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Is There a Better Source of Huperzine A than *Huperzia serrata*? Huperzine A Content of Huperziaceae Species in China

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A precise and selective reversed phase high-performance liquid chromatographic method was developed for quantifying huperzine A (HupA) in samples of the Huperziaceae in China. This method was used to quantify the levels of HupA in samples of *Huperzia serrata* collected from a single population at different times of the year, in different organs of the same *H. serrata* plant, and from different geographical locations of *H. serrata* plants in China. For different species of Huperziaceae, the highest content of HupA was found in *Phlegmariurus carinatus*. Members of the genus *Phlegmariurus* possessed higher levels of HupA than *Huperzia* species. *H. serrata* plants growing in humid forests contained significantly more HupA than plants growing in less humid environments. Finally, HupA content varied significantly by season, with the highest levels being found in mid fall and the lowest levels in early spring, suggesting that HupA is turned over in the plant.

KEYWORDS: Huperzine A; *Huperzia*; *Phlegmariurus*; RP-HPLC; Huperziaceae; traditional medicine; medicinal plant

INTRODUCTION

Huperzine A (HupA) (see **Figure 1**) was first isolated from the traditional Chinese medicine *Qian Ceng Ta* [whole plant of *Huperzia serrata* (Thunb. ex Murray) Trev.] by Chinese scientists in the mid 1980s (*1*−*3*). Since that time, HupA has been extensively evaluated by Chinese researchers for bioactivity, especially for activity toward cholinesterases and for the treatment of Alzheimer's disease (AD). HupA has been found to be a potent, reversible, and selective acetylcholinesterase inhibitor (AChEI) (*4*−*6*). HupA crosses the blood−brain barrier smoothly and shows high specificity for acetylcholinesterase (AChE) over butyrylcholinesterase, with a prolonged biological half-life (*7*). Structural biology investigations (X-ray crystallography and computational modeling) have found that HupA acts against AChE by directly binding to the opening of the active site in this enzyme, thus preventing access to the active site by the normal substrate (*8*). Clinical trials performed with HupA have demonstrated that HupA produces significant improvements in memory deficiencies in aged and AD patients. Most of these studies have been performed in China, where an estimated 100 000 people have been treated with HupA (*9*). Results of these studies indicate that HupA is an effective and safe drug that improves cognitive function. As a result, HupA has attracted the attention of researchers and medical professionals around the world in recent years.

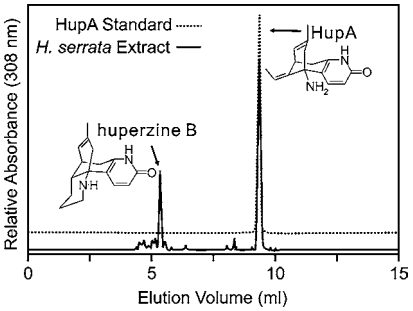

Figure 1. HPLC is an effective means to separate *Lycopodium* alkaloids and quantitate the levels of huperzine A (HupA) in extracts from Huperziaceae species: (- - -) chromatogram of standard HupA compound measured at 308 nm; (–) extract from *H. serrata*. As can be clearly seen, HupA is the most abundant UV-absorbing *Lycopodium* alkaloid in this species and is easily separated by this method from other similar compounds, such as huperzine B, the second most abundant UV-absorbing alkaloid in this species.

The original source plant of HupA, *Huperzia serrata*, belongs to the Huperziaceae family. In addition to being found in *H. serrata*, HupA is produced in other species of the Huperziaceae, which have close taxonomic relationships with *H. serrata* (*10*). Huperziaceae was taxonomically separated from *Lycopodium* (sensu lato) by Rothmaler in 1944 (*11*). This taxonomic system is used in many countries, for example, in China, and has been supported by chemotaxonomic analysis (*10*). *Lycopodium* (s.

