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A study on antioxidative and antimicrobial activities of saliva extract of Indonesian local leeches

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Abstract. Antioxidative and antimicrobial activities of saliva extract of Indonesian leeches were assessed. Leeches were obtained from Situgede Bogor, Sawangan Depok, and Sijunjung Regency, West Sumatra. A completely randomized design with three treatments and five replicates was used. Treatments consisted of three places of leech origins. Measurements were taken on the amount, total protein content, IC50 value, zone of inhibition to bacterial growth, and minimum inhibitory concentration of crude leech saliva extract (CLSE). Results showed that the amount of CLSE obtained was 3.696-4.633g/g BW and total protein content of CLSE was 48.303-64.742 µg/mg. The IC50 value was 47-108 µg/mg while that of ascorbic acid was 3.312 µg/mg. Antimicrobial activity was found to the growth of S. aureus but not to the growth of E. coli with a minimum inhibitory concentration at 2.472-4.528 µg/ml.

1. Introduction

Leech, a freshwater amphibian annelid, commonly has dark-colored body of 10 cm long. There are more than 600 species of leeches belonging to the genus *Hirudo*. Traditionally, leeches have been known and used since ancient times in India and China. This leech medicinal therapy developed well in the Arabian and European regions [1]. Leeching has regained its popularity since early twentieth century. Today, studies on leeches of *Hirudo medicinalis* and *Hirudo verbana* and the active substances in their saliva are extensively done in the USA and Europe. Active substances of leech saliva and leeching have been used in medicinal field. In Asia, *Hirudinaria manillensis* is the species commonly used in leeching and studies [2].

In Indonesia, leeching practices are spreading throughout the country. However, scientific studies on the potential of leech and its active substances for human and animal health is very limited. Meanwhile, recent studies in Malaysia revealed that saliva extract of Malaysian leeches had anticoagulative [3,4], antioxidative [5], antimicrobial [6], and anticancer [7] activities. These biological activities found in leech saliva extract are needed in control of various diseases including mastitis in dairy animals. Therefore, studies on biological activities of saliva extract of Indonesian local leeches deserve to be done. This study was conducted to assess the antioxidative and antimicrobial activities of saliva extract of leeches originating from some places in Indonesia.

2. Materials and methods

2.1. Site of study

The study was done at the Biochemistry, Physiology and Microbiology of Animal Nutrition Laboratory, Faculty of Animal Science, Bogor Agricultural University.

2.2. Leeches

Leeches were taken from three places, namely Situgede, Bogor where leeches were obtained from a river, Sawangan, Depok where leeches were obtained from a leech farm, and Sijunjung Regency, West Sumatra where leeches were obtained from a swamp area. Situgede leeches usually fed themselves by sucking blood from buffaloes which were commonly found to be bathed by their owners in the afternoon. Sawangan leeches were reared for the purpose of leeching practices. They were fed artificial feed. From each place, 1000 leeches were obtained and put in plastic ponds in the laboratory. They were left unfed for about two weeks before they were used as samples.

2.3. Leech saliva extraction

Leech saliva was extracted by using a method used by Abdualkader *et al*. [6] without killing the leech. Leeches were fed a phagostimulatory solution made of a mixture of 0.001 M arginin and 0.15 M sodium chloride solution which was heated at 37°C [8]. This solution was placed in an inverted funnel coated with a parafilm sheet. Leeches were allowed to suck the solution through the parafilm until they were satiated and detached themselves from the parafilm (Figure 1a). The weight of leeches before and after sucking the phagostimulatory solution was recorded.

Figure 1. Leech saliva extraction method

A satiated leech which detached itself from the parafilm was then placed in a small plastic bag (Figure 1b). The plastic bag with a leech in it was closely tied and put in a box containing ice cubes for 15-20 minutes until the leech got unconscious and vomitted all the solution it sucked (Figure 1c). The leech body was then carefully squeezed from posterior to anterior to take all the remaining sucked solution out (Figure 1d). Only clear uncolored liquid was collected and put in plastic containers. The milked leech was then put into warm water (37˚C) for 25-20 minutes until it got its consciousness back.

Collected liquid was centrifuged at 4˚C, 9000 rpm for 10 minutes. Supernatant obtained after centrifugation was refered to as crude leech saliva extract (CLSE). Only fresh CLSE was used in this study.

2.4. Estimation of total protein

Total protein content of CLSE was estimated by using a Bradford protein assay method using *bovine serum albumin* (BSA) as a standard [9]. Phagostimulatory solution was used a blank. A series of BSA standard and CLSE solutions were prepared. Absorbances (A595) of these solutions were measured with the existence of Bradford reagent.

