Studies on Extraction and Activity of Peptides from *Haemadipsa hainana*

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Abstract [**Objectives**] Peptides was extracted from *Haemadipsa hainana* and its activity was studied. [**Methods**] Electric stimulation, water extraction and ultrasonic extraction were used to extract the peptides from *H. hainana*. Then the protein content and molecular weight distribution of *H. hainana* peptides were detected by the BCA method and SDS-PAGE method, respectively. The antithrombin activity and analgesic activity of the three peptide extracts of *H. hainana* were detected by Markwardt thrombin titration method and mouse hot plate experiment, respectively. [**Results**] There extraction methods of electric stimulation, water extraction and ultrasonic extraction were used to extract the peptide extract of *H. hainana*, and the yields were as follows: water extraction > electrical stimulation > ultrasonic extraction. The three peptide extracts from *H. hainana* had antithrombin activity, and the antithrombin activity was as follows: water extraction > ultrasonic extraction > electrical stimulation. Through the hot plate experiment in mice, it was verified that the three peptide extracts of *H. hainana* had analgesic activity, and the analgesic activity was water extraction > electric stimulation > ultrasonic extraction. The analgesic activity of high-dose (100 mg/kg) group of *H. hainana* obtained by water extraction was slightly weaker than that of tramadol. [**Conclusions**] This study confirmed that the peptide extract of *H. hainana* had certain antithrombin and analgesic activity, laying a foundation for the subsequent development and application of *H. hainana*.

Key words *Haemadipsa hainana*; Extraction method; Antithrombin activity; Analgesic activity

Haemadipsa hainana, commonly known as "Shanmahuang", belonging to *Haemadipsa* of Haemadipsidae, is a local endemic species in Hainan. It mainly inhabits springs, streams, bamboo forests and areas with relatively flat terrain in the central and eastern mountainous areas of Hainan Island, China. It prefers to hide in damp and muddy places, such as in damp gravel, decayed wood, dry cattle excreta, grass joints, and damp areas with stagnant water[1]. *H. hainana* has high potential medicinal value due to its unique geographical location and climatic conditions.

Haemadipsa and *Hirudo* are bloodsucking leeches, and their salivary glands contain anticoagulant substances, namely haemadin and hirudin[2]. Richardson *et al.*[3] found that haemadin and hirudin have very similar overall structures, but their binding methods with thrombin are significantly different in that the C-terminals of haemadin and hirudin bind to different binding sites of thrombin respectively. Tan *et al.*[4] extracted the haemadin gene from *H. hainana* and amplified and expressed it, and the expressed product had anticoagulant activity. Tan *et al.*[5] found that the cDNA sequence homology of haemadin between *H. hainana* and *Haemadipsa sylvestris* was greater than 99.9%, while haemadin in *H. sylvestris* has been confirmed to have antithrombin activity[6-7].

Haemadipsa makes a living by sucking on the blood of humans and animals, and it is difficult for animals to detect them during the feeding process. From the perspective of survival strategy, the feeding behavior of *Haemadipsa* can be assumed to the reason that their saliva has analgesic or anesthetic functions which keep them undetected by hosts[8]. Wang *et al.*[9] isolated and purified for the first time a peptide HSTX-I with selective inhibition on NaV1.8 and NaV1.9 channel activity from *H. sylvestris*, indicating that it may be a promising precursor molecule for the development of analgesic drugs.

Based on this, it can be seen that small molecule peptides in *Haemadipsa* may exert analgesic effects by inhibiting sodium channels, which has great potential value for the development of painkillers using haemadin extract. Therefore, in this study, we focused on extracting peptides from *H. hainana* and further verifying its antithrombin and analgesic activity, hoping to expand the pharmacological application of *H. hainana* and lay a foundation for further research.

Materials and Methods

Materials

Animals Fresh individuals of *H. hainana* were collected from Wuzhishan City, Hainan Province in August 2021, and its species was identified as *Haemadipsa* of Haemadipsidae. Adult Kunming female rats, 4 -6 weeks old, weighing about 20 g, were provided by Hunan SJA Laboratory Animal Co., Ltd., with certificate number SCXK (Xiang) 2019-0004.