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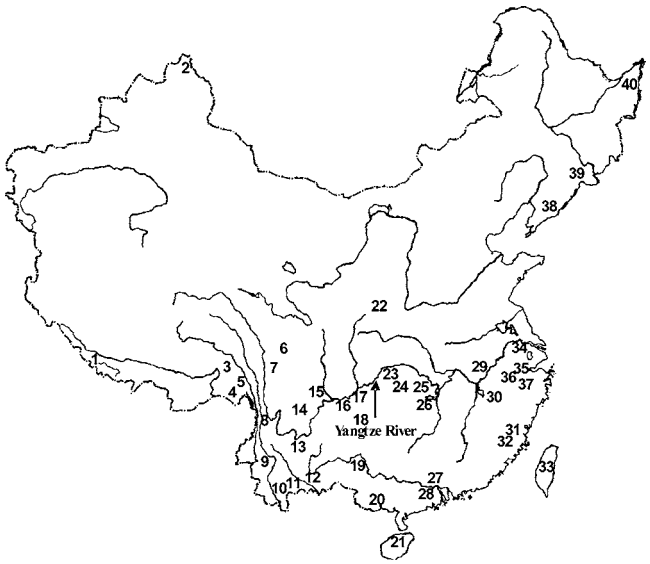

Figure 2. Location of populations of Huperziaceae species evaluated in this study. Collection positions are geographically numbered from west to east. The Yangtze River was specially marked because it is an important geological feature for plotting the distribution of the Huperziaceae plants in this area.

l.) is a large genus with ~500 species that are commonly known as club mosses (*12*). The Huperziaceae is composed of two genera, *Huperzia* and *Phlegmariurus*, with a total of ~150 species; 58 of these species are found in China, with 36 being endemic to China (*13*). The distribution of this plant family is global, but these plants are found in relatively greater abundance in tropical habitats of America than in other areas. In China, they are found predominantly in the regions near the Yangtze River and in the southern parts of China (see **Figure 2**). Nevertheless, these plants are not abundant and are only found in very specialized habitats. Because HupA cannot be chemically synthesized in an industrially feasible manner, the natural plants are the only realistic source of this alkaloid in the foreseeable future. This is likely to lead to endangerment of the native populations of these plants.

The goals of this investigation were to determine the content of HupA (i) in *H. serrata* and its close taxonomic relatives, (ii) in *H. serrata* during different times of the year, and (iii) in different organs of *H. serrata*. Reversed phase high-performance liquid chromatography (RP-HPLC) was used to determine the HupA content of Huperziaceae specimens.

MATERIALS AND METHODS

Chemicals and Materials. Authentic HupA (purity = 98.97%) was from one of the author's (D.Z.) laboratory. Ammonium acetate, glacial acetic acid, and HPLC grade methanol were from Shanghai Chemical Co. Water was double distilled.

High-Performance Liquid Chromatography. The HPLC instrument consisted of a Waters 515 HPLC pump, a Waters 2487 (UV) detector, and an injector loop (Waters). Separation was accomplished over an Alltima C18 column (250 mm × 4.6 mm i.d., 5 μm bead size). The elution conditions were as follows: flow rate, 1 mL/min; column temperature, 50 °C; injection volume, 20 μL; detection, UV absorption (identification, scan mode, 200−500 nm; quantification, 308 nm). The solvent system used was isocratic methanol/0.08 M ammonium acetate/acetic acid buffer (pH 6.0) (32:68). The detection limit for HupA with this method was 0.2 ng (S/N ≥ 3).

Standard Preparation. Stock solutions of HupA standard were prepared at a concentration of 1 mg/mL. Given the expected HupA concentration of the samples to be analyzed, a six-point calibration curve was prepared using diluted standard HupA at concentrations ranging from 1.33 to 80 μg/mL. Samples were diluted as needed to fall within this calibration curve range.

Sample Preparation. The plant specimens were collected from the wild in China beginning in 1999. Except for the specimens used in experiments to evaluate HupA content over the course of the year, all specimens were collected during the months of June, July, and August. One of the authors (X.M.) identified all of the specimens according to their respective species. Voucher specimens (numbers shown in **Tables 1** and **2** and in captions of **Figure 3** and **4**) were deposited in the herbarium of Shanghai Institute of Matria Medica, Chinese Academy of Sciences.

Natural populations of Huperziaceae species were selected for sampling. To evaluate the differences between HupA content in species of the Huperziaceae, populations from *Huperzia* (including 26 species, 1 variety, and 2 forma) (**Table 1A**) and 11 species of *Phlegmariurus* were selected (**Table 1B**). To evaluate geographical and ecological differences in HupA content, samples of *H. serrata* were collected from 21 counties or areas of 16 provinces (see **Table 2**). To determine the seasonal variation in HupA content, samples of *H. serrata* were collected from the same location (Shengzhou, Zhejiang). To compare the HupA accumulation levels in different organs, samples of *H. serrata* were collected from different organs of the same plant from Linlin, Hunan.

