ISSN: 0067-2904

Biochemical analysis of the saliva extracted from two different leech species

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Received: 23/3/2024 Accepted: 28/7/2024 Published: 30/7/2025

Abstract

Leech saliva contains numerous compounds, primarily consisting of proteins and peptides. This research focused on two leech species, *Hirudo verbana* and *Hirudo orientalis*, which were obtained from various alternative medicine clinics and scientific centers located in Baghdad, Iraq. Saliva samples were collected from the leehches after 5 and 6 weeks of food deprivation. Bradford proteins assay, UV absorption technique, Gel electrophoresis and RP-HPLC were used to analyze their saliva chemically. Anticoagulant activity of their saliva was also assessed using prothrombin time assay. Results showed that the concentration of saliva proteins were higher in the 5 weeks of starvation than the concentration of proteins in the 6 weeks of starvation for both species. The gel electrophoresis analysis revealed the presence of up to 16 distinct bands. Three molecular weights were found to be closely related to known proteins from leech species through comparison of the gel electrophoresis data with the literatures. Additional proteins found in the saliva extract by gel electrophoresis may possibly be associated to totally new biologically active proteins and peptides in the saliva extract or to a modification (isoforms) of the present ones or to a mixture of both. It was found that the crude saliva secretions of both *H. verbana* and *H. orientalis* have anticoagulant activity. The active action of *H. verbana* was stronger than the *H. orienralis*. The means of prothrombin time for *H. verbena* were higher than the means of prothrombine time for *H. orientalis* although no significant differences were noticed between them.

Keywords: Anticoagulant activity, Gel electrophoresis, medicinal leeches, proteins

التحليل البايوكيميائي للعاب المستخلص من نوعين مختلفين من العلق

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الخلاصة

يحتوي لعاب العلق الطبي على العديد من المركبات، التي تتكون أساسًا من البروتينات والببتيدات. في هذا البحث تم دراسة نوعين من العلق الطبي *Hirudo verbana* و*Hirudo orientalis*، والتي تم الحصول عليها من مختلف عيادات الطب البديل والمراكز العلمية الموجودة في بغداد، العراق. تم جمع عينات اللعاب من العلق بعد 5 و 6 أسابيع من الحرمان من الطعام. تم تحليل اللعاب المستخلص كيميائياً بواسطة طريقة برادفورد, تقنية امتصاص الاشعة البنفسجية, الترحيل الكهربائي بالهلام و طريقة كروماتوكرافيا السائل عالي الدقة. كما تم تقييم النشاط المضاد للتخثر في لعابهم من خلال اختبار زمن البروثرومبين. اظهرت النتائج ان تراكيز البروتينات كانت اعلى في الاسبوع الخامس من التجويع مقارنة بالاسبوع السادس من التجويع و لكلا النوعين. كشف

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الترحيل الكهربائي بالهلام وجود ما يصل الى16 حزمة مختلفة. تم العثور على ثلاثة أوزان جزيئية ترتبط ارتباطًا وثيقًا بالبروتينات المعروفة من أنواع العلق من خلال مقارنة بيانات الترحيل الكهربائي للهلام مع قاعدة بيانات البروتين. قد تكون البروتينات الإضافية الموجودة في مستخلص اللعاب عن طريق الفصل الكهربائي للهلام مرتبطة ببروتينات وببتيدات نشطة بيولوجيًا جديدة تمامًا في مستخلص اللعاب أو بتعديل (الأشكال المتماثلة) للبروتينات الموجودة أو في خليط من الاثنين معًا. وقد وجد أن إفرازات اللعاب الخام لكل من *H. verbana* و*H. orientalis* لها نشاط مضاد للتخثر. كان النشاط المضاد للتخثر للنوع *H. verbana* اقوى من النشاط المضاد للتخثر للنوع *H. orientalis*. حيث ان معدلات زمن البروثرومبين للنوع *H. verbana* كانت اعلى من معدلات زمن البروثرومبين للنوع *H. orientalis* على الرغم من عدم وجود فروق معنوية بينهما.