Reagents The dry powder of *Hirudinaria manillensis* extract, provided by Guangxi Natural hirudin Biotechnology Co., Ltd.; normal saline, RIPA cell lysis buffer, glycine, hydrochloric acid, SDS-PAGE protein loading buffer (5X), SDS-PAGE gel preparation kit, bovine serum standard protein, BCA protein concentration determination kit, Thrombin 1000 U and fibrinogen (bovine blood), all of which were from Biosharp company.

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Lizi SHEN (1986 -), female, P. R. China, pharmaceutical engineer, devoted to research about haemadin of *Haemadipsa hainana*.
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Instruments SCIENTZ-ID ultrasonic cell grinder (Ningbo Scientz Biotechnology Co., Ltd.); DYY-8C electrophoresis instrument power supply (Beijing Liuyi Biotechnology Co., Ltd.); vertical protein electrophoresis instrument (Beijing Liuyi Biotechnology Co., Ltd.); Benchtop 6.0K EL freezing vacuum dryer (VirTis, USA); YLS-6B intelligent hot plate instrument (Jinan Yiyan Technology Development Co., Ltd.); SSW-600-2S electric constant temperature water bath (Shanghai Boxun Industrial Co., Ltd.).

Methods

Extraction methods of *H. hainana*

Electrical stimulation Twenty live individuals of *H. hainana*, about 40 g, were washed and placed in distilled water. Electric stimulation was adopted to stimulate the land leeches to produce saliva secretions. The collected saliva secretions were collected and centrifuged to obtain the supernatant, which was freeze-dried under a vacuum condition to obtain dry powder of *H. hainana* secretions for later use.

Water extraction method Twenty live individuals of *H. hainana*, about 40 g, were made into a homogenate in a high-speed stirring grinder. Next, 10 times of distilled water was added, and the mixture was placed in an ice bath in a constant-temperature magnetic stirrer overnight. After centrifuging at 4 ℃ and a power of 9 000 r/min for 15 min, the precipitate was discarded, and the supernatant was required as the land leech extract, which was stored at −20 ℃ and freeze-dried under vacuum to form a powder for later use.

Ultrasonic extraction method About 35 g of precipitate obtained after centrifugation under water extraction method was added with an appropriate amount of distilled water, and ultrasonically pulverized at 70% power for 10 min. Next, the obtained solution was centrifuged at 9 000 r/min for 15 min to obtain the supernatant, which was then freeze-dried under vacuum into a powder for later use.

Protein contents and molecular weights of polypeptide extracts from *H. hainana* First, 1.0 mg of *H. hainana* polypeptide extracts obtained by electrical stimulation method, water extraction method and ultrasonication extraction method and 1.0 mg of *H. manillensis* extract were accurately weighed, dissolved in 1 ml of distilled water. Their protein contents were determined by the BCA method, and the absorbance values of the extracts were measured at a wavelength of 562 nm in three repetitions. Gels were prepared according to the SDS-PAGE kit instructions, and electrophoresis was performed. After electrophoresis, each gel was added with Coomassie Brilliant Blue G-250 staining solution to dye it overnight, and it was decolorized with a decolorizing solution the next day until the background was clear. The molecular weights of the samples were checked in a gel imaging system.

Study on antithrombin activity of polypeptide extracts from *H. hainana* The antithrombin activity of polypeptide extracts from *H. hainana* was detected by the thrombin titration method proposed by Markwardtt[10]. The principle is to use the amount of hirudin with unknown activity to judge its antithrombin effect on thrombin with certain activity, and fibrinogen is an indicator. Hirudin can directly act on thrombin, making the activity of thrombin disappear, so as to play an antithrombin role. The two are combined in equal amounts, so the antithrombin activity of hirudin can be judged by the amount of thrombin consumed. The results will be in units of ATU, that is each thrombin unit (U) consumed is equivalent to one antithrombin unit (ATU).

