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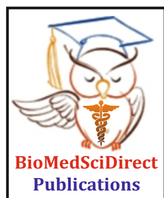

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Original Article

Preliminary test on the effectiveness of Protein Fraction of *Aedes aegypti* larvae against Bacteria Growth of *Escherichia coli*

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A R T I C L E I N F O

Keywords:
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Fractination
Fat

A B S T R A C T

Background & Objectives: Aedes aegypti is the main vector of dengue and chikungunya diseases. This mosquito is known to have proteins and fats that play a role in the process of life. This study aims to determine the fraction of protein and fat content of Aedes aegypti larvae and its ability to inhibit the growth of Escherichia coli bacteria. Methods: Fractionation of protein with ammonium sulfate at levels of 0-30%, 30-40%, 40-60%, and 60-80%. Determination of protein content by Lowry method and fat by standard laboratory operations. Test of effectiveness by measuring the diameter of inhibition zone around the paper disc. Result: The results of protein content measurements using 0.2 ml with 10 times dilution showed that 30-40 % of fraction contains highest levels of total protein 5.26 mg and lowest in the 0-30 % fraction 3.76 mg. The results of protein content measurements using 0.02 ml of sample extract with 100 times dilution showed that 40-60 % of fraction contains the highest total protein content 27.03 mg and lowest in the 0-30% fraction 4.88 mg. Fat content is only found in the protein fraction of 0-30% and 30-40 %. Result of effectiveness test showed that protein fraction of 30-40% and 60-80 % are bakteriocide. Fraction of 30-40 % has 0.6 % fat content and the fraction of 60-80 % have no fat content. Crude extract, fractions of 0-30 %, and 40-60 % are bacteriostatic. Protein fraction of 0-30 % has fat content of 0.8%, while the crude extract and fractions of 40-60 % do not have fat content. Interpretation & Conclusion: Protein fraction of Ae.aegypti showed inhibition to the growth of E. coli. Fat content does not give effect to bacteriostatic or bacteriocide of bioactive compounds of *Ae.aegypti.*

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1. Introduction

Dengue Haemorrhagic Fever, also called as DHF, is a tropical disease caused by the dengue virus and transmitted by the mosquito of Aedes aegypti Linnaeus as the main vector and Aedes albopictus Skuse as secondary vector[1, 2]. Dengue Haemorrhagic Fever (DHF) in Indonesia was reported for the first time in Surabaya and in Jakarta in 1968 and 1969. In Makassar city, dengue disease was firstly discovered in 1975 and then spread to several districts/cities in South Sulawesi[3].

Ae.aegypti is one of members of the family Culicidae which is a disease vector to human and animal. This mosquito is also known as the yellow fever mosquito, and is a domestic mosquito species that can live long at adult stage. This mosquito is also vector of chikungunya disease4, 5. Adult mosquito breeding supported by nutrition derived from natural herbs in the form of liquid/flower nectar for males and females in the form of protein obtained through the blood of the host. Proteins contained in the blood is used by female mosquitoes to maturing the eggs[6]. For larval stages, food in the form of tiny particles present in the water, including bacteria and fungi[4].

Larvae of *Ae. aegypti* was found living in densely populated settlements, both in urban and rural[7, 8] on various container such as water bath, water drums, jars, buckets[9], ponds, and tree holes[4], flower pots in the yard, and vases of flowers in the house[10]. Recently Ae. aegypti larvae found breeding in the well both indoor and outdoor[11, 12]. Adult mosquitoes suck the blood in the morning until late afternoon, now it was also found to be active in dusk until night to feed on blood[13,14].

Ae. aegypti is commonly found laying eggs in the container that contains Acinetobacter calcoaceticus, Enterobacter cloacae, Pseudomonas gladialli and Pseudomonas alcaligenes

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bacterium[15,16]. The presence of bacteria affects the percentage of egg laying of Ae. aegypti[17]. Various studies indicate that both larval and adult stages of Ae. aegypti contain bacteria in the gut. Types of bacteria found in the larval midgut, such as Escherichia coli and Aeromonas are known to influence the susceptibility of adult mosquitoes to dengue viruses[18, 20]. Bacteria presenting in the mosquito midgut is also known to stimulate immune activity that protects the mosquito from dengue viral infection19.

