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Peptides from two sanguinovorous leeches analyzed by ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometric detector

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ABSTRACT

Background: *Hirudo nipponica* Whitman and *Poecilobdella manillensis* Lesson fall into the family of *Hirudinidae* Whitman, both of them are sanguinovorous leeches and used a anticoagulant medicines in China. Their medicinal parts are the dried bodies. However, the peptides in the dried body of the two leeches have not been very clear up to now. **Objective:** To analyze the peptides from two sanguinovorous leeches, *H. nipponica* and *P. manillensis.* **Materials and Methods:** In this article it is reported that the peptides were obtained from anticoagulant active extracted parts of dried bodies of the two leeches and their molecular weights were analyzed by ultra-performance liquid chromatography with electrospray ionization quadrupole time-of-flight mass spectrometry mass spectrometric detector online. **Results:** Three peptide components were identified from *H. nipponica* with their molecular weight separately 14998, 15988, and 15956, six peptide components were identified from *P. manillensis* with molecular weight 9590, 13642, 14998, 17631, 15988, and 16567. Two of peptides from *P. manillensis* have the same molecular weight 14998 and 15988 as that in *H. nipponica*. **Conclusion:** And the two peptides are the main peaks in the base peak ion chromatogram because they occupied a large ratio of total base peak area. Hence the composition of the extracted active part of the two leeches are very close, difference is in that the extract of *P. manillensis* has more small peptide peaks, but the extract of *H. nipponica* has not. Furthermore, the tryptic digestion hydrolysates of the extracted active part of each sample were analyzed and the results showed that there were four peaks which only exist in *P. manillensis*, but not in *Hirudo nipponia*. They may be the identified peak between the two leeches. This work support the viewpoint that *P. manillensis* can be used as a medicinal leech as *H. nipponia* and these peptide components of dried bodies of the two species leeches are a basis for their chemical identification and further investigations in active action.

Key words: *Hirudo nipponia* Whitman, leech, peptide, *Poecilobdella manillensis* Lesson, ultra performance liquid chromatography-electrospray ionization quadrupole time-of-flight

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INTRODUCTION

Hirudo nipponia Whitman has been one important medicine in Traditional Chinese Medicine, which effects is promoting blood circulation and removing blood stasis. Now it is mainly used in cerebral thrombosis and cerebral apoplexy in clinical treating and recorded in China Pharmacopoeia. *Poecilobdella manillensis* Lesson we is also used as a medicinal leech in the folk with the same function as *H. nipponia* Whitman, but it has not been recorded in China Pharmacopoeia. Both of them fall into the famliy of *Hirudinidae* Whitman and are sanguinovorous leeches. Hirudin is a well-known and the strongest thrombin inhibitor in the world, which was isolated from saliva of *Hirudo nipponica* Whitman.[1] Bufrudin was isolated from saliva of *P. manillensis* Lesson, which has the same effect and 50-60% same amino acid sequence as Hirudin.[2] Baskova reported the proteins and peptides which was separated from the secretion of *Hirudo verbana*'s, *Hirudo medicinalis*'s and *Hirudo orientalis*'s salivary gland analyzed by SELDI mass spectrometry (MS) and two-dimensional electrophoresis in 2008.[3] In China, the dried whole bodies of the two leeches are the medicinal parts in Traditional Chinese Medicine for many years, but up to now the

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dried whole bodies of these leeches have yet seldom been analyzed to identify their biologically active part and peptide compounds. In this paper, we found an active part, the extract solution, and used the methods of ultra-performance liquid chromatography (UPLC) coupled with electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometric detector to have determined the molecular weight of some peptide compounds in the extracted active part of the two leech species. No doubt, these peptides do not cover the whole compounds of the biologically active part of the dried bodies, but it can provide with new method and clue for the two sanguinovorous leeches' chemical identification and discovering new active compounds.

