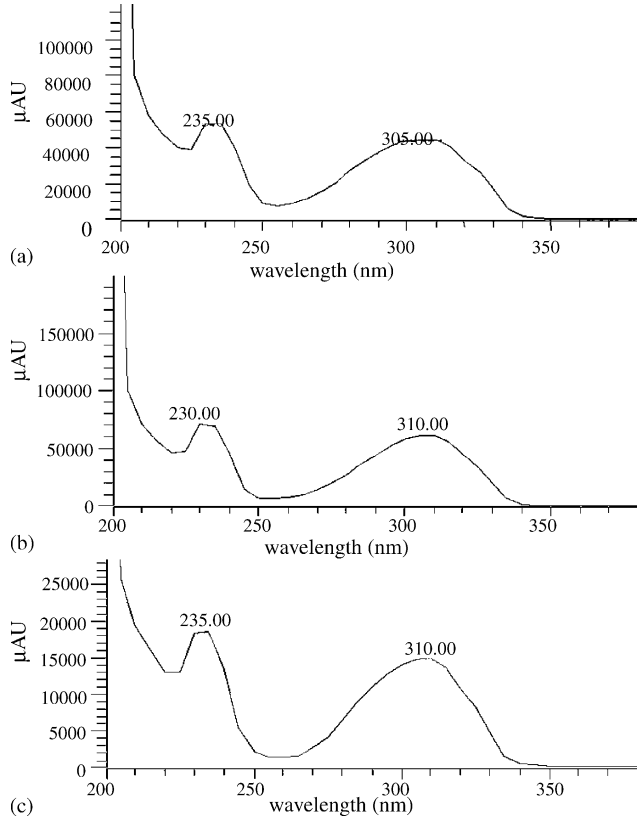

Fig. 2. UV spectra of 6 β-hydroxyhuperzne(a), A huperzine B(b) and *N*-methylhuperzine B(c).

Table 3
HPLC-DAD-ESI-MS-MS identification

Compound	t_R (min)	$[M+H]^+$ (*m/z*)	UV λ_{max} (nm)	Identification
1	6.90	264	230,285	Lycodoline[a]
2	7.80	259	235,305	6 β-hydroxyhuperzne A
3	8.18	257	230,310	Huperzine B
4	10.29	248		Lycopodine
5	12.36	308		Acetylannofoline or its isomer
6	14.60	243	230,310	Huperzine A
7	16.18	289	230	A novel lycopodine type alkaloid
8	16.85	308		Acetylannofoline or its isomer
9	19.60	271	235,310	*N*-methyl-huperzine B
10	23.20	319	230	A novel lycopodine type alkaloid
11	24.11	260	325	Huperzine E*

[a] Tentative identification.

injections were 1.25, 0.66 and 0.96%, respectively. The inter-day precisions (3 days, $n = 5$) expressed as R.S.D. were 0.87, 1.51 and 0.53%, respectively. The accuracy was assessed by a recovery experiment. Huperzine A reference standards were added to a sample of dried plant material known huperzine A content and the mixture was processed as described above. The recovery was calculated by comparing the found amount of standards to those of added. The results obtained can be seen in Table 1. The limits of detection (LOD) were 60 ng/ml ($s/n = 3$).

When the test solutions of *H. serrata* extracts were analyzed by HPLC under the selected conditions, the calibration curves were used for quantitative analysis. The extractions of different parts of *H. serrata* showed identical profiles in the analysis of their alkaloid content (not shown). Table 2 reports the yields in