1. Introduction

Leeches, as significant and well-recognized invertebrates, have garnered considerable attention across multiple scientific disciplines. Various leech species have become the subject of extensive research in areas such as taxonomy, environmental studies, healthcare, medicine, and pharmacology [1, 2]. Leeches have been used since ancient times for medical and therapeutic purposes [3]. These invertebrates present many different chemicals in their saliva [4]. Physicians from different ancient nations used these animals to treat different diseases. These diseases involve many systematic disorders, such as skin disease, gastrointestinal dysfunctions, neurological disorder, respiratory diseases and genitourinary tract problems, in addition of using the saliva secretion of leeches to treat clotting issues [5]. Many important chemical components have recently been known in leech saliva secretions [6]. *Hirudo* Linnaeus, 1758, is among the various genera of leeches that have piqued interest in a variety of human activities. This genus was originally known in Asia and then quickly spread to Europe. Five closely related living species, *Hirudo medicinalis* Linnaeus, 1758; *H. verbana* Carena, 1820; *H. troctina* Johnson, 1816; *H. orientalis* Utevsky & Trontelj, 2005; and *H. sulukii* Saglam, Saunders, Lang & Shain, 2016, are used in a range of medical actions [7]. The therapeutic leeches of the genus *Hirudo* are well-known invertebrates that have been the subject of in-depth biological and physiological researches because of their common use in medicine and surgery [6, 7]. In addition many taxonomical, ecological and genetic researches were achieved on them [8]. Large amounts of medicinal leeches were collected from the environment during the eighteenth and nineteenth centuries, but at the end of that period, leeches were becoming rare in different areas [9]. The salivary glands of these leeches secrete many chemical compounds and the majority of these compounds are peptides and proteins, which differ in their chemical properties, biological activities, and therapeutic uses. [10]. These peptides, which have direct antithrombin activity, include hirudin, which was taken from the European leech *H. medicinalis*, *H. orientalis* [11], and hirudin-like peptide, which was extracted from the medicinal Australian leech *H. verbana* [12]. All of these chemicals pose a significant challenge to formulation scientists because of their complicated protein-based structure, which made them unstable. There is a notable lack of research focused on the chemical composition of leech saliva extract. Additionally, few studies have been conducted to compare the saliva of different leech species, particularly those utilized in alternative medicine practices. This study aimed to demonstrate the active compounds in the saliva of two different leech species that used in alternative medicine clinics in Baghdad.

2. Materials and methods

2.1 Leech collection and identification

A total of 13 leeches were included in the study. These leeches were sourced from multiple medical facilities and scientific institutions in Baghdad, Iraq. These involved: Alhwya centres

for studies, researches and training, Al-Yermok; Rafi center for chines medicine, Al Monsoor and Al-Huda Centre for Complementary medicine, Baghdad Aljadeeda. These clinics and centres were certified by General Syndicate for Complementary and Herbal Medicine, Iraq.

Leeches were obtained between January and March 2022. They were characterized and identified based on morphological characterization using morphological keys [13, 14]. Two species were identified as *Hirudo verbena* (n=7) and *Hirudo orientalis* (n=6). All the morphological characterizations of the specimens were recorded as a part of different project [15].

2.2 Extraction of saliva from leeches

During the entire duration of the experiment, the leeches were maintained in plastic bottles filled with non-chlorinated tap water. Water was changed every 72 hours [15]. Five individual of *H. verbana* and two individuals of *Hirudo orientalis* were starved for five weeks. The other four individuals of *Hirudo verbana* and two individuals of *Hirudo orientalis* were starved for six weeks. Then saliva was obtained following the method described by [16]. Hard pressing of the Parafilm membrane was done on a funnel that had 0.15 M saline solution and 0.001 M arginine in it. Leeches were permitted to suck the solution over the membrane until they were satiated while the solution was kept at 37°C. Leeches were submerged in ice for seven minutes in a plastic container as soon as they emerged from the membrane. This method induces the leeches to regurgitate the entirety of their ingested contents. Leeches were carefully squeezed from the posterior region toward the anterior region to finish the saliva collection. The vomited fluids were collected in clean test tubes (bloody fluids were disposed of) and spun using a cooled centrifuge for 12 minutes at 2800 rpm. During the experimentation, supernatant was extracted and utilized.

2.3 Assessment of protein concentration from saliva secretion

To assessment of the saliva protein contents the Bradford proteins technique and UV absorption techniques were used. The protein content was quantified by measuring absorbance at 280 nm using ultraviolet (UV) spectroscopy [17]. The protein concentration was also measured using the Bradford protein technique [18], that uses the wavelength of 595 nm as a calibration curve and uses bovine serum albumin (BSA) as a reference.

2.4 Gel Electrophoresis of saliva secretion

For protein identification, one-dimensional (1D) electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques were employed [20]. A mixture of peptide markers measuring 1.02–26 kD (Sigma) and saliva extract proteins (about 15 µg) were applied using a Mini Protein Tetra cell instrument (Bio Rad) and power supply. For all tests, 6 x 10 cm gels (1 mm thick) were utilized. The conventional protocol for Coomassie blue staining was followed [22]. The gels were visualized with a gel documentation apparatus from Bio-Rad.