2.5. Antioxidative activity

Antioxidative activity of CLSE was assessed by using a DPPH method [10] as used by [5]. In this antioxidative test, 1,1-diphenyl-2-picrylhydrazyl. (DPPH) was used as a free radical. Using this method, antioxidative activity of CLSE was measured based on its ability to catch hydrogen released from DPPH.

CLSE was diluted in methanolic solvent and the solution was referred to as 3 times CLSE concentration (3 CLSE). Three two-fold serial dilutions of methanolic CLSE were prepared first by dissolving 100 µL CLSE in 100 µL methanol. 100 µL of this methanolic CLSE solution was then dissolved in another 100 µL methanol. The same step was repeated once again until methanolic CLSE solutions with concentrations of 1:2, 1:4, and 1:8 were obtained. Then, into each tube methanol solution was added until a final volume of 300 µl was reached. Simultaneously, DPPH was diluted in methanol to make 0.002 M DPPH methanolic solution. 15 µl DPPH methanolic solution was added into each tube and after 15 minutes, A_{516} absorbance reading was done. Phagostimulatory solution was used as a negative control. As a control reading, 15 µl DPPH was diluted in 300 µl methanol and its A_{516} absorbance reading was done. The same procedures were used to prepare three two-fold serial dilutions of methanolic L-ascorbic acid (50 µg/ml) as positive control. Antioxidative activity was calculated by using the following formulation.

$$\% \text{ antioxidative activity} = \frac{Control\ absorbance - Sample\ absorbance}{Control\ absorbance} \times 100$$

The concentration of ascorbic acid and CLSE protein (µg/ml) needed to inhibit free radical activity of DPPH 50% (IC50) was estimated from the curve obtained by plotting % antioxidative activity to concentration.

2.6. Antimicrobial activity

Antimicrobial activity of CLSE was assessed by using a disc diffusion test and minimum inhibitory concentration test methods as described by [11].

2.6.1. Disc diffusion test

In this test, gram positive and gram negative bacteria were used. These gram positive and gram negative bacteria were *Staphylococcus aureus* (B2647) and *Escherichia coli* (B2743), respectively. Inocula of these bacteria were obtained from Bacteriology Laboratory of Indonesian Research Centre for Veterinary Science (IRCVS), Bogor. Tetracycline antibiotic (30 µg) and phagostimulatory solution were used as positive and negative controls.

Inoculum was prepared in a tube filled with 5 ml saline solution. The turbidity of the suspension was adjusted to McFarland standard 3.0 (9 x 10^8 CFU/ ml). This adjustment was done by placing the tubes containing inoculum and McFarland standard in front of a piece of white paper with black lines on it. Inoculum was used immediately before 18-24 hours after it was prepared.

Bacterial inoculum of 50 µl was added to a Mueller-Hinton agar (MHA) plate. The plate was left at room temperature so that the excess water favor could be absorbed by the agar medium. This was done by placing the plate in an incubator and the cover of the plate was left ajar. MHA should be 4 mm thick. After the medium was solidified, four wells with diameter of 0.5 cm were made. CLSE solution was injected into two wells and tetracycline and phagostimulatory solution were injected into the other two wells. The plate was then incubated at 37 °C for 18-24 hours. Zone of inhibition formed around each well was measured by using a caliper.

2.6.2. Minimum inhibitory concentration test.

Minimum inhibitory concentration test was performed to determine minimum concentration of CLSE which could inhibit the growth of bacterium isolate. The test was performed by referring to the procedures described by [11].

A two-fold serial dilutions of CLSE solution was prepared in 96-well microtiter plates. Mueller-Hinton Broth of 100 µl was placed into eight wells from the first to the eleventh columns of 96-well

microtiter plate. CLSE solution of 100 µl was added into ten (CLSE of each treatment was added into two adjacent wells) wells containing the broth in the first row. Dilution was done by vertically transfering 100 µl aliquot from ten wells in the first row to ten wells in the second row. Similar procedures were applied for the six wells in row three to the last row. Solution containing bacterial inoculum of 10 µl (10^7 *CFU*/ml) was added into each well in 10 columns. Wells in the eleventh column was left without bacterial inoculum and used as control. Microtiter plates were covered and incubated at 37ºC for 24 hours.

After 24 hour incubation, the plates were taken out from the incubator. No contamination should be found in the purity plate and there should be a minimal growth of 2 mm in the positive control well and negative control well should be clean. Afterall, minimum inhibitory concentration of CLSE could be determined.

2.7. Statistical analysis

Measurement on antioxudative and antimicrobial activities were done to Indonesian local leeches originating from three places, namely 1) Situgede Village, Bogor, where leeches were obtained from a river, 2) Nagari Kampung Dalam, Lubuk Tarok District, Sijunjung Regency, West Sumatra where leeches were obtained from a swamp area, and 3) Bedahan Village, Sawangan District, Depok where leeches were obtained from a leech farm.