The results of the study of larval protein of Ae. aegypti indicates that larvae infected by Varvaria culicis and Edhazardia aedis parasites has [17] types of proteins which several proteins identified such as actin and ferritin are known to play a role to protect the host from patogen attack[22]. Antibacterial protein has several advantages that are very promising because it can be well accepted by the human body and cause fewer side effects because proteins are formed naturally, so the research began to be developed step towards treatment by using protein[23].

Various studies concerning of distribution of Ae. aegypti[10], larval mortality using extracts of neem and ylang plant24 as well as various research concerning these mosquitoes have been carried out. However, studies that determine the extent to which the effectiveness or inhibition of larval protein of Ae. aegypti against bacterial growth to date has not been done. This research is an effort and early step in finding the benefits of larval protein of Ae. aegypti and its ability to inhibit the growth of E. coli. This study is based on the idea to find a natural compound material of animal origin which may be used as raw material for medicine. There is a possibility that larvae protein of *Ae. aegypti* can be used as raw material for drugs later in the future.

Material and Methods

This is an experimental research which conducted in March to July, 2011. Samples of Aedes aegypti larvae taken from the village of Kampung Beru, Regency of Takalar and Pa'lanassang village, Makassar. Sampling sites is the place where most of mosquitos acquire and is the centre of laboratory stock. The samples obtained were washed with water and examined under a microscope to determine the species. They are then kept in petridish and stored in a freezer at -20C. Fractionation of larvae protein of *Ae.aegypti* is done in the Laboratory of Biochemistry Department of Chemicals and the test of effectiveness is conducted in the Laboratory of Microbiology Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar.

1. **Material research :** 50 g Aedes aegypti larva, Escherichia coli bacteria, Agar Nutrient (NA) medium, Muller Hinton Agar (MHA), Buffer A (0,1 M Tris HCl pH 8,3, 0,01 M CaC_{l2}, *B* merkaptoetanol 1 %, Triton-X 100 0,5 %), Buffer B (0,1 M Tris HCl pH 8,3, 0,2 M NaCl 0,01 M CaC_{l2}), Buffer C (0,01 M Tris HCl pH 8,3, 0,2 M NaCl 0,01 M CaC_{l2}), lowry A (folin, aquades), lowry B (Na_2CO_3 2 %, NaOH 0,1 N, $CuSO_4$ 1 %, $NaKC_4H6OH.4H_2O$), aquadest, aluminium foil, Bovine Serum Albumin (BSA).

1. **Preparation of sample** includes the preparation of crude extract of larval protein of Ae. aegypti and E. coli bacteria. Crude extract is done by smoothing the larva of Ae. aegypti by using mortar then added with buffer A. After being mixed, it was filtered with a Buchner funnel to produce a filtrate/crude extract. Preparation of bacteria by rejuvenating the bacteria25 through inoculating E. coli from pure cultures (1 loop) and scratched on oblique medium of Nutrient Agar (NA). Bacterial culture on oblique agar is incubated at 37°C for 15 hours. Preparation of bacterial suspension for test is done by taking each of the E. coli as much as 1 Ose then suspended in sterile distilled water solution. Dilutions of the bacterial suspension up to 25% transmittance of the blank solution of sterile distilled water at a wavelength of 520 nm is then conducted. Control solution, as positive control, antibiotic ampicillin trihydrate (0.04 g) and Bovine Serum Albumin (BSA) as negative control (0.01 mg/ml.) are used. Preparation of paper disc for test the effectiveness, paper discs (= 0.5 cm) as much as 7 sheets are poured with 0.2 ml of protein fraction 0-30 % (J1) , protein fraction 30-40 % (J2), fraction protein 40-60 % (J3), protein fraction 60-80 % (J4) , crude extract (J5), BSA as negative control (K-), ampicillin trihydrate as positive control (K+) .