2.5 RP-HPLC of saliva secretion

RP-HPLC of saliva secretion was done according to [23]. An Agilent reversed-phase C18 column (4.5 mm x 150 mm, 5 µm particle size) was connected to an automated HPLC injector system. This setup was used to analyze fresh saliva samples. The mobile phases were buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile). For the elution, a linear gradient of 5–90% buffer B is applied over 45 minutes. At 214 nm, the absorbance detection was fixed.

2.6 Prothrombin time (PT) assay

The prothrombin time assay was achieved based on the method of [24]. A volume of 100 µl of citrated plasma was pipetted into a coagulation tube provided with the coagulometer and then incubated at 37°C in the coagulation analyzer for about three minutes. Subsequently, 10 µl of the reconstituted thrombin reagent was introduced to the sample. Using a coagulometer, the time elapsed until the initiation of coagulation was recorded. Different dilutions of the crude saliva (10%, 20% and 30%) from each *H. verbana* and *H. orientalis* extract were mixed with the citrated plasma to yield a final volume of 100 µl. A control plasma test was also achieved using the above procedure. This operation was repeated 3 times for each sample and the mean value was taken as the final result.

2.7 Statistical analysis

The data of prothrombin time was statistically analysed using the Statistical Analysis System - version 9.1(SAS. 9.1). One-way ANOVA was used to analyse the differences between the efficacy of crude saliva belonged to *H.verbana* and *H. orientalis* in different dilutions and during different starvation period. The significance of differences between pairs of group was assessed by Tukey post hoc analysis. A P-value of less than 0.05 is reflected statistically significant.

3. Results

3.1 Saliva Collection

Leeches of both species (Figure 1-a and 1-b) were smoothly extracted by squeezing them from the rear toward the anterior sucker (Figure 2). Thirteen leeches were used to collect the saliva. The body weights of the leeches ranged from 0.3 to 5 grams. Saliva collection involved leech suction from solution volumes of 0.7 to 3 ml. Some leeches have been reported to exude a blood-like saliva extract. The leech's body weight was connected in some way to this extract of bloody saliva. Bloody saliva extracts were more likely to be given by heavy body mass leeches than by lighter ones. It's interesting to see that the volume of fluid sucked was not much impacted by the starving period.

(a). **(b)**

Figure 1: The whole mount of *Hirudo verbana* (a), and whole amount of *Hirudo orientalis* (b)

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Figure 2: Leech while sucking the phagostimulatory fluid over the parafilm membrane.

3.2 Concentration of saliva proteins

The UV absorbance measurements at 280 nm were conducted on various leech saliva extracts, and the results are summarized in Table 1. The means of absorbance at A280 were (0.1309±0.007) and (0.1529±0.012) for *H.verbana* and *H. orientalis* respectively after 5 weeks of starvation while the means of absorbance at A280 showed certain changes after 6 weeks of starvation. They were (0.1261±0.0055) and (0.0236±0.0019) for *H.verbana* and *H. orientalis* respectively. The results of saliva protein concentration by Bradford method are also showed in Table 1. The mean concentrations of saliva protein were (8.96±1.18 mg/ml) and (2.4±0.28 mg/ml) for *H.verbana* and *H. orientalis* respectively after 5 weeks of starvation. Declining in the concentrations of saliva protein was noticed after 6 weeks of starvation for *H.verbana* and *H. orientalis* they were (3.6±0.46 mg/ml) and (2.3±0.28) mg/ml respectively.

Table 1: A280 for crude saliva secretion and concentration of saliva protein by Bradford method

No	Number of leeches	Species	Volume range of crude saliva taken from leeches (ml)	Total volume collected (ml)	Period of starvation	Absorbance at 280 nm (mean±SD)	Concentration of saliva protein by Bradford method (mean ±SD)mg/ml
1	5	*H. verbana*	0.5-1.03	7	5 weeks	0.1309±0.007	8.96±1.18
2	2	*H. orientalis*	1.01-1.49	2.5	5 weeks	0.1529±0.012	2.4±0.28
3	4	*H. verbana*	0.18-2.15	5	6 weeks	0.1261±0.0055	3.6±0.46
4	2	*H. orientalis*	0.69-1.31	2	6 weeks	0.0236±0.0019	2.3±0.28