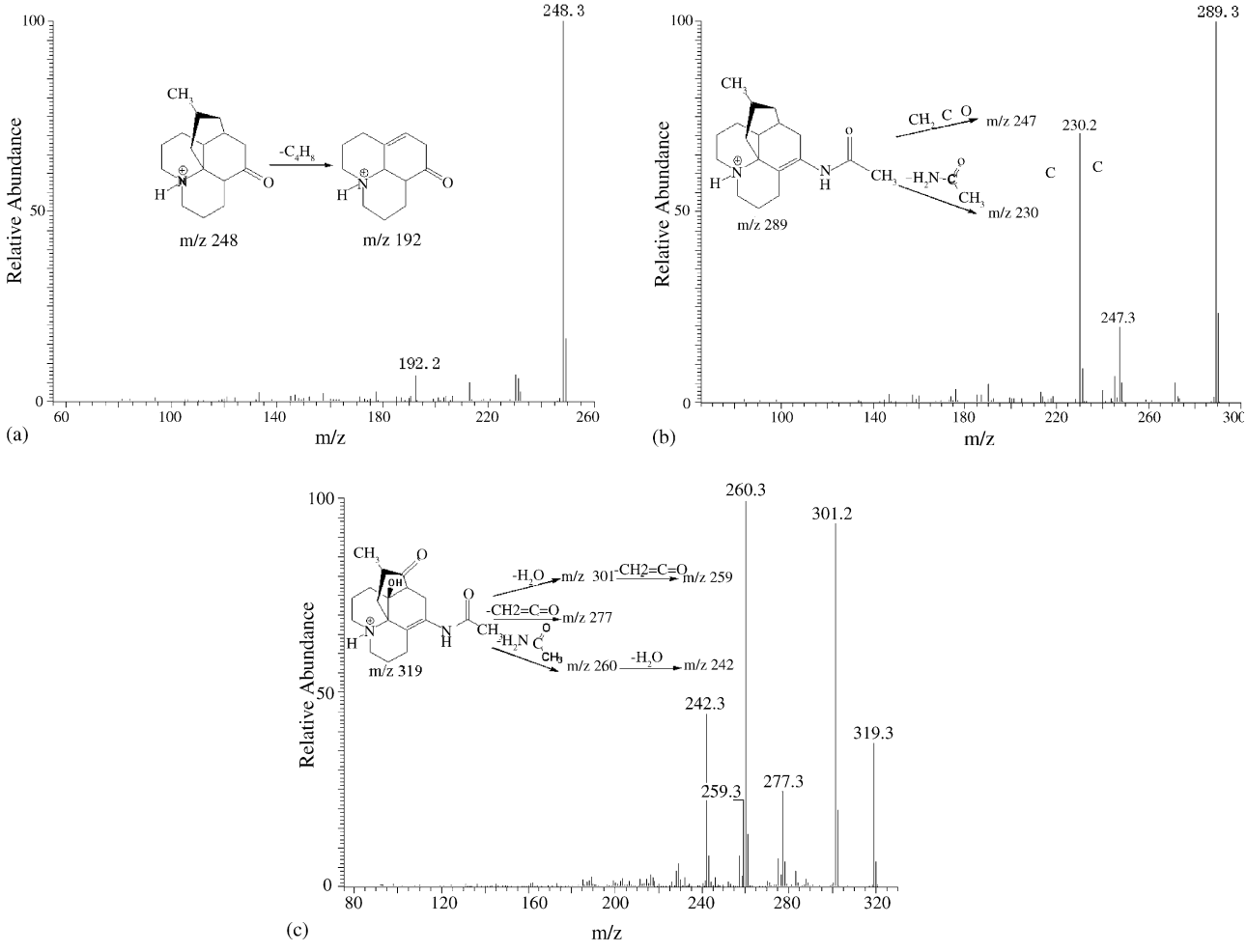

Fig. 3. Positive ESI-MS–MS spectra of compound 4(a), compound 7(b) and compound 10(c).

huperzine A of the different parts: leaves gave the most abundant yield.

3.2. HPLC-DAD-MS-MS analysis of the alkaloid extracts of Huperzia serrata

By careful analysis of the chromatograms at different wavelengths in the range of 200–500 nm, it was found that the chromatograms at 230 nm together with 310 nm could well represent the profile of the constituents. Representative chromatograms are shown in Fig. 1a and b.