Whole plants were collected and air-dried. Samples for HupA content determination were prepared by bulking ~20 plants from each population and grinding the dried plants to obtain ~100 g of powder. This grinding process was done during the field collection before samples were delivered to the laboratory and mimicked the process used to collect *H. serrata* for use in traditional Chinese medicine. The collected powder was stored with silica gel to stabilize the chemical constituents.

Standard Extraction Method for HupA Determination. Three samples of 2 g each of powdered plant material were extracted three times by adding 2% aqueous tartaric acid (50 mL) and sonicating for 30 min. Each extract was removed by filtration over cellulose filter paper (Whatman filter paper no. 1). The combined filtrates for each sample were concentrated to ~$^1/_{10}$ of the original volume by lyophilization and then extracted once with 20 mL of $CHCl_3$ (discarded). The aqueous layer was adjusted to pH >10 with aqueous ammonia and extracted five times with $CHCl_3$. The combined $CHCl_3$ extracts were filtered over Whatman no. 3 paper, and the filtrates were evaporated to dryness using dry nitrogen gas. The residue was then dissolved in methanol and passed through a 0.45-μm Millipore poly(tetrafluoroethylene) (PTFE, 0.45 μm) syringe filter into a 2.5-mL measuring flask and adjusted to volume for RP-HPLC analysis. Statistical analysis was performed using Microsoft Office Excel 2003.

Extraction Method Development. Two extraction methods were evaluated to determine the optimal method for HupA extraction and determination from Huperziaceae species. Two grams of a powdered sample of *H. serrata* collected from Dongkou, Hunan, was used for each sample analyzed.

Method 1: Acidic Water Extraction—2% Aqueous Tartaric Acid. This method was the same as the standard extraction method, described above, using the same identical plant material evaluated for the second method.

Method 2: Organic Solvent Extraction—Benzene, Chloroform, Ethyl Acetate, and Methanol. A small amount of 10% aqueous ammonia was used to wet each sample powder at first. Extractions in 3 volumes (v/w) of one of the organic solvents either with shaking at room temperature for 24 h or by Soxhlet with reflux for 4 h were performed, followed by filtration over Whatman no. 3 paper. Each filtrate was then extracted three times with a one-third volume of 2% aqueous tartaric acid. Each combined 2% tartaric acid extract was adjusted with ammonia to pH 10 and extracted three times with chloroform. The combined chloroform extract was dried, and the residue was dissolved in methanol for further analysis.

RESULTS AND DISCUSSION

RP-HPLC Method To Detect and Quantify HupA. The identification of HupA in the samples was achieved by

Table 1. HupA Content in Different Species in the Huperziaceae[a]