METERIALS AND METHODS

Instrument and reagent

Chromotographic analysis was conducted on a Waters series ultra-fast HPLC system Waters ACQUITY UPLC-Xevo G2-Q-TOF(ACQUIT UPLC Online Community). Chromatographic column, ACQUITY UPLC BEH C_{18}(1.7 μm, 100 × 2.1 mm), was used for analysis. Milli-Q Water Purification System was used to purify the water for a test. Trypsin was from Promega Corporation. Sysmex CA-500 Coagulation analyzer was from Sysmex Corporation. Thrombocheck activated partial thromboplastin time (APTT) reagent and Thrombocheck prothrombin time (PT) reagent were from Sysmex Corporation. Acetonitrile and formic acid (chromatographic grade) were from TEDIA, USA.

Preparetion of sample

All dried samples of the bodies of *H. nipponia* Whitman and *P. manillensis* Lesson were get rid of the internal organs, and then were ground into powder. Transfer about 1-g sample powder to a flask, add 50 ml 0.9% sodium chloride and swirling for 1 h at 37°C, centrifuge for 5 min at 10,000 rpm, to obtain the extract solution from the supernatant liquor.

Anticoagulant effect *in vitro* determination

Human plasma 100 μL were added to an eppendorf tube, and 100 μL the extract solution of each sample or 0.9% sodium chloride, and then 100 μL APTT or PT reagent were added into the tube. The value of APTT and PT of the mixture were detected by Sysmex CA-500 Coagulation analyzer. This operation was repeated 5 times and the mean value was taken as the final result.

Tryptic digestion of the sample

Samples were divided into two groups according to species, *P. manillensis* Lesson and *H. nipponia* Whitman, each group have four batches of samples. The extract method of each batch samples is as the same as the "preparation of sample". Transfer 50 μL of the extract solution to an eppendorf tube, diluted with 450 μL 1% NH_4HCO_3, add 10 μL 100 mM DTT in 1% NH_4HCO_3 and incubate the solution at 60°C for 15 min, add 50 μL trypsin in 1% NH_4HCO_3 and leave the digested solution overnight at 37°C.

Liquid chromatography conditions

Acetonitrile: Water (2:98) with 0.2% formic acid was used as mobile phase A, and acetonitrile: Water (98:2) with 0.2% formic acid was used as mobile phase B, gradient elution, 0-60 min, 100% A → 100% B. The column temperature was set at 65°C. The flow rate was set at 0.2 mL/min, and the injection volume was fixed at 1 μL.

Mass spectrometry conditions

The MS detection was performed on an ESI-Q-TOF MS System. The ionization mode was positive electrospray (ESI^+). The desolvation temperature was fixed at 350°C with desolvation gas flow was fixed at 600 L/h, and the source temperature was fixed at 120°C. The cone voltage was fixed at 40 V, and the capillary voltage was fixed at 3 kV. The collision energies were fixed as 6 eV for low-energy scan, and 20-40 eV for high-energy scan. The LC-MS data acquisition mode was MS^E, which was controlled by MassLynx 4.0 Mass Spectrometry Software.(Waters SCN871).

Data processing

The extract solution and digest solution of the samples were injected into the UPLC at the above chromatography condition to acquire the MS data. Postacquisition data processing was performed by Mass Ent 1 and MarkerLynx XS, which are the "application manager" for MassLynx software. (Waters SCN871). Mass Ent 1 was used to transform multiple charges mass peak into a single charge mass peak. MarkerLnyx XS were used to identify the difference through the principal component analysis (PCA).

RESULTS AND DISCUSSION

The anticoagulant effect *in vitro* of the extracted solutions of samples

Prothrombin time APTT. Both of them are widely used in evaluating the anticoagulant effect. The results showed that the extracted solutions of two leech species have an anticoagulant effect. The active action of *P. manillensis* is stronger than the *H. nipponia*'s [Table 1].

Results of time-of-flight mass spectrometry analysis

Electrospray ionization, a soft ionization technology, is an effective measure to analyze the protein and peptide,[4-8] in which ESI source is coupled with LC online. When a molecule of protein or peptide is analyzed by ESI source, a series of multiple-charge icons peaks will be detected by TOF mass spectrometric detector. The molecular

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weight of every peak (M) can be calculated according to the mass-to-charge ratio (m/z) and the ion charges (e): m/z = (M + e)/e. MassEnt 1 can transfer the multiple charges mass peaks into a single charge mass peak and calibrate the calculated molecular weights.