3.3 Gel electrophoresis of saliva extract

Figure 4 shows one-dimensional electrophoresis of separation of high molecular weight proteins and peptides isolated from the crude saliva of both *H.verbana* and *H. orientalis*. One-dimensional electrophoresis showed the presence of 16 proteins and peptides. Eight of them were isolated from *H.verbana* and the other eight were isolated from *H. orientalis.* These

proteins and peptides ranging from 26.302-151.359 kDa for *H. verbena* after 5 weeks of starvation. While the separated proteins and peptides of *H. orientalis* after 5 weeks of starvation ranged between 28.183-154.881 kDa. On other hand the protein and peptide gained by both *H.verbana* and *H. orientalis* after 6 weeks ranged between 25.703-151.359 kDa and 27.542-158.489 kDa respectively (Table-2). Analysis revealed that the primary constituents of saliva secretions in both *H. verbana* and *H. orientalis* are high molecular weight proteins, exceeding 53 kDa. Table 3 illustrates molecular weights of known proteins isolated from leech species. Comparison of these data with previous literatures revealed coincidence of three known leech proteins. The proteins were Hyaluronidase I, Hyaluronidase II and γGlutamyl transpeptidase. Hyaluronidase I was detected only in *H. orientalis* while Hyaluronidase II was found only in *H.verbana.* Finally, γGlutamyl transpeptidas was detected in both species. Other noticed bands might represent modification of known proteins and also unknown biologically active components of the saliva.

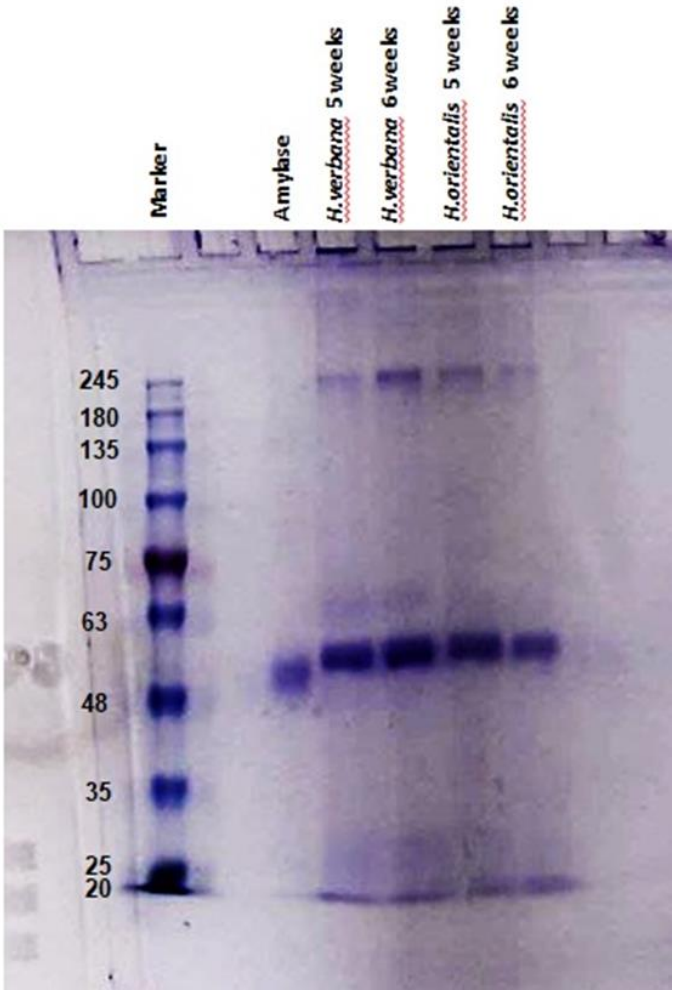

Figure 3: Separation of protein of medicinal leech (*H.verbana* and *H. orientalis*) saliva extraction by Tricine-SDS-PAGE 16%,

Table 2: Molecular weights of isolated protein from saliva extractions of *H. verbena* and *H.orientalis*

No	Number of leeches	Species	Period of starvation	Proteins separated from leeches	Molecular weight (kDa)
1	5	*H. verbana*	5 weeks	P1	151.359
				P2	66.69
				P3	53.703
				P4	26.302
2	2	*H. orientalis*	5 weeks	P1	154.881
				P2	66.69
				P3	52.48
				P4	28.183
3	4	*H. verbana*	6 weeks	P1	151.359
				P2	75.857
				P3	54.954
				P4	25.703
4	2	*H. orientalis*	6 weeks	P1	158.489
				P2	70.794
				P3	60.255
				P4	27.542