species	specimen no.	collection date	collection site: county province, numbered position on map (**Figure 2**)	HupA content (μg/g ± SD)
		(**A**) Genus *Huperzia*		
H. austrosinica Ching	HH00-0204	June 25, 2000	Longsheng, Guangxi, 19	96.03 ± 0.20
H. bucahwangensis Ching	HH99-0152	Aug 9, 1999	Jinping, Yunnan, 11	168.72 ± 0.33
H. chinensis (Christ) Ching	HH99-0109	July 1, 2000	Taibashan, Shannxi, 22	105.83 ± 0.47
H. chishuiensis X. Y. Wang et P. S. Wang	HH99-0112	Aug 1, 1999	Chishui, Guizhou, 16	104.19 ± 0.16
H. crispate (Ching) Ching	HH99-0122	July 20, 1999	Leigongshan, Guizhou, 18	135.53 ± 0.28
H. emeiensis (Ching et H. S. Kung) Ching et H. S. Kung	HH99-0134	June 29, 1999	Emeishan, Sichuan, 15	88.52 ± 0.60
H. herteriana (Kümm.) Sen et Sen	HH99-0113	Aug 17, 1999	Nielamu, Tibet, 1	254.58 ± 0.22
H. kangdingensis (Ching ex H. S. Kung) Ching	HH99-0125	July 16, 1999	Kangding, Sichuan, 7	74.88 ± 0.18
H. kunmingensis Ching	HH99-0132	July 25, 1999	Kunming, Yunnan, 13	91.09 ± 0.21
H. laipoensis Ching	HH99-0171	July 10, 1999	Leipo, Sichuan, 14	116.72 ± 0.25
H. lajouensis Ching	HH99-0163	Aug 13, 1999	Chayu, Tibet, 4	58.23 ± 0.23
H. leishanensis X. Y. Wang	HH99-0195	July 20, 1999	Leigongshan, Guizhou, 18	95.06 ± 0.06
H. liangshanica (H. S. Kung) Ching et H. S. Kung	HH99-0103	July 10, 1999	Leipo, Sichuan, 14	72.58 ± 0.26
H. lucidula (Michx.) Trev.	HH00-0238	Aug 2, 2000	Changbaishan, Jilin, 39	54.94 ± 0.21
H. miyoshiana (Makino) Ching	HH00-0256	Aug 2, 2000	Changbaishan, Jilin, 39	68.44 ± 0.21
H. nanchuanensis (Ching et H. S. Kung) Ching et H. S. Kung	HH99-0124	July 4, 1999	Nanchuan, Chongqing, 17	92.83 ± 0.23
H. nylamensis Ching et S. K. Wu	HH99-0190	Aug 16, 1999	Nielamu, Tibet, 1	94.47 ± 0.15
H. obscure-denticulata Ching	HH99-0107	July 8, 1999	Maerkang, Sichuan, 6	56.94 ± 0.16
H. ovatifolia Ching	HH99-0119	Aug 8, 1999	Jinping, Yunnan, 11	189.40 ± 0.24
H. quasipolytrichoides (Hayata) Ching	HH00-0263	July 5, 2000	Alishan, Taiwan, 33	89.36 ± 0.20
H. rubicauli S. K. Wu et X. Cheng	HH99-0109	July 30, 1999	Gongshan, Yunnan, 8	65.74 ± 0.41
H. selago (L.) Bernh. ex Schrank et Mart.	HH00-0298	July 10, 2000	Altai, Xinjiang, 2	114.58 ± 0.24
H. selago (L.) Bernh. ex Schrank et Mart. var. *appressa* (Desv.) Ching	HH99-0137	Aug 20, 1999	Basu, Tibet, 5	109.70 ± 0.23
H. serrata (Thunb. ex Murray) Trev.	HH99-0104	June 15, 1999	Shangzhi, Hunan, 24	80.16 ± 0.17
H. serrata (Thunb. ex Murray) Trev. f. *intermedia* (Nakai) Ching	HH99-0107	Aug 6, 1999	Jinpin, Yunnan, 11	148.30 ± 0.21
H. serrata (Thunb. ex Murray) Trev. f. *longipetiolata* (Spring) Ching	HH99-0110	July 12, 1999	Leipo, Sichuan, 14	182.55 ± 0.18
H. sutcheniana (Herter) Ching	HH99-0113	July 3, 1999	Nanchuan, Chongqing, 17	46.85 ± 0.16
H. tibetica (Ching) Ching	HH99-0106	July 28, 1999	Gongshan, Yunnan, 8	96.24 ± 0.16
H. whangshanensis Ching	HH00-0218	Aug 2, 2000	Huangshan, Anhui, 29	57.82 ± 0.20
		(**B**) Genus *Phlegmariurus*		
Ph. cancellatus (Spring) Ching	HP99-0179	Aug 16, 1999	Bomi, Tibet, 3	358.44 ± 0.16
Ph. carinatus (Desv.) Ching	HP00-0293	June 20, 2000	Fangchen, Guangxi, 20	560.46 ± 0.21
Ph. fargesii (Herter) Ching	HP99-0185	July 20, 2000	Leigongshan, Guizhou, 18	273.60 ± 0.13
Ph. fordii (Bak.) Ching	HP00-0278	July 13, 2000	Luofushan, Guangdong, 27	376.18 ± 0.23
Ph. guangdongensis	HP00-0281	July 15, 2000	Xinyi, Guangdong, 27	269.58 ± 0.23
Ph. henryi (Bak.) Ching	HP99-0175	July 28, 1999	Pinbian, Yunnan, 12	286.54 ± 0.47
Ph. mingcheensis Ching	HP00-0247	Aug 12, 2000	Chongan, Fujian, 31	498.58 ± 0.16
Ph. phlegmaria (L.) Holub	HP99-0184	Aug 11, 1999	Jinping, Yunnan, 11	345.23 ± 0.18
Ph. pulcherrimus (Wall. ex Hook. et. Grev.) Löve et Löve	HP00-0277	June 25, 2000	Longsheng, Guangxi, 19	342.57 ± 0.20
Ph. squarrosus (Forst.) Löve et Löve	HP99-0192	Aug 2, 1999	Xishuangbann, Yunnan, 10	378.83 ± 0.33
Ph. yunnanensis Ching	HP99-0194	July 30, 1999	Gongshan, Yunnan, 8	241.84 ± 0.24