Three peptide components named H1 ~ H3 were detected from *H. nipponia* and their molecular weight were 14998 (H1), 15988 (H2) and 15956 (H3), six peptide components named P1 ~ P6 weredetected from *P. manillensis* and their molecular weight were 9590 (P1), 13642 (P2), 14998 (P3), 17631(P4), 15988 (P5), and 16567 (P6). Figure 1 is the base peak ion chromatogram (BPI) of extracting solution of two leeches and 0.9% sodium chloride solution. Figure 2 is the multiple charges mass peak given by the ESI^+ (left) and a single charge mass peak given by MassEnt 1 (right) of H1 ~ H3, P1 ~ P6. Table 2 shows the MS information of each peptide peak: The mass-to-charge ratio (m/z), the ion charges (e) of the multiple-charge ions peaks given by the ESI^+, the calculated molecular weights and the weight given by MassEnt 1.

From Figure 1 it is noticed that H1 (detected from *H. nipponia*) and P3 (detected from *P. manillensis*) have the same molecular weight, and H2 (detected from *H. nipponia*) and P5 (detected from *P. manillensis*) also have the same molecular weight. And the two peptide peaks are the main peaks in the BPI of each species because they occupied a large ratio of total base peak area. Hence, the main composition of the extracted active part of the two leeches are very close or similar, difference is in that the extraction of *P. manillensis* has more small peptide peaks, but the extract of *H. nipponica* has not.

Analysis of tryptic digestion of samples

The results of TOF-MS analysis showed that *P. manillensis and H. nipponia* are close to each other in main peptide composition of dried bodies. Because of the complexity of composition, it is different to discriminate them in the dried bodies and the extraction only by vision observation.

The method of digestion was used to analyze the protein hydrolysates of the samples in order to identify them. The extracted solution of *P. manillensis* and *H. nipponia* were digested with trypsase, then, the hydrolysates were analyzed by LC-MS. Figure 3 shows the BPIs obtained from hydrolysates of *P. manillensis* and *H. nipponia*. Because of the complexity of the sample, it is difficult to discriminate them only by visual observation of the two chromatograms. Hence, the data were processed by MarkerLnyx XS software. The software converts each data point into an exact mass/retention time (EMRT) pair and tabulates the results into a two-dimensional matrix, after a two-dimensional matrix is obtained, the extended statistics module PCA can be carried out. From the PCA plot, any two groups of samples can be paired for an orthogonal partial least squares data analysis (OPLS-DA).[9] The data of samples

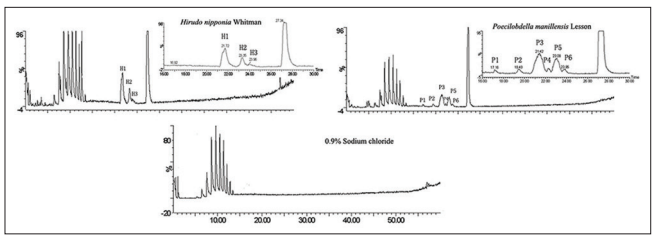

Figure 1: Base peak ion chromatograms of extracted solutions of two leeches and 0.9% sodium chloride solution

Table 1: The anticoagulant effect of samples *in vitro* (X±S, *n*=5)

Group	Name	APTT	PT
sample	*Hirudo nipponia* Whitman	80.7±2.02*	28.4±2.12*
sample	*Poecilobdella manillensis* Lesson	93.4±1.65*	38.5±1.78*
negative	0.9% sodium chloride solution	28.03±2.14	11.3±1.82