Table 3: Comparison of molecular weights of the medicinal leech saliva secretion with literature data on calculated and experimentally detected molecular weights of protein produced by different leech species

Proteins isolated from different leech species	Molecular weight in kDa -Literature data [reference]	Calculated molecular weight in kDa from the present study (species-weeks)
Hyaluronidase I	28.5220 [25]	28.183 (*H.orientalis*-5 weeks) 27.542 (*H.orientalis*-6 weeks)
Hyaluronidase II	55.090 [4]	53.703(*H.verbana*-5 weeks) 54.954 *(H.verbana*-5 weeks*)*
γGlutamyl transpeptidase	66.6541 [25]	66.69 (*H.verbana*-5 weeks) 66.69 (*H.orientalis*-5 weeks)

3.4 RP-HPLC of saliva secretion

The analytical chromatogram of the crude saliva secretions for *H. verbena* after 5 and 6 weeks of starvation revealed the presence of only two peaks with good resolution (Figure 4, 5). The peaks represented the following absorption value (2.30 and 3.58) for *H. verbena* after 5 weeks of starvation and (2.37 and 3.55) for *H.verbana* after 6 weeks of starvation. The same results were obtained for *H. orientalis* after 5 and 6 weeks of starvation (Figure 6, 7) as tow peaks with good resolution were noticed. The peaks of the 5 weeks of starvation for *H. orentalis* showed absorption values of (2.32 and 3.54). While the peaks of the 6 weeks of starvation for *H.orientalis* showed absorption values of (2.32 and 3.57).

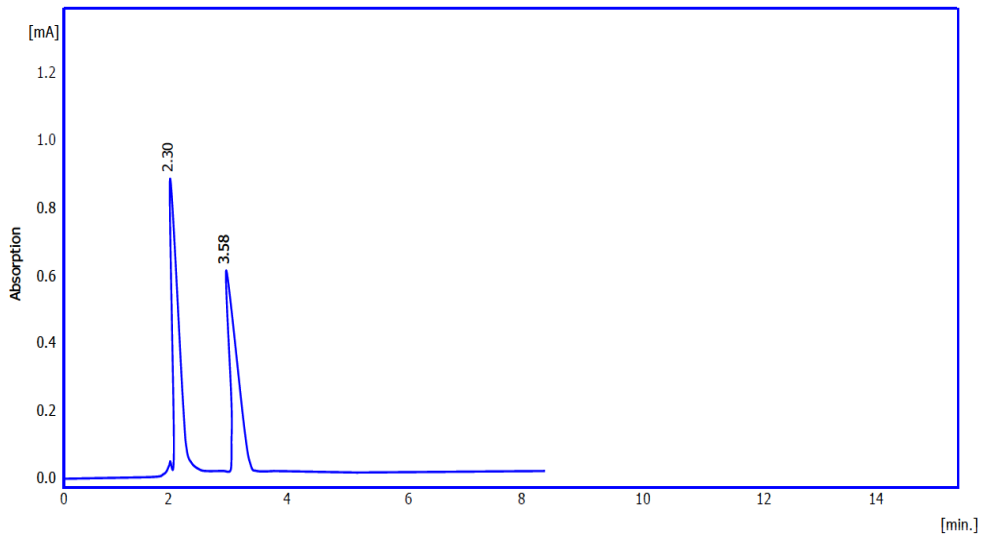

Figure 4: Analysis of crude saliva secretions from *H. verbana* after 5 weeks of starvation using RP-HPLC