A completely randomized design was used. Places of leech origins were used as treatments and five replicates were allocated into each treatment. Data were subjected to an analysis of variance and a Duncan test by referring to the procedures of [12].

3. Results and discussion

3.1. Extraction of leech saliva and estimation of its total protein

One-hundred-fifty leeches with an average initial body weight (BW) of 1.24 ± 0.62 g/head were used. The amount of CLSE obtained and its protein content are listed in Table 1. No difference was found (P>0.05) in the amount of CLSE obtained. No difference was either found in total protein contents of CLSE.

Table 1. Amount and total protein content of CLSE (±SEM)

Leech Origin	Amount (g/g BW)	Total Protein (µg/g)
Situgede	3.854 ± 0.465	48.303 ± 4.838
Sawangan	4.633 ± 0.371	49.439 ± 7.515
West Sumatra	3.696 ± 0.458	64.742 ± 6.017

Note: P>0.05, SEM: Standard Error of Mean

Before their saliva was milked, leeches were allowed to suck phagostimulatory solution until they were satiated and voluntarily detached themselves from the parafilm. Leeches were then made unconscious and vomit the solution they sucked. The vomitted solution was mixed with saliva. This was why, as listed in Table 1, the amount of CLSE obtained was higher than their initial BW. This was in line with the finding that in nature, a leech might be able to suck blood in the amount equals to 10 times of its initial BW [13].

Total protein contents of CLSE (48.303-64.742 µg/mg) obtained in this study were lower than those obtained by [6] (62.602-79.962 µg/ml) and [5] (78.753 µg/ml). These researchers used leeches from a river in Kuantan and a natural lake in Trengganu, Malaysia. Meanwhile, leeches from Pahang region [14] were found to have saliva extract with total protein range of 39-105 µg/ml with an average of 67.918 µg/ml.

3.2. Antioxidative activity

Antioxidative activities of CLSE in this study is listed in Table 2. IC50 (Inhibitory Concentration 50) defined as the concentration of tested substance causing the loss of DPPH activity by 50% was used as a parameter. The loss of DPPH activity as a free radical occurs as the tested substance donates a hydrogen (H) atom to DPPH [15] as illustrated in Figure 2.

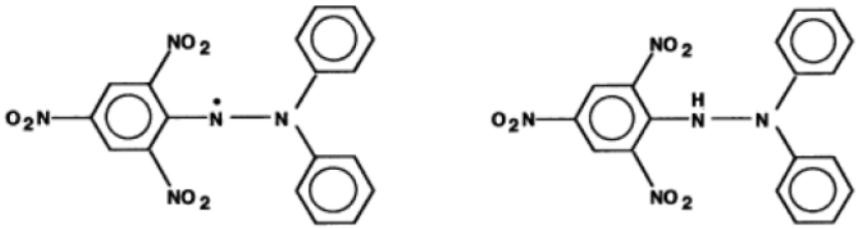

Figure 2. Molecular structure of DPPH as free radical (left) and non radical (right)

Table 2. IC50 L-ascorbic acid and CLSE

Substrate	IC50 (µg/g) ± SEM
Situgede CLSE	108.048 ± 0.465
Sawangan CLSE	47.425 ± 10.252
Sumatera Barat CLSE	88.437 ± 5.413
L-ascorbic acid	3.312

Note: $P>0.05$, SEM: Standard Error of Mean

Results in this study showed that CLSE obtained from leeches of several places in Indonesia had some antioxidative activities. There was no different IC50 values of CLSE. However, there was a tendency ($P<0.10$) that Sawangan leeches produced CLSE with higher antioxidative capacity (47.425 µg/mg) than those from West Sumatra (88.437 µg/mg) and Situgede (108.048 µg/mg).

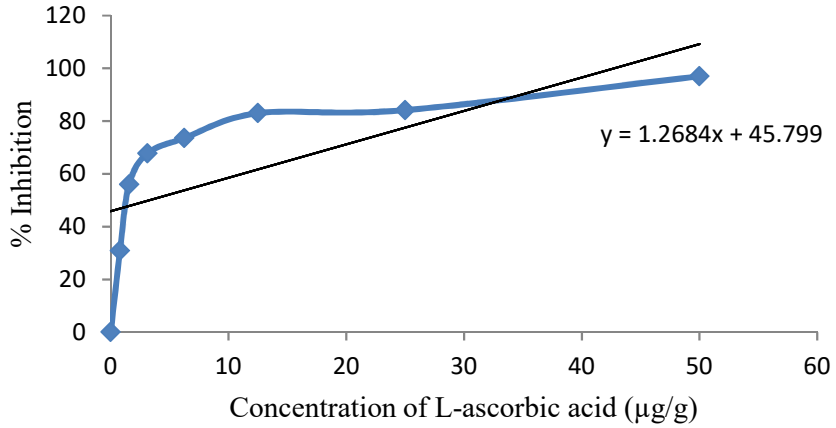

Figure 3. Curve of DPPH inhibition by L-ascorbic acid (N=8)