Bovine fibrinogen was accurately weighed and dissolved in a Tris-HCl buffer solution to prepare a 0.5% bovine blood fibrinogen solution. Next, 200 μl of 0.5% bovine blood fibrinogen solution was accurately measured and added into a small hole of ELISA plate. Each of the crude extracts of *H. hainana* (0.1 g) obtained by three methods: electrical stimulation, water extraction, and ultrasonic extraction, was accurately weighed and added in a test tube, and mixed and completely dissolved with 500 μl of physiological saline. Each sample was soaked for half an hour at room temperature. After shaking evenly, centrifugation was performed and the precipitates were discarded to obtain solutions to be tested. Next, 1 000 U of thrombin was dissolved with 1 ml of physiological saline and diluted 25 times to obtain a 40 U/ml of thrombin solution. The prepared solutions were sampled and titrated using a microsyringe with a sampling amount of 5 μl each time. Whether fibrinogen has coagulated was determined within an interval of 1 min, *i. e.* whether the liquid level is turbid, with clots or fibrin present. If so, it indicated that the titration endpoint had been reached, and the amount of thrombin consumed was calculated and recorded according to the following formula:

$$U = C_1 V_1 / C_2 V_2$$

In the formula, U is the antithrombin activity unit (ATU/g) per 1 g of substance; C_1 is the concentration of thrombin solution (U/ml); C_2 is the concentration of test solution (g/ml); V_1 is the volume of thrombin solution consumed (μl); and V_2 is the amount of test solution added (μl).

Study on the analgesic activity of *H. hainana* The analgesic effect was tested using a mouse hot plate experiment. The mice were placed on a hot plate instrument to measure the pain threshold, with a temperature set at (55 ± 0.5) ℃, and the mice licking their hind feet served as an observation indicator. If there was no pain response within 60 s, the mice should be promptly removed, and the pain threshold value should be calculated based on 60 s.

Preliminary screening of injection method Six adult Kunming female mice were randomly divided into two groups, namely the intraperitoneal injection group and the intramuscular injection group, where 25 mg/kg of *H. hainana* peptide extract was injected at a dose of 10 ml/kg. The pain threshold was measured at 15, 30, 45, 60, 75, 90, 105 and 120 min after intraperitoneal injection, and it was measured in the first 15 min at an interval of 5 min and in the last 75 min at an interval of 15 min after intramuscular injection, so as to select the optimal injection method.

Comparison on analgesic activity of *H. hainana* polypeptide extracts obtained with different extraction methods Twelve adult Kunming female mice were randomly divided into four groups: electric stimulation group, water extraction group, ultrasound extraction group, and normal control group, with three mice in each group. The electrical stimulation group, water extraction group, and ultrasonic extraction group were injected with 25 mg/kg of *H. hainana* peptides extracted by the electrical stimulation method, water extraction method, and ultrasonic extraction method, respectively. The normal control group was injected with physiological saline. The dosage of each group was 10 ml/kg, and the injection method and pain threshold test time could be found under item "Preliminary screening of injection method". The optimal extraction method for *H. hainana* peptide extract with analgesic activity was selected by comparing the pain threshold.

Comparison on analgesic activity of *H. hainana* polypeptide extract at different doses Twenty four adult Kunming female mice were randomly divided into 6 groups: positive control group, normal control group, *H. manillensis* group, and low-, medium-, and high-dose groups of *H. hainana*, with 6 mice in each group. The low-, medium-, and high-dose groups of *H. hainana* were injected with 25, 50, and 100 mg/kg of *H. hainana* peptide extract (obtained by the best extraction method selected under item "Comparison on analgesic activity of *H. hainana* polypeptide extracts obtained with different extraction methods"). The positive control group was injected with 100 mg/kg tramadol; the normal control group was injected with physiological saline; and the *H. manillensis* group was injected with 100 mg/kg of *H. manillensis* extract. The dosage of each group was 10 ml/kg, and the injection method and pain threshold test time could be found under item "Preliminary screening of injection method".