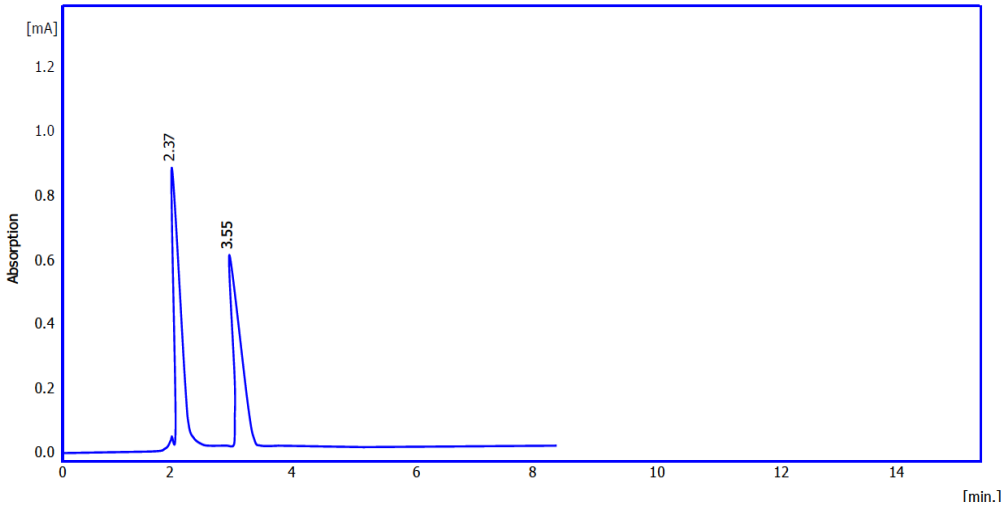

Figure 5 : Analysis of crude saliva secretions from *H. verbana* after 6 weeks of starvation using RP-HPLC

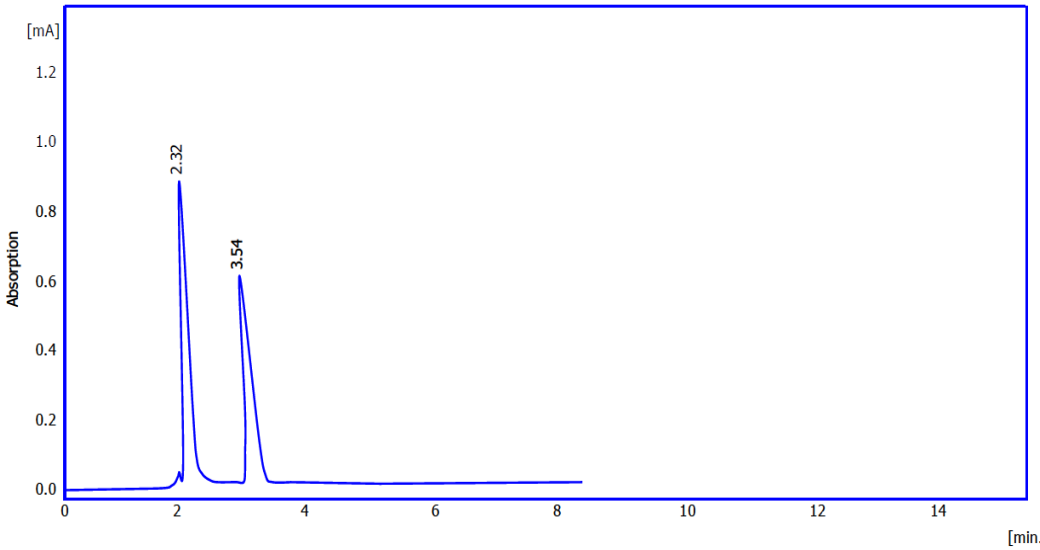

Figure 6: Analysis of crude saliva secretions from *H. orientalis* after 5 weeks of starvation using RP-HPLC

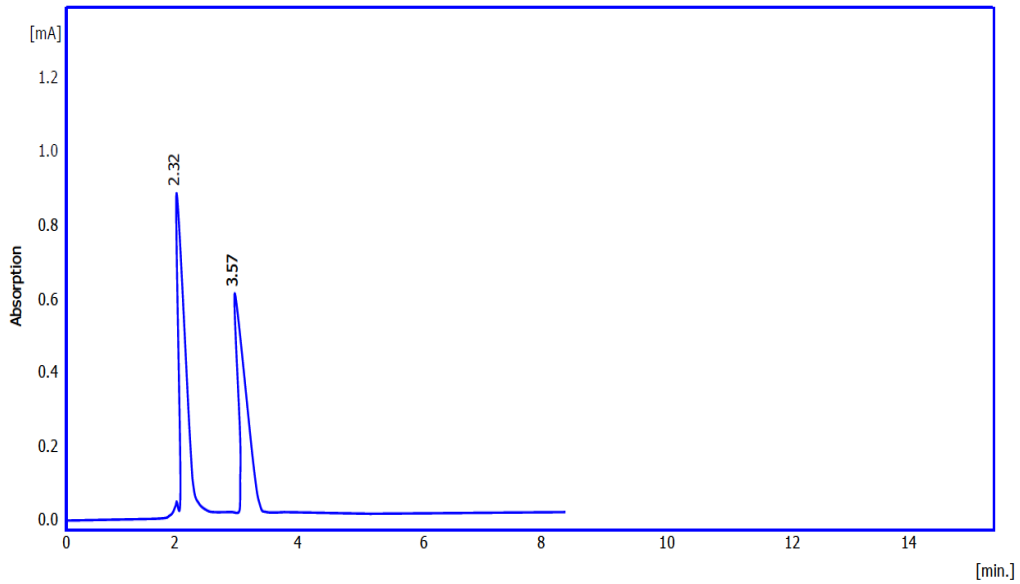

Figure 7: Analysis of crude saliva secretions from *H. orirntalis* after 6 weeks of starvation using RP-HPLC