[a] All samples are from whole plant and from triplicate tests.

comparing retention times and UV spectra. The HupA standard, scanned from 200 to 500 nm, showed absorption maxima at 228 and 308 nm in MeOH. To reduce interference from other plant constituents, 308 nm was chosen for detection and quantification. Typical chromatograms from HupA standard and a tested sample (*H. serrata*, Dongkou, Hunan) are shown in **Figure 1**, where HupA has good peak shape and is well-separated from other *Lycopodium* alkaloids.

A linear relationship exists between the peak area (measured at 308 nm) and the concentration of HupA for concentrations ranging from 1.33 to 80 μg/mL in the sample injected into the HPLC. Linear regression analysis of the data yielded a correlation coefficient (R^2) of 0.9999 in the equation $Y = -1.131 \times 10^4 + 4.326 \times 10^4 X$, where Y equals the concentration of HupA (in μg/mL of the original sample) and X equals the peak area in the UV chromatogram at 308 nm. The precision of the method was determined by analyzing a sample of *H. serrata*, and the relative standard deviation (RSD) of replicate samples ($n = 5$) for HupA content was 0.27 (<2%). Intraday precision was determined by analyzing three concentrations of standard HupA ranging from 1 to 5 μg/mL in five replicates on the same day. Interday precision was determined by measuring the same five replicates on two separate days (**Table 3**), again with very good stability of HupA in the extraction solvent and very good reproducibility of the method to quantitate the HupA in these samples. In addition, recovery of HupA in the extraction procedure was evaluated by adding HupA (20 μg added, five replicates measured) to ground plant powder at the beginning of the standard extraction procedure (see below). This yielded a recovery rate of 96.9 ± 1.12% (mean ± SD). Thus, this method appeared to be quite accurate and precise in measuring the concentration of HupA, with very little loss of HupA due to the extraction procedure.

Evaluation of Methods To Extract HupA. Two general extraction methods were evaluated for their efficiency and reproducibility in extracting HupA from tissues of Huperziaceae

Table 2. HupA Content in *H. serrata* Collected at Different Geographical Locations in China[a]

specimen no.	collection date	collection site: county, province, numbered position on map (**Figure 2**)	HupA content (μg/g ± SD)	ecological niche	elevation (m)
HH01-04	Aug 12, 2001	Jinpin, Yuannan, 11	132.58 ± 0.37	humid forest, mossy and rocks	1900–2400
HH01-12	June 5, 2001	Dianchangshan, Yunnan, 9	92.63 ± 0.42	humid forest	2600–2900
HH01-08	June 10, 2001	Nanchuan, Chongqing, 17	114.45 ± 0.48	humid forest, shady	1600–2200
HH01-21	Aug 12, 2001	Leigongshan, Guizhou, 18	100.89 ± 0.40	humid forest	1700–2100
HH01-05	June 28, 2001	Longsheng, Guanxi, 19	98.42 ± 0.34	humid forest close to ridge	800–1300
HH01-17	June 18, 2001	Baisha, Hainan, 21	79.85 ± 0.35	valley side shrubs and forest	400–900
HH01-02	July 20, 2001	Enshi, Hubei, 23	67.77 ± 0.31	forest close to ridge	1700–2000
HH01-01	July 1, 2001	Shangzhi, Hunan, 24	79.32 ± 0.43	humid forest close to ridge, shady	1800–2400
HH01-10	June 10, 2001	Dongkou, Hunan, 25	76.39 ± 0.43	valley side forest	1600–2400
HH01-15	July 10, 2001	Linlin, Hunan, 26	82.47 ± 0.60	humid forest	1400–1900
HH01-14	May 5, 2001	Xinyi, Guangdong, 28	46.35 ± 0.37	less humid forest	600–800
HH01-18	July 27, 2001	Huangshan, Anhui, 29	64.13 ± 0.24	valley side forest	1400–1800
HH01-20	Aug 2, 2001	Lushan, Jiangxi, 30	76.33 ± 0.32	humid forest close to ridge	1000–1400
HH01-19	June 1, 2001	Lianchen, Fujian, 32	69.26 ± 0.36	forest close to ridge	400–800
HH01-11	May 23, 2001	Yixin, Jiangsu, 34	75.70 ± 0.45	less humid forest	400–600
HH01-07	June 20, 2001	Linan, Zhejiang, 35	54.27 ± 0.30	shrubs close to ridge	1000–1500
HH01-13	July 16, 2001	Shengzhou, Zhejiang, 36	69.55 ± 0.32	less humid bamboo forest	750–1000
HH01-03	May 15, 2001	Xianju, Zhejiang, 37	105.70 ± 0.34	humid bamboo forest, valley side	800–1200
HH01-06	Aug 12, 2001	Songkao, Liaonin, 38	68.65 ± 0.43	less humid forest	700–1000
HH01-09	May 10, 2001	Changbaishan, Jilin, 39	53.98 ± 0.37	less humid forest	1500–2100
HH01-16	July 5, 2001	Raohe, Heilongjiang, 40	60.43 ± 0.37	less humid forest	800–1200