Based on the above experimental data, the increase in pain threshold of each group of mice was calculated according to the following formula.

Increase of pain threshold = [(Average pain threshold after administration − Average pain threshold before administration)/Average pain threshold before administration] × 100%

Data processing All experimental data were analyzed and processed using GraphPad Prism 8.0.2, and charts were drawn.

Results and Analysis

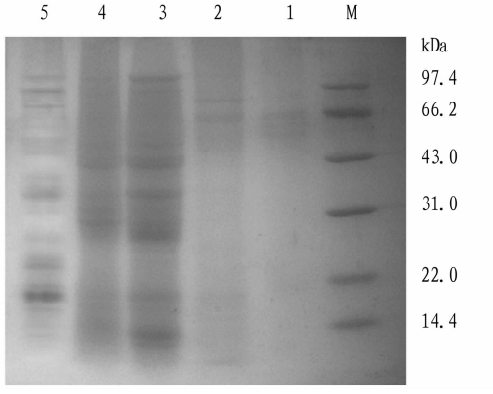
Effects of three extraction methods on the yield of *H. hainana* polypeptide extract

This study used three extraction methods: electrical stimulation, water extraction, and ultrasonic extraction to extract *H. hainana* polypeptides. The yields were as follows: water extraction (14.39%) > electrical stimulation (9.14%) > ultrasonic extraction (2.62%).

Protein contents and molecular weights of *H. hainana* polypeptide extract

The protein contents of *H. hainana* polypeptides determined by BCA method, electrical stimulation method, water extraction method, and ultrasonic extraction method were 23.84% ± 0.07%, 4.58% ± 0.16%, 23.94% ± 0.20%, and 15.69% ± 0.17%, respectively.

The molecular weight distribution of *H. hainana* polypeptide extracts obtained by the electrical stimulation, water extraction and ultrasonic extraction methods and that of *H. manillensis* extract are shown in Fig. 1. The electrophoretic patterns of the stimulation method, water extraction method, and ultrasonic extraction method had high overall similarity. Among them, the bands of *H. hainana* polypeptide extracts obtained by the water extraction method and ultrasonic extraction method were clearer, while the bands of *H. hainana* polypeptide extract obtained by the electric stimulation method were more blurry. The image analysis results showed that within the range of 66.2 − 97.4 kDa, the polypeptide extracts of *H. hainana* obtained by the electrical stimulation, water extraction, and ultrasonic extraction methods all had band distribution. Within the range of 14.4 − 43.0 kDa, both the *H. hainana* polypeptide extracts obtained by the water extraction and ultrasonic extraction methods showed band distribution, while that of the electric stimulation method did not show band distribution. The extract of *H. manillensis* showed the highest number of bands, reaching 12, and it was different from the *H. hainana* polypeptide extracts in distribution positions, and showed significant differences in terms of molecular weight distribution.



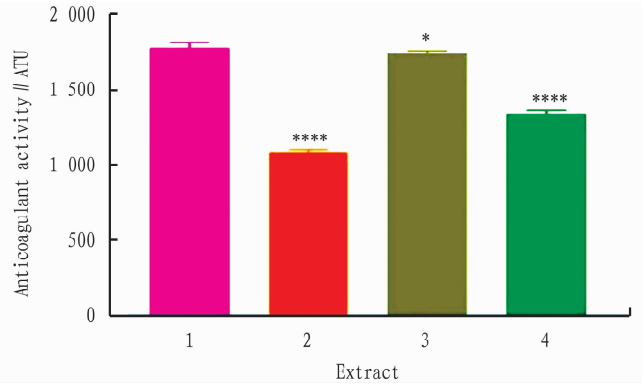
M is a low-molecular-weight protein marker; 1 and 2 are polypeptide extracts of *H. hainana* at concentrations of 20 and 100 mg/ml, respectively (electrical stimulation method); and 3 − 5 are polypeptide extracts of *H. hainana* (water extraction method), polypeptide extract of *H. hainana* (ultrasound extraction method) and *H. manillensis* extract of 20 mg/ml, respectively.