3.5 Prothrombin time (PT)

Results revealed that the prothrombin time of the crude saliva extract of both *H.verbana* and *H.orientalis* after the two periods of starvation (5 and 6 weeks of starvation) increased with the increase of the saliva volume (Table 3). The highest average prothrombin times were observed with the 30% saliva solution dilution, at 50.66±11 seconds and 38.8±15.02 seconds for H. verbena after 5 and 6 weeks of fasting respectively. These values were higher than those found for other saliva solution dilutions and the control. Statistical analysis using ANOVA showed significant differences among the different dilutions and control (Table 3). The results of the *H. orientalis* did not differ from results of *H.verbana* regarding prothrombin time, as the dilution of 30% crude saliva was distinguished by showing the highest means of prothrombin time in weeks 5 and 6 (44.33±39.42 second) and (35.06±20.69 second) respectively compared to the other dilutions and the control group, although there were no significant differences among them (Table 3). The results illustrated that the saliva crude solutions of the two leech species had an anticoagulant effect. The active action of *H. verbana* was stronger than the *H. orienralis*. The means of prothrombin time for *H. verbena* were higher than the means of prothrombine time for *H. orientalis* although no significant differences were noticed between them.

Table 3: Prothrombin time values of the crude saliva extracted from *H. verbana* and *H. orientalis*

	Protrombin time (second)				
Group	**0% saliva Control- (100 µl plasma citrate)**	**10% saliva (10 µl of saliva+90 µl plasma citrate)**	**20% saliva (20 µl saliva +80 µl plasma citrate)**	**30% saliva (30 µl saliva +70 µl plasma citrate)**	**F-test (p-value)**
Hirudo verbena (5 weeks)	15.2±0.05^{A}	13.06±2.43^{B}	15.46±5.69^{C}	50.66±11.73$^{A, B, C}$	7.41(0.01)*
Hirudo verbena (6 weeks)	15.2±0.05	27.66±11.37	32.26±22.59	38.8±15.02	0.45(0.71)NS
Hirudo orientalis (5 weeks)	15.2±0.05	20.2±10.66	35±22.1	44.33±39.42	0.9(0.44)NS
Hirudo orientalis (6 weeks)	15.2±0.05	18.13±10.53	23.33±15.4	35.06±20.69	0.3(0.7)NS
F-test (p-value)		0.15(0.6)	0.3(0.8)	0.14(0.9)	

Means with a similar capital letter in same row significantly different (P<0.5)

4. Discussion

4.1 Saliva collection and protein concentrations

Leeches were starved for a certain amount of time in order to produce a leech saliva extract with a high concentration of proteins and peptides [26]. It was discovered that the ideal time frame was between three and sixteen weeks [11]. In this instance, five and six weeks of starvation periods were used. The mean concentrations of saliva protein were higher for *H.verbana* and *H. orientalis* after 5 weeks of starvation compared to the concentrations of saliva protein after 6 weeks of starvation for *H.verbana* and *H. orientalis*. The decrease in the concentration of protein in the saliva after the sixth week is supposed to be associated to the exhaustion of the leeches. Regarding the effects of time intervals on the recovery of protein in the leech saliva extraction, there is disagreement in some literature. It is important to note that leeches can produce a good quantity of protein after a one-week gap after the initial collection, as shown by Rigbi and his team [16]. While the research of Baskova (1984) reported starving leeches for two weeks at a time in order to extract a useful amount of protein [27]. The results also disagreed with Alaama *et al*., (2014) who showed that the concentration of proteins and peptides in the leech saliva increased linearly until the twelfth week of the starvation period [26]. The discrepancies between this study and previous research can likely be attributed to the variation in leech species examined across different investigations. Numerous techniques for examining the salivary content of various leech families have been reported. Most of these techniques are highly intrusive and involve scarifying leeches by either grounding and fusing their anterior regions or the entire body regions [28], or surgically excising salivary glands [29]. A starving leech can be made to regurgitate its saliva by submerging it in 8% ethanol, which is another way that still needs to scarify the animal [30]. A highly gentle technique that preserves leeches' lives while enabling the extraction of the required amount of saliva from them was reported [12]. After multiple tries, it was discovered that placing the leeches in an ice bath in a plastic container makes it much easier to collect the saliva. The freezing temperature causes the leech to vomit the sucked solution and immobilizes it, which makes it easier to squeeze the animal to extract as much saliva as possible. Finally, the animal is not killed by the ice; all that is needed to restore its activity and allow it to depart properly for an indeterminate amount of time is to place it back in a warm body of water.