Table 2: The peptide analysis of two species leech

Sample	Peak number	Retention time (min)	m/z (Mass-to-charge ratio)	e (Ion charges)	Calculated weights	Weights by Mass Ent1
Hirudo nipponia Whitman	H1	21.72	790.3231 $[M+19H]^{19+}$ 834.1744 $[M+18H]^{18+}$ 883.2406 $[M+17H]^{17+}$ 938.3778 $[M+16H]^{16+}$ 1000.8737 $[M+15H]^{15+}$ 1072.3583 $[M+14H]^{14+}$ 1154.8523 $[M+13H]^{13+}$	19 18 17 16 15 14 13	14997.14 14997.14 14998.09 14998.04 14998.10 14999.01 15000.08	14998
	H2	23.35	842.4532 $[M+19H]^{19+}$ 889.2002 $[M+18H]^{18+}$ 941.4465 $[M+17H]^{17+}$ 1000.2249 $[M+16H]^{16+}$ 1066.8977 $[M+15H]^{15+}$ 1142.9635 $[M+14H]^{14+}$ 1230.8134 $[M+13H]^{13+}$	19 18 17 16 15 14 13	15987.62 15987.64 15987.59 15987.60 15988.46 15987.49 15987.57	15988
	H3	23.96	840.7157 $[M+19H]^{19+}$ 887.4274 $[M+18H]^{18+}$ 939.4463 $[M+17H]^{17+}$ 998.2278 $[M+16H]^{16+}$ 1064.6343 $[M+15H]^{15+}$ 1140.6901 $[M+14H]^{14+}$ 1228.3045 $[M+13H]^{13+}$	19 18 17 16 15 14 13	15954.60 15955.69 15953.59 15955.64 15954.51 15955.66 15954.96	15956
Poecilobdella manillensis Lesson	P1	17.16	872.6291 $[M+11H]^{11+}$ 959.9008 $[M+10H]^{10+}$ 1066.4421 $[M+9H]^{9+}$ 1199.8658 $[M+8H]^{8+}$ 1370.9940 $[M+7H]^{7+}$	11 10 9 8 7	9587.92 9589.01 9588.98 9590.93 9589.96	9590
	P2	19.43	910.5386 $[M+15H]^{15+}$ 975.5080 $[M+14H]^{14+}$ 1050.4532 $[M+13H]^{13+}$ 1137.9208 $[M+12H]^{12+}$ 1241.1839 $[M+11H]^{11+}$ 1365.2053 $[M+10H]^{10+}$ 1516.8870 $[M+9H]^{9+}$	15 14 13 12 11 10 9	13643.08 13643.11 13642.89 13643.05 13642.02 13642.05 13642.98	13642
	P3	21.42	790.2769 $[M+19H]^{19+}$ 834.1862 $[M+18H]^{18+}$ 883.2527 $[M+17H]^{17+}$ 938.3275 $[M+16H]^{16+}$ 1000.8867 $[M+15H]^{15+}$ 1072.3717 $[M+14H]^{14+}$ 1154.8862 $[M+13H]^{13+}$	19 18 17 16 15 14 13	14996.26 14997.35 14998.30 14997.24 14998.30 14999.20 15000.52	14998
	P4	22.26	1176.4211 $[M+15H]^{15+}$ 1260.3048 $[M+14H]^{14+}$ 1357.2592 $[M+13H]^{13+}$ 1470.2544 $[M+12H]^{12+}$ 1603.7512 $[M+11H]^{11+}$ 1764.1368 $[M+10H]^{10+}$	15 14 13 12 11 10	17631.31 17630.27 17631.37 17631.05 17630.26 17631.37	17631
	P5	23.08	842.4561 $[M+19H]^{19+}$ 889.1513 $[M+18H]^{18+}$ 941.4591 $[M+17H]^{17+}$ 1000.1730 $[M+16H]^{16+}$ 1066.9244 $[M+15H]^{15+}$ 1142.9081 $[M+14H]^{14+}$ 1230.7559 $[M+13H]^{13+}$	19 18 17 16 15 14 13	15987.67 15986.72 15987.80 15986.77 15988.87 15986.71 15986.83	15988
	P6	23.85	1275.3938 $[M+13H]^{13+}$ 1381.5940 $[M+12H]^{12+}$ 1507.1006 $[M+11H]^{11+}$ 1657.6050 $[M+10H]^{10+}$ 1841.6814 $[M+9H]^{9+}$	13 12 11 10 9	16567.12 16567.13 16567.11 16566.05 16566.13	16567