Fig. 1 Electrophoretic analysis of peptide extracts from *H. hainana*

Study on antithrombin activity of *H. hainana*

The thrombin titration method proposed by Markwardtt was applied to detect the antithrombin activity of *H. hainana* polypeptide extracts, and the results are shown in Fig. 2. In 8 parallel tests, there were statistical differences in the antithrombin activity of *H. hainana* polypeptides extracted by different extraction methods. The bufrudin secreted by the salivary gland of *H. manillensis* is one of the strongest natural antithrombin substances found in the world. The anticoagulant activity of *H. hainana* polypeptides

extracted by the water extraction method was equivalent to it, and the *H. hainana* polypeptides extracted by ultrasonic extraction method took the second place, while the anticoagulant activity of the *H. hainana* polypeptides extracted by the electrical stimulation method was the lowest.



1 is the extract of *H. manillensis*; 2 is the polypeptide extract of *H. hainana* (electric stimulation method); 3 is the polypeptide extract of *H. hainana* (water extraction method); and 4 is the polypeptide extract of *H. hainana* (ultrasonic extraction method). * indicates a statistical difference ($P<0.05$); * * indicates a significant statistical difference ($P<0.01$); and * * * indicates an extremely significant statistical difference ($P<0.001$).

Fig. 2 Antithrombin activity of *H. hainana* polypeptide extracts

Study on analgesic activity of *H. hainana*

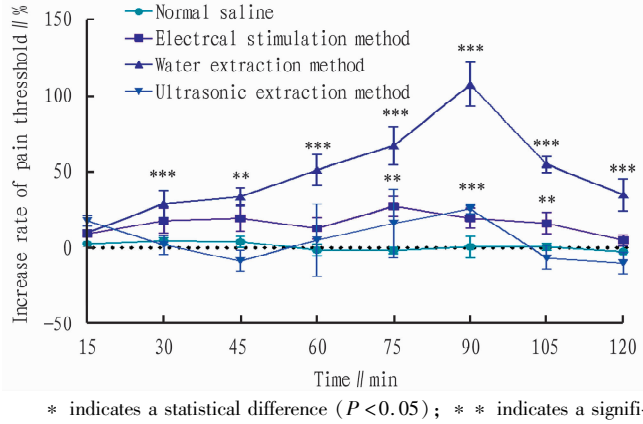
Initial screening by injection method After the same amount of *H. hainana* peptide extracts were intraperitoneally and intramuscularly injected into mice, respectively. The pain threshold was significantly increased after intraperitoneal injection, and the increase was greater than 100% in the period from 75 to 90 min. However, the maximum increase in pain threshold in mice was less than 30% after intramuscular injection, as shown in Table 1. Therefore, the subsequent hot plate experiment adopted the intraperitoneal injection method.

Comparison on analgesic activity of *H. hainana* polypeptide extracts obtained by different extraction methods The pain threshold increase rates of the three *H. hainana* peptide extract are shown in Fig. 3. It could be observed that the pain threshold increased to varying degrees after intraperitoneal injection of *H. hainana* peptides extracted by the three extraction methods, and the impacts to the pain threshold value ranked as water extraction method > electrical stimulation method > ultrasonic extraction method. In specific, the difference in the change curve of pain threshold improvement rate between the electric stimulation method and the ultrasonic extraction method was not significant, and the pain threshold improvement rates were relatively low, with a maximum value less than 30%; and the pain threshold improvement rate of the *H. hainana* peptide extract obtained by the water extraction method reached its maximum value at 90 min, and the increase was greater than 100%. Therefore, it was speculated that the water extraction method was the best extraction method for the analgesic peptides of *H. hainana*, which had less damage to *H. hainana* peptides and could increase the pain threshold in mice to a greater extent.

