Antibacterial and Hemolytic Activity of the Coelomic Fluid of *Dendrobaena veneta* (Oligochaeta, Lumbricidae) Living in Different Localities

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Abstract

Earthworms are able to protect themselves against invading microorganisms through their immune systems. Although there are many studies about defense systems of *Dendrobaena veneta*, this is the first report focusing on the immune properties of earthworms belonging to the same species, which live in different localities. Thus, it was aimed to compare antibacterial and hemolytic activities of earthworms collected from different localities of Istanbul (Vezneciler, Süleymaniye and Beykoz). Both activities were studied with dilution and agar diffusion techniques. In antibacterial activity assays with dilution technique, it was observed that all suspensions of coelomic fluid of *D. veneta* living in Beykoz had significantly strong activity, while no results could be determined in diffusion assays. Nevertheless, in dilution assays for determination of hemolytic activity, coelomic fluid of earthworms living in Süleymaniye was the most effective group. In dilution assays, hemolytic activity were in the order of (S) > (V) > (B) for human erythrocytes. As a conclusion, coelomic fluid of Beykoz earthworms was a potential agent, which can be used as an alternative drug, since this coelomic fluid was effective against bacteria but not on erythrocytes.

Keyworlds: *Dendrobaena venata*, earthworms, coelomic fluid, antibacterial activity, hemolytic activity.
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Introduction

Earthworms have been living with the aid of their defense system since the early phases of evolution, although they always face the invasion of pathogen microorganisms in their environments (Lassegues et al. 1981; Engelmann et al. 2004). The studies which have been continued for about 50 years showed that earthworms have humoral and cellular immunity mechanisms (Kauschke et al. 1997; Beschin et al. 1998; Hanusova et al. 1999; Bilej et al. 2001; Field et al. 2004). It has been found that coelomic fluid of the earthworms contains more than 40 proteins and exhibits several biological activities as follows: cytolytic, proteolytic, antimicrobial, hemolytic, hemagglutinating, tumorolytic, mitogenic activities (Çotuk and Dales 1984; Lange et al. 1997; Lange et al. 1999; Cooper and Roch 2003).

These investigations with earthworms have usually intensified with *Eisenia foetida, Lumbricus terrestris* and *Dendrobaena venata* (Dales and Kalaç 1992; Milochau et al. 1997; Eue et al. 1998; Furlong et al. 2002; Kalaç et al. 2002; Koening et al. 2003). The hemolytic action of coelomic fluid of *E. foetida* was first defined by Du Pasquier and Duprat. They demonstrated that hemolytic factor was active against sheep red blood cell and various other vertebrate erythrocytes (Du Pasquier and Duprat 1968). Lassegues et al. (1981) found that this hemolytic factor also inhibited the growth of different bacterial species which were isolated from nature. They showed that these

bacteria antigens were common with sheep red cells.

The coelomic fluid of *Eisenia foetida andrei* was demonstrated to possess an antimicrobial activity against *Aeromonas hydrophila* and *Bacillus megaterium* which are known as earthworm pathogens (Valembois et al. 1982; Pan et al. 2003). Afterwards, Milochau et al. (1997) obtained two proteins, named Fetidins, from dialyzed coelomic fluid of earthworms and confirmed that this antibacterial activity was due to fetidins. Cho et al. (1998) found that *Lumbricus rubellus* also has two antibacterial agents named Lumbricin 1 and Lumbricin 2. Recently, two types of antibacterial factors which include lysozyme-like molecules with hemolytic activity as well as a pattern recognition protein named coelomic cytolytic factor (CCF) have been identified in *Eisenia foetida* earthworms (Kohlerova et al. 2004). Bruhn et al. (2006) stated that lysenin which was a different protein of *Eisenia foetida* and lysenin-like proteins had several cytolytic activities which exerted hemolytic, antibacterial and membrane-permeabilizing properties.

Since, previous studies suggested that environmental factors can change the chemical content and quantity of coelomic fluid of earthworms, it was aimed to test and compare the antibacterial and hemolytic effects of coelomic fluids of *D. veneta*, which were collected from three different districts of Istanbul: Vezneciler, Süleymaniye and Beykoz.

Material And Methods

Earthworms and harvesting of coelomic fluid

Dendrobaena venata was collected from three different localities in Istanbul: Vezneciler, Süleymaniye and Beykoz (Fig. 1). After the collection, three animal groups were separately washed with Holtfreter's earthworm saline solution and dried on Whatman paper. Coelomic fluid from 20 earthworms was harvested by mild electrical stimulation (5V) and pooled in polypropylene centrifuge tubes. Following the harvesting, coelomic fluids were centrifuged immediately at 10.000 rpm for 15 minutes. Cell free supernatant was then passed through a 0.2 μm-pore size Millipore membrane filter. The sterile supernatants were stored at $^{-}20^{o}$C until used.