[a] All samples are from whole plant and from triplicate tests.

Table 3. Stability of HupA and Reproducibility of HPLC Analysis of HupA Quantitation

comparison	concentration (μg/mL)	precision (% RSD)	*N*
intraday	1.0	1.50	5
	2.5	0.52	5
	5.0	0.50	5
interday	1.0	1.35	5
	2.5	0.69	5
	5.0	0.30	5

species. This analysis was performed to ensure that precious plant samples obtained in many cases from very difficult to reach places were not wasted in inefficient or inaccurate extraction procedures. For example, the population at Jinpin Yuannan is located high in the mountains on the border near Vietnam, with many land mines still present in the area. It would be practically impossible to collect samples from this or other regions again. In these tests, a large sample of *H. serrata* obtained from Dongkou, Hunan, was dried and powdered. Replicate aliquots of the powder were extracted according to two different methods, which had been previously used to extract compounds such as HupA from these plants (*13*).

As described in detail under Materials and Methods, the first method involved direct extraction of *Lycopodium* alkaloids from the powder by 2% aqueous tartaric acid, followed by cleanup. The affect of sonication on the extraction efficiency was evaluated for this extraction method, as compared to extraction with simple shaking. As can be seen in **Table 4**, sonication allowed for the rapid and efficient extraction of HupA. Although shaking at room temperature was also able to achieve essentially the same yield of HupA, it required significantly longer extraction time (20 h compared to 30 min).

The second extraction method evaluated involved extraction first with various organic solvents (benzene, chloroform, ethyl acetate, and methanol were evaluated) followed by fractionation with 2% aqueous tartaric acid and cleanup. As shown in **Table 5**, this approach was not able to achieve the same yield as the standard extraction method when performed at room temperature. Even Soxhlet-based extraction with solvent reflux, which is tedious and expensive to perform compared to the other methods, was not able to improve on yield compared to the first method.

Table 4. Extraction Efficiency for Standard Extraction Method (Method 1) with Shaking or Sonication over Time

time (h)	HupA content (μg/g ± SD)	*N*
shaking at room temperature		
5	31.42 ± 0.22	5
10	58.26 ± 0.31	5
15	70.81 ± 0.24	5
20	75.12 ± 0.20	5
sonication at room temperature		
0.17	55.91 ± 0.18	5
0.25	64.54 ± 0.25	5
0.33	72.62 ± 0.32	5
0.50	76.39 ± 0.43	5

Table 5. Efficiency of HupA Extraction with Method 2: Initial Organic Solvent Extraction

solvent	method	time (h)	HupA content (μg/g ± SD)	*N*
benzene	shaking at room temperature	24	67.39 ± 0.24	5
	Soxhlet with refluxing	4	72.63 ± 0.15	5
chloroform	shaking at room temperature	24	67.02 ± 0.25	5
	Soxhlet with refluxing	4	72.54 ± 0.28	5
ethyl acetate	shaking at room temperature	24	59.14 ± 0.19	5
	Soxhlet with refluxing	4	70.38 ± 0.24	5
methanol	shaking at room temperature	24	66.92 ± 0.22	5
	Soxhlet with refluxing	4	72.45 ± 0.26	5