2. **Fractination of Protein and Dialysis**

Crude extract is fractionated and dialyzed. Protein fractionation is performed using ammonium sulfate with saturation levels of 0 – 30 % (F1), 30 – 40 (F2), 40 – 60 % (F3), 60 – 80 % (F4). Deposition results of the fractionation of each ammonium sulfate saturation levels are put in a cellophane bag and further dialyzed with buffer B and then with buffer C. Cellophane that has been filled with the protein fraction is put into a beaker containing a solution of buffer B and then stirred with a magnetic stirrer.

The dialysis process was continued by replacing the buffer solution B with buffer C until the buffer solution become colorless again. Dialysis process is performed to purify the protein from small molecules and minerals that are not needed. To determine the protein content of each fraction, Lowry method is applied by using serum albumin Bovin (BSA) as a standard solution. We then measured the fat content of each protein fraction and crude extract. The final stage is to test the effectiveness of the protein fraction of larval Ae . aegypti and crude extract towards the growth of E. coli bacteria.

3. **Test of effectiveness** is conducted in vitro by agar diffusion method using paper disc. Suspension bacterial test of transmittance 25% put in a petridish as much as 1 ml, then added with MHA sterile medium (which is cooled at a temperature of 40C - 45C) as much as 20 ml aseptically and homogenized. After that, as much as 7 sheets of paper disc are placed aseptically with sterile forceps on the surface of medium with the distance from one to another disc is 2-3 cm from the edge of the petridish. Furthermore, it is incubated at 37°C for 12 hours, 24 hours, 36 hours, and 48 hours. Observations were made by measuring the diameter of bacterial growth inhibition around the paper discs using calipers. To see the ability of the protein compounds of Ae. aegypti in inhibiting the growth of E. Coli, measurement is performed in the incubation period of 12 hours, 24 hours, 36 hours and 48 hours.

Result

The results of the content of crude protein extract of Ae. aegypti larvae, the protein fraction of 0-30%, 30-40%, 40-60%, 60-80% and fat as well as the inhibitory ability against the growth of *E. coli* bacteria are presented as follows:

1. The content of protein and fat of Aedes aegypti larva

The content of protein extract of *Ae . aegypti* larvae is done by determining the maximum uptake (maximum λ), the absorbance of standard solution (BSA) and the equation of the calibration curve standard solutions. The results of measurements of maximum absorption (maximum λ) are presented in Table 1.

Table 1 shows that the maximum λ at BSA concentration of 0.06 mg / ml was 690 nm.

The results of measurements of the absorbance of standard solutions are presented in Table 2. Table 2 shows that the absorbance of standard solution concentration (BSA) increases due to increasing concentration of the standard solution.

The results of the determination of the calibration curve equation is presented in Figure 1. Based on the Figure 1, the regression of standard solution with line equation : $Y = 3.225 x + 0.110$ is known.

The results of protein concentration measurements using 0.2 ml sample extract with 10 times dilution are presented in Table 3. Table 3 shows that the highest of total protein fractions is obtained from 30-40 % (5.26 mg) and the lowest fraction is from 0-30 % (3.76 mg) .

The results of protein concentration measurements using 0.02 ml of sample extract with 100 times dilution are presented in Table 4. Table 4 shows that the highest of total protein fraction is obtained from 40-60 % (27.03 mg) and the lowest fraction is from 0-30 % (4.88 mg). The volume of the sample with the smallest value of 0.02 ml shows that the protein level is greater than 0.2 ml of sample volume.

Fat measurement results are presented in Table 5. Table 5 shows that the fat content is only found in the protein fraction contained 0-30 % and 30-40 % .

1. Test of effectiviness of protein extract and fat of *Aedes aegypti* againt the growth of *Escherichia coli*

Measurement of the effectiveness of Ae. aegypti extracts against E. coli is seen through the presence of a clear zone (zone of inhibition) around the paper disc. The result of the effectiveness test is presented in Table 6 and Figure 1. Table 6 shows that the highest average of inhibition zone for incubation period of 24 hours is shown by the crude extract (J5) (11.46 mm), and the lowest fraction of 0-30 % (J1) (6.16 mm). The highest average of inhibition zone for incubation period of 48 hours is shown by the crude extract (J5) (11.70 mm), and lowest in the 40-60 % of fraction (J3) (6.31 mm). The highest average of inhibition zone for incubation period of 72 hours is shown in the crude extract (J5) (11.43 mm), and the lowest fraction of 40-60 % (J3) (5.85 mm). The measurement results indicate that the negative control (BSA) (K-) showed no inhibition zone, whereas the positive control (ampicillin) (K+) in the incubation period of 24 hours showed inhibition zone of 10.25 mm, further decline due to incubation period.