4.2 Gel electrophoresis of saliva extract

In the current study, the result shows that the high molecular weight of the saliva secretion of the two species of leech contained 16 proteins ranged between 25.703-158.489 kDa. The molecular mass ranges observed in this investigation align with those reported in several earlier studies. The range was between 8-400 kDa in the study of Rigbi and his group, who studied the saliva secretion of *H. medicinalis* and *H. granulosa* [16].While it was between 11-483 kDa in the study of Baskova and his group, who investigated the saliva secretion of *H. medicinalis* [25]. On the other hand, the ranges of molecular weights obtained in the current study did not fall within the limits that appeared in the study of Alaama and his group (3.7-80 kDa), they obtained their data on the saliva of *Hirudinaria manillrnsis* [26]. This difference may be due to the species of leeches used, the method of collecting saliva, and the concentrations of isolated proteins. Comparison of gel electrophoresis data with proteins from the literatures exposed the closeness of three molecular weights to known proteins from different leech species. HyaluronidaseI and HyaluronidaseII were both detected from *H. orientals* and *H.verbana* respectively. These proteins are common in different leech genera. It was found in *Helobdella*, *Glossiphonia*, *Theromyzon*, *Placobdella*, *Erpobdella*, *Nephelopsis*, *Haemopis* and *Hirudo* [31]. Hyaluronidase is responsible for penetration of salivary gland secretion over tissue barriers by disintegrating hyaluronic acid, a key piece of the intercellular matrix [32]. The other detected

band was γ-Glutamyl transpeptidase. It was also detected previously by Baskova and his team [25]. γ-Glutamyl transpeptidase is involved in preservation of liquid state of host blood by cleaving endo-ε-(γ-Glu)-Lys isopeptide bonds in stabilized fibrin and D-dimer. Nevertheless, these proteins cannot account for the wide functional spectrum of medicinal leech salivary secretion [25].

4.3 Prothrombin time (PT)

Both of *H.verbana* and *H.orientalis* showed anticoagulant activity as their crude saliva led to a clear prolongation of the prothrombin time at different concentration. The results also showed that there was a linear relationship between the PT and the volume of saliva used, as the PT time increased with increasing saliva volume. This result agreed with the results obtained by Abdulkader and his team [24]. They showed that the antithrombin activity of the crude saliva extract was a linear function with the volume of the crude saliva mixed with the citrated plasma. Both H. verbena and H. medicinalis are known to secrete enzymes that can inhibit blood clotting. One of these enzymes is Hirudin which is an active constituent in the leeches' salivary gland secretion and plays as an active anticoagulant. It is a protein irreversibly binding to thrombin resulting in the use of antithrombin activity and active thrombin. This enzyme was not detected in the current study because it may be present in undetectable concentrations. In conclusion the active action of *H. verbana* was more effective than the *H. orienralis*. The means of prothrombin time for *H. verbena* were higher than the means of prothrombine time for *H. orientalis* although no significant differences were noticed between them. This result may be evidence of the convergence of these two species in saliva content, as the current study did not show significant difference in the ability of their saliva in blood clotting using PT assay.

Conclusion

Three molecular weights were found to be closely related to known proteins from leech species by comparing gel electrophoresis data with a protein database. Additional proteins found in the saliva extract by gel electrophoresis may be bound to completely new bioactive proteins and peptides in the saliva extract, to modifications (isoforms) of existing proteins, or to a mixture of both. It has been found that the raw saliva secretions of both *H.verbana* and *H.orientalis* have anticoagulant activity. The anticoagulant activity of *H.verbana* was stronger than the anticoagulant activity of *H.orientalis*. The prothrombin time rates of *H.verbana* were higher than the prothrombin time rates of *H.orientalis*, although there were no significant differences between them.

4.6 Acknowledgements

Acknowledgment is extended to the staff of the Alhwya centres for studies, researches and training; Rafi center for chines medicine and Al-Huda Centre for Complementary medicine for their help in providing leech specimens.

4.7 Ethics approval

This research project was approved by Ethics Committee-College of Science, University of Baghdad (CSEC/1121/0089).

4.8 Conflict of interest statement

The authors have no conflicts of interest to be mentioned.

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