Analysis of Huperziaceae Specimens for HupA Content. Once developed, the standard extraction method was applied to determine the levels of HupA in Huperziaceae plants collected in China. The level of HupA in different species and genera within the Huperziaceae was initially evaluated. Samples were collected from 26 species, 1 variety, and 2 forma of *Huperzia* and 11 species of *Phlegmariurus* (see **Table 1**) to determine

the level of HupA. The samples were collected from very diverse locations across China (see **Figure 2**). All determinations were performed in triplicate from the prepared plant material powder. In the genus *Huperzia*, the amount of HupA varied from 46.85 to 254.58 μg/g (dry weight), on a whole plant basis, with the lowest and highest concentrations being found in *H. sutcheniana* and *H. herteriana*, respectively. The average concentration of HupA in *Huperzia* species was 103.82 μg/g, with a standard deviation of 46.47. *H. serrata* is used as the main and well-known source of HupA because it has been used as a medicinal herb in China for over a thousand years. It is widely distributed in the Yangtze River area, and it is the most (relatively speaking) abundant species of the Huperziaceae in China. Nevertheless, the natural resources of the Huperziaceae in China are not abundant. In fact, the other species in the Huperziaceae (except for *H. serrata*) are only found in very special ecological niches. Species of the genus *Phlegmariurus* are even more restricted in distribution and abundance than species of *Huperzia*. *Phlegmariurus* species grow (nonsaprophytically) on trees in the threatened tropical or subtropical rainforests of China and are very rare. Interestingly, HupA concentrations in *Phlegmariurus* species were remarkably higher than those found in *Huperzia* species (see **Table 1**). HupA content ranged from 241.84 (*Ph. yunnanensis*) to 560.46 (*Ph. carinatus*), with *Ph. yunnanensis* being the only species in *Phlegmariurus* to have a lower concentration of HupA than *H. herteriana* (the species of *Huperzia* with the highest HupA concentration). The average concentration of HupA in *Phlegmariurus* species was 357.44 μg/g, with a standard deviation of 94.6. This average was almost 3.5-fold higher than the average for *Huperzia* species (103.82 μg/g) and was very significantly different (P value of 6.11×10^{-39} in one-way ANOVA).

H. serrata is the only species found to be widely distributed in China (see **Table 2** and **Figure 2**). HupA content in *H. serrata* samples from 21 different locations across 17 provinces of China was determined and varied from 46.35 to 132.58 μg/g (see **Table 2** and **Figure 2**). There was no apparent significant correlation between increasing HupA concentration and increasing elevation from which a given plant specimen was collected ($R^2 = 0.1586$). This suggests that HupA is not likely to be involved in protecting the plant from UV damage. This role is generally attributed to flavonoids in plants. In addition, no trend was observed for HupA content compared to geographical origin. Although there appears to be a slightly higher concentration among samples collected in the western parts of China, samples with higher and lower HupA content were found across China, with samples containing some of the highest HupA content being found in samples from far inland to the west and from near the coast in the east. Instead of being based on geographical location, higher HupA contents appear to be associated with an ecological niche. Samples taken from humid forests had significantly higher average levels of HupA (98.09 μg/g) than samples taken from less humid environments (65.53 μg/g) (P value from one-way ANOVA of 4.50×10^{-5}). This suggests that unknown ecological factors (such as pathogens or pests that could not be observed when the plant samples were taken, but which would grow in more humid environments) are also likely to be involved in affecting the levels of HupA production in *H. serrata*. This may explain, in part, why *H. serrata* has broader ecological adaptability than the other species in this plant family.

To determine whether the levels of HupA varied throughout the year, or remained constant, in the tissues of *H. serrata*, samples were taken from a single location once each month for one year (2001, see **Figure 3**). This location (Shengzhou, Zhejiang) was chosen because it contained HupA concentrations that were close on average to the average concentration in populations across China. Furthermore, it is located at the center of one of the two major regions of HupA-rich populations in China. The concentration of HupA varied by ~33% throughout the year. Samples collected in October had the highest levels (97.59 μg/g), whereas the lowest levels (~64−70 μg/g) were observed in late winter through spring. Differences between all consecutive months were significant (based on P values of <0.02 for all comparisons). Interestingly, *H. serrata* plants are evergreen and extremely slow growing, generally requiring several years to reach a few centimeters in height and adding very little new tissue each year. Thus, differences throughout the year in HupA content cannot be attributed solely to new growth that had not yet accumulated significant amounts of HupA. Instead, it appears that HupA is metabolized in *H. serrata* during the winter (drop in concentration in the months December through March). HupA does not seem to be a likely nitrogen or energy storage molecule. One possible explanation for this drop in HupA content is that HupA is normally turned over in the plant and during the winter, the production of HupA is attenuated, leading to a general drop in concentration.