Protein fractions of 0-30 % (J1) showed an increasing and decreasing of average of inhibition zone during in the incubation period of 24 to 72 hours. Similarly, the crude extract (J5) is also increased and decreased in terms of average inhibition zone at incubation period of 24-hour of 11,46 mm, at 48-hour incubation of 11.70 mm, and at 72 hours incubation of 11.43 mm. The protein fraction of 30-40 % (J2) shows an increase in the average of inhibition zone during incubation period. At the 24-hour incubation, inhibition zone is 6.20 mm, incubation period of 48-hours is 6.55 mm, and incubation period 72-hour is 7.00 mm. Similarly, the fraction of 60–80% (J4) shows the increase of inhibition zone average during incubation period. The 24-hour incubation period is 7,91 mm, period 48-hours is 8,08 mm, and 72-hours incubation is 9,50 mm. Otherwise, the protein fraction of 40 – 60% (J3) shows a decrease of average inhibition zone due to the increase of incubation period.

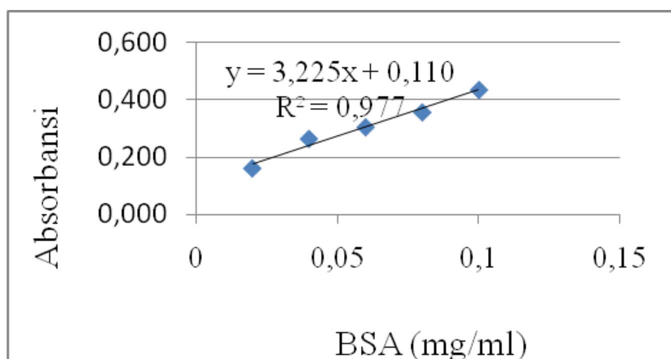
The result of effectiveness test shows that protein extracts prove properties of bacteriocide is protein fraction of 30-40 % (J2) and the fraction of 60-80 % (J4). Fraction of 30-40 % (J2) has 0.6 % of fat content and the fraction of 60-80 % (J4) has no fat content. The highest fat content of protein fractions is indicated by 0-30 % (J1) where the fraction of 0-30 % (J1) is bacteriostatic only.

Wattimena (1991)29 states that if the inhibition zone formed after an incubation period of 48 hours remains the same or diameter of the inhibition zone is increased, it can be said that the extract are bacteriocide. Whereas if the inhibition zone formed on the 24-hour incubation period is covered back with population of bacteria and the decrease of inhibition zone appears at 48-hour incubation period, it can be said that the extract is bacteriostatic. An active compound is said to be bacteriostatic if the active compound efficaciously inhibit microbial growth but does not kill microbes.

Table 1. Maximum absorbance (λ maximum) at standard solution of absorbance of concentration of 0,06 mg/ml

Wave length (nm	Absorbace (A)
650	0,274
655	0,276
660	0,278
665	0,281
670	0,282
675	0,282
680	0,282
685	0,284
690	0,285(λ max)
695	0,284
700	0,282

Table 2: Knowledge and practices regarding menstruation in the study population.



Graphic 1. Calibration curve of protein standard solution (BSA) in maximum wave length (ɹmax) of 690 nm

Table 2. Absorbance of solution (BSA) in maximum wave length (ɹmaks) at 690 nm

Standard of concentration [BSA] (mg/ml)	Absorbance (A)
0,02	0,160
0,04	0,264
0,06	0,305
0,08	0,357
0,1	0,436

Table 3. Protein level with 0,2 ml of samples (10 times dilution)

Protein fraction	$Y_1 = A$	X= mg/ml	Dilution factor	Total of protein (mg)
0-30%	1,334	0,376	10	3,76
30-40%	1,823	0,526	10	5,26
40-60%	1,773	0,511	10	5,10
60-80%	1,772	0,495	10	4,95
Raw extract	1,770	0,510	10	5,10