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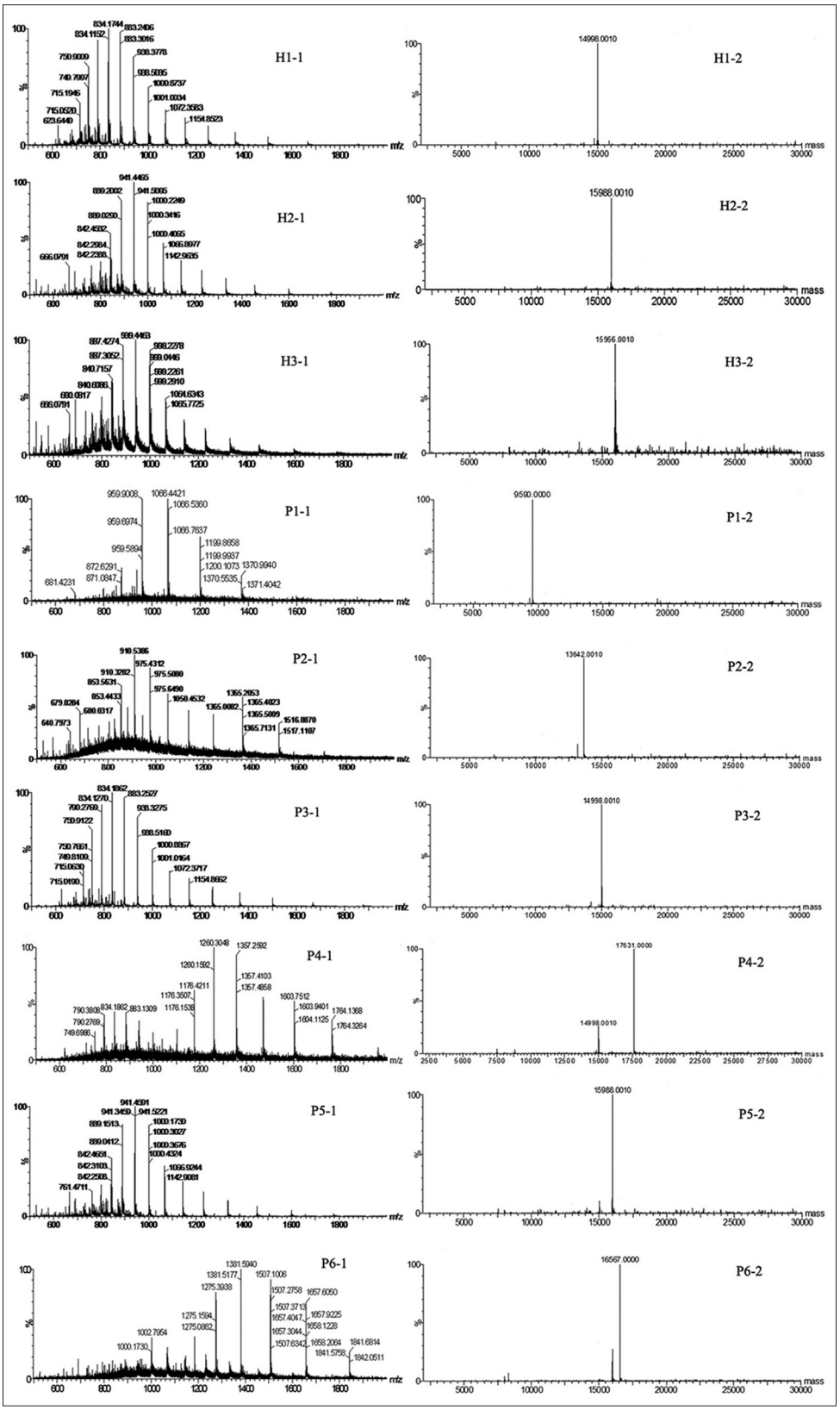