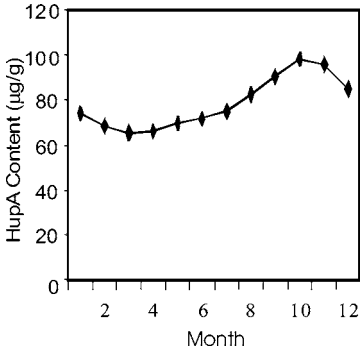

Figure 3. HupA content varies throughout the year in *H. serrata* total plant tissues. Samples ($n = 5$) were collected once each month for one year, from January to December 2001, in Shengzhou, Zhejiang (voucher specimens HH01-0101; position 36 in **Figure 2**). Standard error for these measurements was smaller than the graph markers for all months.

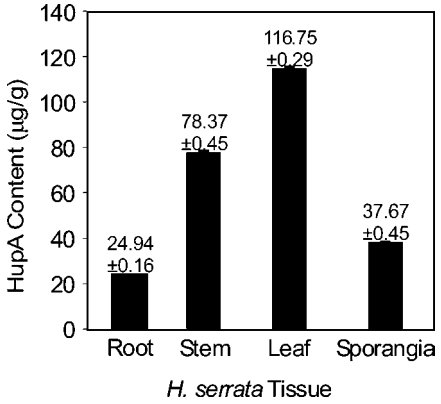

Figure 4. HupA content varies dramatically by plant organ in *H. serrata*. Leaf and stem tissues are significantly better sources of HupA than is root tissue, suggesting that aerial harvest would be more productive than whole plant collection for these plants. Samples were collected on February 10, 2001, from Linlin, Hunan (voucher specimen HH01-0077; position 26 in **Figure 2**).

The content of HupA varied dramatically by tissue in *H. serrata* (see **Figure 4**). Plants for this investigation were

collected at Linlin Hunan, because it, too, is located at the center of one of the two major regions of HupA-rich populations in China. The highest content was found in the leaves (116.75 ± 0.17 μg/g), whereas the roots and sporangia had much lower levels (24.94 ± 0.09 and 37.67 ± 0.26 μg/g, respectively). The stems had intermediary levels (78.37 ± 0.26 μg/g). Analysis of variance determined that these differences were very significant, with P values ranging from 1.27×10^{-6} to 1.18×10^{-10} for pairwise comparisons. Thus, the best tissues for HupA isolation are the leaves, followed by the stems. This suggests that the whole plant need not be harvested, but that cuttings could be made instead, perhaps allowing the root system to regenerate new aerial parts of the plant. If this practice were to be introduced by those that collect *H. serrata* for medicinal use, the wild populations of these plants may be spared, even if collection were to continue. However, due to the ease by which the whole plant is harvested, and due to traditional medicine practice, where the whole plant is used as the medicine, it is very unlikely that the practice of taking only cuttings during harvest would be accepted, especially by poachers. For use as a dietary supplement to treat Alzheimer's disease symptoms, however, preparations made solely from *H. serrata* leaves would likely be most efficacious.

In conclusion, our RP-HPLC method for determining HupA is simple, fast, accurate, and relatively inexpensive. This method may be used for determining HupA content in natural resources and also for quality control of products and formulations claiming to contain significant levels of HupA. Furthermore, the HupA content of different Huperziaceae species varies dramatically. Species of *Phlegmariurus* contain much higher levels of HupA than *Huperzia* species. Because these plants are very restricted in their distribution and are rare where they do grow, they should not be collected from the wild as a source of HupA. Determination of the HupA content of *H. serrata* by habitat and organ provides very important information for HupA raw material collection. Because the HupA content in *H. serrata* roots is very low compared to the stem and leaf, entire plants of *H. serrata* should not be harvested for use in preparation of HupA for medicinal use. Furthermore, plants growing in less humid environments are not likely to be good sources for HupA. Finally, these results establish a reference point for HupA natural resource development and protection, and suggest that methods such as cultivation and in vitro propagation should be pursued with more vigor to avoid decimation of wild populations of *H. serrata* and related species.

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