Note: Y_1 = absorbance, X = concentration

Table 4. Level of Protein with 0,02 ml of samples (100 times dilution)

Protein fraction	$Y_2 = A$	X = mg/ml	Dilution factor	Total of Protein (mg)
0-30%	0,269	0,049	100	4,88
30-40%	0,352	0,074	100	7,43
40-60%	0,990	0,270	100	27,03
60-80%	0,589	0,150	100	14,99
Raw extract	0,889	0,242	100	24,24

Note : Y_1 = absorbance, X = concentration

Table 5. Fat level of Aedes aegypti

Type of Sample	a	b	Fat level (%)
Fraksi 0-30	10,150	10,154	0,8
30-40%	13,597	13,600	0,6
40-60%	9,852	9,862	0
60-80%	11,485	11,485	0
Raw extract	11,595	11,595	0

Note: a = weight of empty bottle, b = weight of sample after being in the oven

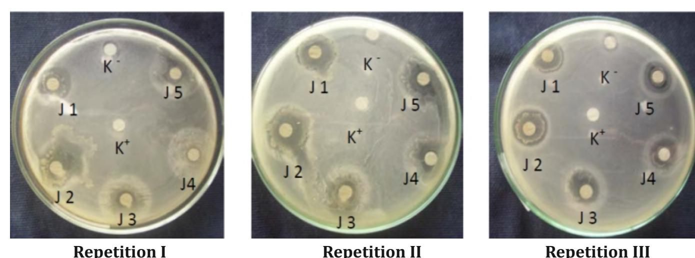
Table 6. Average inhibition zone of protein fraction of *Aedes aegypti* and control to the growth of *Escherichia coli* (mm)

Material of test	Average inhibition zone (mm) in 3-time repetitions (hour)		
	24 hour	48 hours	72 hour
Negative Control (K^-)	0	0	0
Positive Control (K^+)	10,25	7,85	7,66
Fraction of 0-30% (J1)	6,16	7,80	6,43
Fraction of 30-40% (J2)	6,20	6,55	7,00
Fraction of 40-60% (J3)	6,35	6,31	5,85
Fraction of 60-80% (J4)	7,91	8,08	9,50
Raw extract (J5)	11,46	11,70	11,43

Note: K^-: Bovine serum albumin (BSA), K^+: ampicillin

Figure 1. Average inhibitory zone of protein fraction and larval crude extract of *Aedes aegypti* towards the growth of *Escherichia coli* bacteria

Note: K- : negative control (BSA), K+: positive control (Ampicillin), J1: fraction 0 – 30 %, J2: fraction 30 – 40%, J3: fraction 40 – 60%, J4: fraction 60 – 80%, J5: crude extract



Discussion

1. Effect of extract volume of *Aedes aegypti* to the level of protein

The results showed that the larvae of *Ae . aegypti* has a protein content. Sample volume of 0.2 ml and 0.02 ml affect the amount of total protein content. Differences in levels of the protein may be caused by sample turbidity. The more turbid the sample, the less light absorption of the sample and thus the absorbance shown by a spectrophotometer is too small. Conversely, the bright sample will facilitate the absorption of light so that the absorbance of the sample

is easily legible. Effect of sample thickness affects the absorbed light. It is in line with the law of Lambert beer26. The absorbed light is measured as absorbance (A) while the scattered light is measured as transmittance (T) which expressed by Lambert - beer law saying that the amount of visible light radiation (ultraviolet, infrared, etc.) that is absorbed or transmitted by a solution is an exponential function of concentration and thickness solution.

Food larval of Ae. aegypti in the form of tiny particles contained in the water including bacteria, fungi and protozoa4. Larvae obtains protein from the substances contained in the water. Proteins contained in the larval body is from the mother mosquito obtained from the blood of the host in this case is a human as a source of food. Larval development is affected by the formation of the protein, fat, and carbohydrate27. The results shows that feeding with valine, leucine, isoleucine, phenylalanine, histidine, tryptophan, threonine, and lysine methionin will stimulate the growth of larvae26.