Table 1 Effects of different injection methods on hot plate reaction in mice ($\bar{x}\pm s$, $n=3$)

Group	Dosage	BPTBA	BPTAA // s									
	%	s	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min	105 min	120 min
I. P.	0.25	20.34 ± 1.67	–	–	20.95 ± 1.67	25.52 ± 2.49	26.75 ± 3.00	32.88 ± 2.90	42.54 ± 2.08	43.19 ± 3.14	29.47 ± 1.88	28.33 ± 1.33
I. M.	0.25	21.13 ± 4.44	19.97 ± 1.82	27.30 ± 2.29	24.40 ± 4.74	25.59 ± 1.62	27.27 ± 4.34	25.13 ± 2.64	24.88 ± 5.55	24.62 ± 6.73	–	–

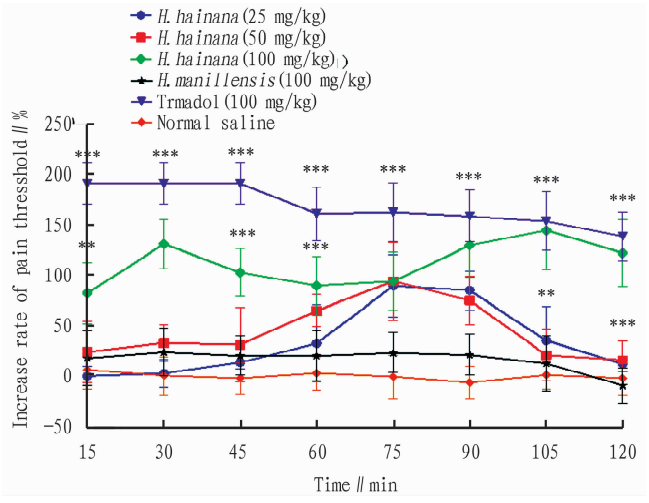
BPTBA is basic pain threshold before administration; BPTAA is basic pain threshold after administration; I. P. is intraperitoneal injection; I. M. is intramuscular injection.



* indicates a statistical difference ($P<0.05$); * * indicates a significant statistical difference ($P<0.01$); and * * * indicates an extremely significant statistical difference ($P<0.001$).

Fig. 3 Comparison on analgesic activity of *H. hainana* polypeptide extracts obtained by different extraction methods

Comparison on analgesic activity of *H. hainana* polypeptide extract at different doses The increase rates of pain threshold after low-, medium-, and high-dose administration of *H. hainana* polypeptide extract (water extraction method) are shown in Fig. 4. From the data, it could be observed that after intraperitoneal injection, the pain thresholds of different doses all showed an upward trend. The differences in the change curves of pain threshold increase rates between the low-dose and medium-dose groups of *H. hainana* polypeptide extract were not significant, while the high-dose group had the greatest impact on the pain threshold increase rate, and the maximum pain threshold increase rate was greater than 100%, which was only second to the positive control (tramadol). There was no significant difference in the change curve of pain threshold increase rate between the extract of *H. manillensis* and physiological saline (normal control), which might be due to the fact that the extract of *H. manillensis* does not have analgesic activity. Compared with the normal control (physiological saline), the pain threshold increase rate of *H. hainana* extract significantly increased, indicating that the *H. hainana* extract had analgesic activity. However, the analgesic activity of *H. hainana* extract was slightly weaker than that of tramadol serving as the positive control.



* indicates a statistical difference ($P<0.05$); ** indicates a significant statistical difference ($P<0.01$); and *** indicates an extremely significant statistical difference ($P<0.001$).