Figure 2: Multiple charges mass peak (left) and single charge mass peak (right) of H1~H3, P1~P6

were divided into two groups: Group 1 for *P. manillensis* and group 2 for *H. nipponia*. The resulting scatter plot (S-plot) from the OPLS-DA analysis can clearly display significant markers that can differentiate the sample groups. Figure 4 shows the loading plot (S-plot) of the OPLS-DA result. The points on the Figure 4 are the EMRTs plotted by

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covariance (X-axis) and correlation (Y-axis) values. The point the farther away from the X-axis is the greater the contribution to the difference between the two groups, while, the farther along the Y-axis is the higher the reliability of the analytical result. Every point is an EMRT pairs, which nearest the upper right corner of the S-plot are the leading contributing markers from the *P. manillensis* group, while, nearest the lower left corner of the S-plot are the leading contributing markers from the *H. nipponia* group [Figure 4].

Table 3 shows the combined list of the top 15 leading EMRT pairs obtained from the S-plot for both sample groups and the factor of change for each of the markers identified. The value of a factor of change indicates the intensity of difference between the peaks. No. 2-7 peaks are the markers which are highest in concentration in the *P. manillensis* group, and No. 10-15 peaks are the markers which are highest in concentration in the *H. nipponia* group. This table also can be displayed as a column plot, as shown in Figure 5. As a result, the markers tend to stretch out more toward the end of the S-plot and show the highest factor of change between the two groups. Hence, according to the highest factor of change and higher peak intensities, four EMRT pairs, which were detected only in *P. manillensis* and weren't in *H. nipponia*, can be regarded as useful for discriminating two species leeches: Peak 2 (24.74_ 492.2604), peak 3 (34.74_633.4192), peak 4 (22.30_711.8679), peak 5 (19.67_611.9691) [Table 3].

Trypsin digests the amino acid sequence in the peptide bond between lysine and arginine. Four peaks from the trypsin hydrolysate were detected in *P. manillensis* and weren't in *H. nipponia*, so that they can be taken as the discriminating peaks between the two species leeches.

Table 3: Top contributing markers from the two sample groups

Peak number	Retention Time	Mass	p[1]P	p(corr)[1]P	Factor of Change	peak intensities of *H. nipponia*	peak intensities of *P. manillensis*
1	3.01	120.081	0.11	0.99	2.9	97.6	279.8
2	24.74	492.2604	0.11	0.99	10000	1.63E-06	167
3	34.74	633.4192	0.09	0.87	10000	3.68E-06	139.3
4	22.30	711.8679	0.09	0.95	10000	7.47E-07	138.6
5	19.67	611.9691	0.08	0.94	10000	6.25E-07	114.3
6	24.73	766.0628	0.08	0.99	10000	1.40E-06	95.7
7	47.82	616.175	0.07	0.99	10000	1.90E-06	85.9
8	44.73	803.873	-0.06	-0.98	14.3	53.7	3.75
9	44.73	703.5149	-0.06	-0.99	17.9	54.8	3.06
10	24.91	492.2604	-0.06	-1.00	10000	62.8	1.44E-06
11	45.63	758.28	-0.06	-1.00	10000	49.2	2.77E-07
12	14.31	437.2334	-0.05	-1.00	10000	43.08	1.08E-07
13	45.6	884.4973	-0.05	-1.00	10000	41	3.07E-07
14	19.81	611.9694	-0.04	-1.00	10000	28.3	9.56E-07
15	44.8	703.515	-0.06	-1.00	10000	54.8	1.22E-06

CONCLUSION

Hirudotherapy is actively used in clinical practice in Russia, USA, Canada, etc., Hirudin is isolated in 1955