2. The result of effectiveness of protein extract of *Aedes aegypti* larva towards the growth of *Escherichia coli*

The results of effectiveness shows that crude extract and protein fractions of Ae. aegypti larvae are able to inhibit the growth of E. coli . Each test sample of both crude extracts and protein fractions during the incubation period of 3 x 24 hours shows a zone of inhibition, except in the negative control which shows no inhibition zone. Capuccino and Sherman (1992)30 state that if the diameter of the inhibition zone indicated by a compound/antibiotics for > 14 mm then it is said to be effective in inhibiting the growth of bacteria, if the inhibition zone diameters of 10-11 mm are obtained it is said to be less effective, and if the diameter inhibition zone ≤ 9 mm obtained the compounds/ antibiotics are not effective in inhibiting the growth of bacteria.

The results of this study indicate that the fractions of 0-30 % (J1) , 30-40 % (J2) , and 40-60 % (J3) are not effective in inhibiting the growth of bacteria while the crude extract is less effective in inhibiting the growth of bacteria which makes it less effective if used as antibiotics. Protein fractions that are less/not effective in inhibiting the growth of bacteria may be caused by the chemical activity of the substance itself, the diffusion of the active ingredient at a slow medium and low concentrations of active substances, and thus the fraction can not optimally inhibit bacteria30. The size of the inhibition zone formed may be influenced by the levels of the active substance in the form of proteins in each fraction and crude extract. Because the number of extracted larvae sample is not too much (50 g), it is possible if the crude extract and proteins fraction will show higher inhibitory response (bacteriocide) if the number of larval samples is also increased.

Bioactivity benchmark indicated by the size of the inhibition zone diameter of a compound/antibiotic is influenced by several factors including: the rate of growth of microorganisms, resistance of bacteria against bioactive substance, the active substance concentration, amount of inoculum bacteria/bacterial density test as well as the concentration of an antimicrobial contained in the sampel31. This is a natural mechanism of bacterial cells in maintaining their life32. During the incubation period, fraction that has an increasing inhibition zone is the fraction of 30-40 % (J2) and the fraction of 60-80 % (J4). This indicates that the fraction of 30-40 % (J2) and the fraction of 60-80 % (J4) is bacteriocide. According to Wattimena (1991)29 if the inhibition zone formed after an incubation period of 48 hours remains the same or an expansion in the diameter of the inhibition zone of the extract appear, it can be said to be bacteriocide. Crude extract (J5), the fraction of 0-30 % (J1), 40-60 % (J3) shows an increase and decrease of inhibition zone during incubation period and around the paper disc overgrown with bacteria. According to Wattimena (1991) if the inhibition zone formed at the 24-hour incubation period covered back with bacteria, diameter of inhibition zone is reduced to 48-hour incubation period, it can be said that the extract is bacteriostatic. An active compound is said to be bacteriostatic if the active compound efficaciously inhibit microbial growth but does not kill mikroba29.

Protein fraction that shows bacteriocide properties are fractions of 30-40 % (J2) and 60-80 % (J4). The highest protein content was obtained from fraction of 40-60 % (J3) but this fraction shows only bacteriostatic effect. This shows that the size of the protein content does not determine whether or not a substance effective influences the growth of bacteria, but may be influenced by the levels of the active substance in the form of protein type in each fraction and crude extract. When viewed from the size of the fat content contained in this Ae.aegypti larvae samples, it can be said that the fat content does not affect bacteriocide or bacteriostatic properties of an active compound.

The results of this study indicate that the larvae of Ae. aegypti has a protein content that shows inhibitory response to the growth of E. coli but can not be used as raw material for medicine/antibiotics given that barrier zone that is displayed is not too large. However, further research is needed to re-examine the content and type of the protein fraction in order to achieve a maximal result.

Conclusion

Based on the research, it can be concluded that:

1. Extract of Aedes aegypti larvae contains proteins that shows inhibition to the growth of Escherichia coli bacteria although it is not effectively used as an antibiotic because the diameter of the barriers obtained is <9 mm.

2. Protein fraction of 30-40%, 60-80% are bacteriocide while crude extract, 0-30%, and the fraction of 40-60% are bacteriostatic.

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