Fig. 4 Comparison of analgesic activity of *H. hainana* polypeptide extract at different doses

Discussion and Conclusions

At present, there is a lot of research on *Hirudo* both domestically and internationally, while there is very little research on *Haemadipsa* that belongs to the same class of leeches. *H. hainana*, as an endemic species in Hainan, has potential medicinal value. However, there is very little research on *H. hainana*, and only the research team of Tan Enguang has successively studied its ecology, morphology, prevention and control measures, and also cloned and expressed the haemadin gene of *H. hainana*, and preliminarily discovered the anticoagulant activity of *H. hainana* haemadin gene[1,4,5,11-16]. However, the research on active polypeptides of *H. manillensis*, which belongs to the same genus of *Haemadipsa*, has yielded fruitful results. Currently, its analgesic and antithrombotic activity have been found[17]. Hence, *H. hainana* should also have potential antithrombin and analgesic activity.

In this study, we used three methods to extract *H. hainana* polypeptides, and the water extraction method had the highest yield and protein content. It might be due to the good water solubility of the active ingredients in *H. hainana*, and the yield of the water extraction method was higher. The electrical stimulation and mechanical effects of electric stimulation and ultrasonic extraction methods had a relatively low degree of decomposition and damage to the living body of *H. hainana*, making the cells less prone to rupture, resulting in more substances in the cells being unable to be decomposed, and the yields of *H. hainana* polypeptide extract were relatively lower. Bufrudin secreted by the salivary gland of *H. manillensis* is a strong natural antithrombin substance found in the world. The anticoagulant activity of *H. hainana* polypeptides extracted by the water extraction method was equivalent to it, and the *H. hainana* polypeptides extracted by ultrasonic extraction method took the second place, while the anticoagulant activity of the *H. hainana* polypeptides extracted by the electrical stimulation method was the lowest, indicating that the antithrombin activity of *H. hainana* polypeptide extract obtained by the water extraction method was optimal.

Through the mouse hot plate experiment, intraperitoneal injection was initially selected as the optimal injection method. Subsequently, the analgesic activity of *H. hainana* polypeptide extracts obtained from the three extraction methods was tested. It was found that the *H. hainana* polypeptide extract obtained by the water extraction method achieved the strongest analgesic activity, and the high-dose group of *H. hainana* polypeptide extract (water extraction method) had slightly weaker pain threshold increase rate than tramadol. It might be due to that the water extraction method had less damage to *H. hainana* polypeptides and could maintain their analgesic activity. The yield and protein content of *H. hainana* polypeptide extract obtained by the water extraction method were the highest, and its antithrombin activity and analgesic activity were also better than those obtained by the electric stimulation method and ultrasonic extraction method, indicating that the water extraction method was the best extraction method for *H. hainana* polypeptide extract. At present, HSTX-I has been isolated from *H. sylvestris*, and it blocks NaV1.8 and NaV1.9 channels, showing significant analgesic effects in animal models[9]. Zheng *et al.*[8] found that PGE1 in the salivary gland of *H. sylvestris* inhibited acute pain in a formalin-induced mouse paw pain model. Due to time constraints, specific polypeptides exerting analgesic effects have not yet been identified from *H. hainana* polypeptide extract, and future experiments will be conducted to identify the substances that truly exert analgesic effects.

In summary, in this study, three methods were used to extract *H. hainana* polypeptides, all of which had antithrombin and analgesic activity. However, the antithrombin and analgesic activity of *H. hainana* polypeptide extracts obtained by different methods varied greatly. Among them, the *H. hainana* polypeptide extract obtained by the water extraction method showed best antithrombin and analgesic activity, and the pain threshold increase rate of the high-dose group of *H. hainana* polypeptide extract (water extraction method) was slightly weaker than that of tramadol, indicating that it has the potential for further development of analgesic drugs.

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In summary, X-ray has important application value in the differential diagnosis of pneumoconiosis and pulmonary tuberculosis. The type of lesions can be preliminarily determined by observing imaging features including the location of lesions, characteristics of lesions, nodule appearance, and accompanying cavity sign, providing important reference for clinical differential diagnosis. However, it should be noted that X-ray imaging examination has certain limitations, and for the diagnosis of some lesions, a comprehensive analysis should be conducted based on patients' condition, medical history, and other imaging examination methods and clinical manifestations.

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