Vitamin C (L-ascorbic acid) was used as a positive control as this acid is a very strong alternative antioxidant. The IC50 value of ascorbic acid was needed as a comparation to the IC50 values obtained from CLSE to determine the strength of CLSE antioxidative capacity. The curve of DPPH activity inhibition is depicted in Figure 3. The regression equation obtained was used to determine the percentage of inhibition of the tested CLSE samples. Results showed that the antioxidative power of CLSE in this study was very low compared to that of the ascorbic acid. With IC50 value ranges of CLSE of 47-108 µg/mg and ascorbic acid of 3.312 µg/mg, the antioxidative activity capacities of CLSE were 3.07, 3.75, and 6.98% of that of the ascorbic acid for CLSEs of Situgede, Sumatera Barat, and Sawangan leeches, respectively.

Antioxidative activity of leech saliva extract was first reported by Malaysian researchers [5]. The CLSE they got from Malaysian local leeches had an IC50 value of 7.282 µg/ml and the IC50 value of the ascorbic acid they used was 5.803 µg/ml. A current study [16] revealed that saliva extract of

Nigerian leech, *Aliolimnatis michaelseni*, also had a high antioxidative capacity. The IC50 value of saliva extract of this leech was 8.169-8.670 µg/ml compared to 5.025 µg/ml of the ascorbic acid they used. These findings meant that saliva extract of Malaysian and Nigerian leeches respectively had about 80 and 58-62% the antioxidative capacity of ascorbic acid they used.

3.3. Antimicrobial activity

Results of disk diffusion test are listed in Table 3. There was no difference found in the inhibition zones of CLSE samples to the growth of *S. aureus*. No inhibition zone was detected to the growth of *E.coli*. These results were in contrast to the findings of [6] with their Malaysian leeches. They used fresh and lyophilized saliva extract obtained from leeches which were fasted for 14, 23, and 25 weeks. They found that only fresh saliva extract obtained from leeches fasted for 14 weeks had antimicrobial activity toward *E.coli* as indicated by an inhibition zone of 25 mm. In this study, fresh CLSE was not found to have an antimicrobial activity to the growth of *E. coli*.

Table 3. Antimicrobial activity of CLSE

Samples	Inhibition Zone (mm) ± SEM	
	*S.aureus**	*E.coli*
Situgede CLSE	2.54 ± 0.44	0
Sawangan CLSE	1.51 ± 0.41	0
West Sumatra CLSE	2.70 ± 0.76	0
Tetracycline (30 µg)	13.82	6.75
Phagostimulatory solution	0	0

Note: *: $P>0.05$, SEM: *Standard Error of Mean*

In this study, CLSE was found to have an antimicrobial activity to the growth of *S. aureus*. This was different from what was found in the study of Malaysian researchers. The fresh leech saliva extract they used did not show any inhibition zone to the growth of these bacteria. This difference might be caused by the different conditions of the environment from where the leeches originated.

Table 4. Minimum inhibitory concentration (MIC) of CLSE

Samples	MIC (µg/ml) ± SEM
Situgede CLSE	$4.528^{A} \pm 0.774$
Sawangan CLSE	$2.472^{B} \pm 0.376$
West Sumatra CLSE	$2.804^{B} \pm 0.189$

Note: Different superscripts indicate significant difference ($P<0.05$), SEM: *Standard Error of Mean*

Results of minimum inhibitory concentration (MIC) test are listed in Table 4. The test was done only on S. aureus inoculum as no antimicrobial activity was detected to the growth of *E. coli*. Results showed that CLSE obtained from Situgede leeches had the highest MIC. This indicated that CLSE obtained from Sawangan and West Sumatra leeches was stronger than that obtained from Situgede leeches in inhibiting the growth of *S. aureus*. However, the MIC figures found in this study were much lower than that (78.253 µg/ml) obtained by [6] from lyophilized leech saliva extract. This difference was suspected to be caused by different concentration of microbe inoculum used in the two studies. In both studies, concentration of microbe inoculum was determined by using an estimate according to MacFarland 3.0 standard (9 x 10^8 CFU/ml).

4. Conclusions

CLSE of Indonesian local leeches had some antioxidative and antimicrobial activities The IC value was 47-108 µg/mg. Antimicrobial activity was found to the growth of *S. aureus* but not to the growth of *E. coli* with a minimum inhibitory concentration at 2.472-4.528 µg/ml.

A furher study is required to assess the effects of treatments on leeches before they are milked for their saliva. These treatments may be in the forms of leech feeding, leech starving before saliva extraction, and other kinds of treatment that might have effect on the quality of leech saliva extract, especially on its biological activities.

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