The MS spectra were detected in both the positive and negative ion mode. In MS spectra, most of constituents exhibited their quasi-molecular ions [M+H]+ in positive ion mode. Based on the *m*/*z* value, UV spectrum, retention time and the comparison with standard compounds, compound 6 was identified as huperzine A. Compound 2, 3 and 9 displayed identical UV spectra (typical of selagine type alkaloids) with maxima near 230 and 310 nm (see Fig. 2). ESI-MS spectra showed their molecular ions [M+H]+ at *m*/*z* 259, 257, 271, respectively. Based on a comparison of their UV spectra and molecular ions with published data, the three compounds were identified as 6 *β*-hydroxyhuperzne A(2), huperzine B(3) and *N*-methyl-huperzine B(9), and were confirmed by their MS/MS spectra. Compound 4 exhibited a [M+H]+ ion at *m*/*z* 248 and its MS/MS spectrum contains a daughter ion at *m/z* 192, corresponding to the neutral loss of the C_4H_8 (four-carbon bridge) confirming it to be a lycopodine type alkaloid (see Fig. 3a). Based on a comparison of its UV spectrum and molecular ion with published data it was identified as lycopodine. The ESI-MS spectrum of compound 7 showed the molecular ion at *m/z* 289 [M+H]+ and has not been reported in the literature before. The MS/MS spectrum displayed similar fragmentation behavior to lycopodine, and its major peaks at *m*/*z* 247 and 230 corresponded to the characteristic losses of CH_2CO (*m*/*z* 42) and CH_3CONH_2 (*m*/*z* 59), respectively (see Fig. 3b). Absorption at 230 nm in the UV spectrum of compound 7 also supported the presence of the acylamino. For these reasons, we have proposed that this compound is a novel 5-acylamino substituted lycopodine type alkaloid that has a similar structure to huperzine G (which was reported in literature, structure in Fig. 4, but was not detected in this analysis). In the same way, the compound 10 was identified as another novel lycopodine type alkaloid (for ESI-MS-MS spectrum see Fig. 3c). Its tentative structure is shown in Fig. 4. Both compound 5 and 8 exhibited a [M+H]+ ion at *m*/*z* 308. They had identical MS/MS spectrum, but had different retention times in their HPLC chromatogram. Based on comparison with the literature data, they were identified as acetylannofoline and its novel isomer. Compound 1 and 11 were tentatively identified as lycodoline (1) and huperzine E (11) by comparing their *m/z* value and UV spectra with the literature data. The results are listed in Table 3 and the structures of these compounds are shown in Fig. 4.

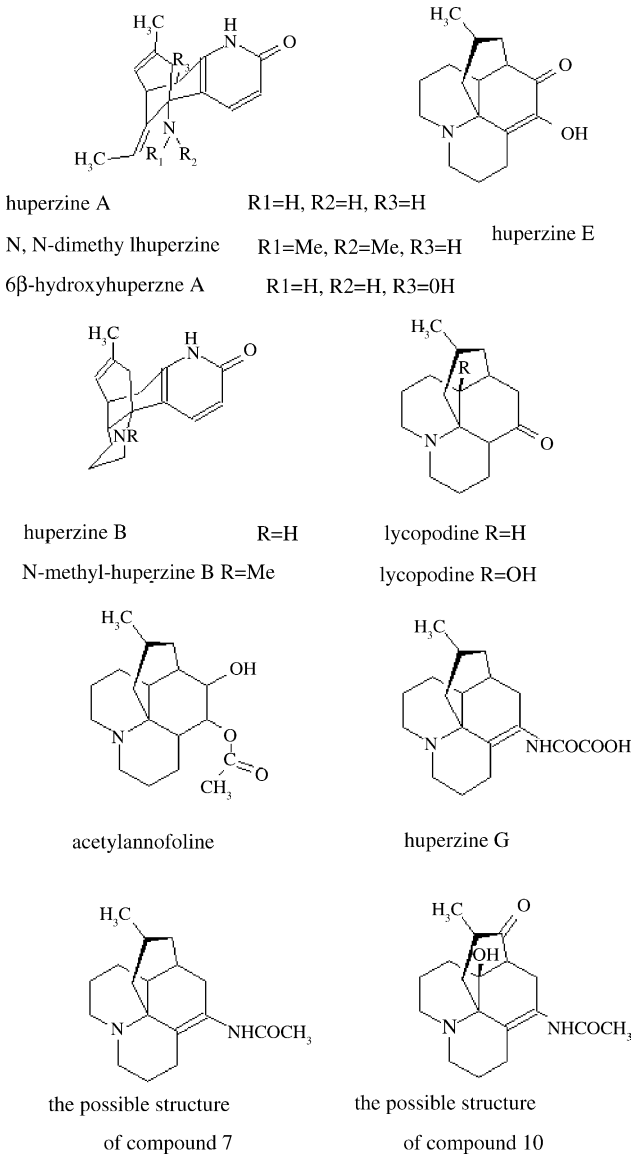

Fig. 4. Structures of some alkaloid constituents in *Huperzia serrata* (structures of compound 7 and 10 are tentative).

4. Conclusion

An HPLC method for determination of huperzine A in *H. serrata* has been developed. For the first time we report the analysis of alkaloid compounds from *H. serrata* of the using HPLC-DAD-ESI-MS-MS. With the help of multi-dimensional information of HPLC-DAD-ESI-MS-MS the alkaloid compounds in *H. serrata* could be identified in a single run successfully. The chromatogram acquired using this method can serve as a fingerprint of the alkaloid components for the quality control of *H. serrata* and its alkaloid extracts.

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