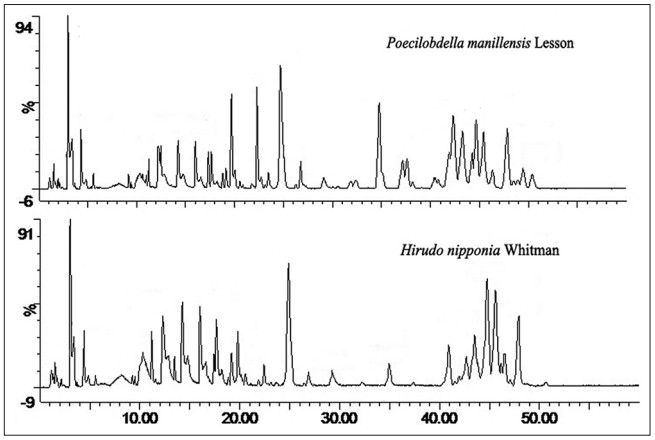

Figure 3: Base peak ion chromatogram from hydrolysates of *Poecilobdella manillensis* Lesson and *Hirudo nipponia* Whitman

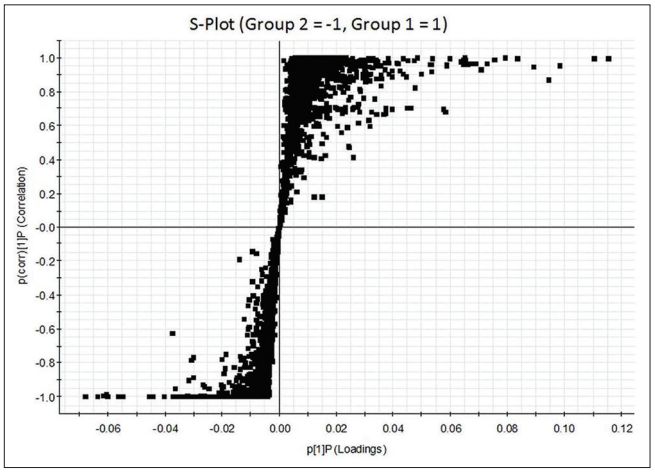

Figure 4: Scatter plot of Group 1 *Poecilobdella manillensis* versus Group 2 *Hirudo nipponia*

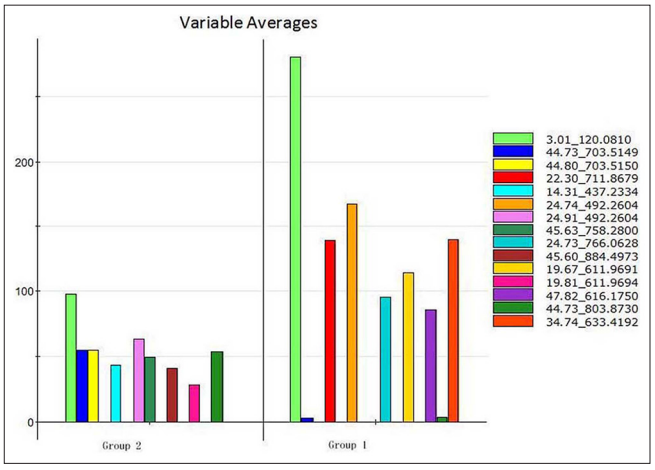

Figure 5: Column plot of the averages of Group 1 *Poecilobdella anillensis* versus Group 2 *Hirudo nipponia*

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from the secretion of *H. nipponia*'s salivary gland and is the main active compound in the humoral agent in hirudotherapy. In this article, anticoagulant active part was found in the dried body of the two species leeches, *H. nipponia and P. manillensis,* and they may have different anticoagulant action and effect from Hirudin. Furthermore, some peptide components were isolated from the anticoagulant active part of the dried body of the two species leeches. Among them, two of the peptides separately come from the two leeches have the same molecular weight, and the two peptide peaks occupied a very large ratio of total base peak area. Hence, the composition of the extracted active part of the two leeches is very close. This work shows that the dried body of the two species leeches have anticoagulant action, and *P. manillensis* may be used as a medicinal leech as *H. nipponia*. The two species leeches and their extraction can be discriminated by tryptic dehydrolytes of the extraction. The peptide components, isolated from the dried body of the two species leeches, are the basis for chemical identification and further investigations in active action.

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