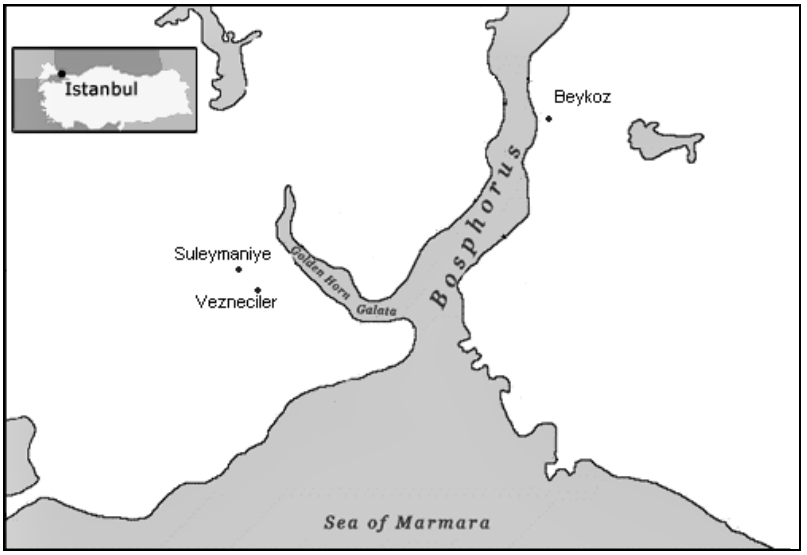

Figure 1. Earthworm harvesting localities in Istanbul.

Test organisms

Test bacteria were isolated from earthworms' environment and identified by used API test kits (BioMérieux, Marcy-l' Etoile, France) according to Bergey's Manual (9th Edition, 1994) (Table 1). All bacteria were stored at -20ºC in appropriate buffer/glycerol suspension until used in antibacterial activity assays.

Determination of antibacterial activity

The antibacterial effect of coelomic fluids of earthworms which were collected three localities [which were named according to earthworm derivation area Vezneciler (V), Süleymaniye (S), and Beykoz (B)] was determined by using microtiter plates. This was performed through dilution and agar diffusion assays. In dilution assays, the test organisms were prepared in physiological salt solution to a McFarland 0.5 Standard [approximately 1×10^8 colony forming units per ml (cfu/ml)] and was diluted 1:10 (v/v) to 1×10^7 cfu/ml in Brain Hearth Infusion (BHI) broth medium. The supernatants of coelomic fluids were diluted at ratios of 1:10; 1:100; 1:250 and 1:500 in Holtfreter's earthworm saline solution. Bacteria suspensions were mixed with non-diluted and diluted coelomic fluids at a ratio of 1:1 (v/v). These aliquots were incubated at 37ºC for 16 hours. After incubation, bacterial counts of all wells were determined by spread plate technique on BHI agar. After 24 hours of incubation at 37ºC viable counts were determined as cfu/ml using a Colony Counter Device (åCOLyte Super Colony Counter, Synbiosis). Bacterial suspensions in Holtfreter's earthworm saline solution (1:1, v/v) were used as control. Coelomic fluid of the earthworms was evaluated as efficient when bacterial inhibition was at least 50%.

In diffusion assay, the test organisms were prepared in physiological salt solution to a McFarland 0.5 Standard [approximately 1×10^8 colony forming units per ml (cfu/ml)] and spread on BHI agar. Then same suspensions of dilution assay were dropped on inoculated agar and plates were incubated at 37ºC for overnight. After the incubation, diameters of inhibition zones were measured.

Determination of hemolytic activity

Hemolytic activity was carried out with rabbit, sheep and human red blood cells. After the cells were washed three times by centrifugation (2000 rpm, 4ºC and 15 minutes) with phosphate buffered salt (PBS) solution, erythrocytes were suspended to a final concentration of 2%. Coelomic fluids were diluted serially two-fold in Holtfreter's earthworm saline solution. Coelomic fluid suspensions and erythrocyte suspensions were mixed (1:1, v/v) and incubated 2 hours at 25ºC. After the incubation period, all aliquots were collected and centrifuged (2000 rpm, 4ºC and 10 minutes) for the separation of free hemoglobin from cell fragment. Following the centrifugation step, supernatants were collected and hemolyse quantities were determined spectrophotometrically at 405 nm. In all spectrophotometric measurements, coelomic fluid suspensions were used as a blank, whereas 0.003% saponin suspension in sterile distilled water and the mixture of Holtfreter's earthworm saline solution:erythrocyte suspensions [1:1 (v/v) ratio] were used as positive and negative controls, respectively. Percentage of hemolysis was calculated by using the following formula:

[(absorbance of sample) / (absorbance of the total hemolysis)] × 100 = % hemolysis

Total hemolysis was obtained by adding saponin.

Hemolytic activity was also carried out with blood agar diffusion method. In this method, 6 mm diameter wells were cut from an agarose gel which contains 1% erythrocytes, and then wells were filled with 20 µl of the same suspensions as spectrophotometric hemolyse assay (dilution assay). After the incubation at 25ºC for 24 hours, diameters of hemolyse zones were measured.

Statistical analysis

All experiments were performed in quadruplicate. Paired student's t-test using SPSS software was performed to evaluate the significance of the data. Differences were considered significant when $P<0.05$.

Results

Determination of the antibacterial activity

Antibacterial activity of coelomic fluids of earthworms was performed by dilution and agar diffusion assays against Gram negative and Gram positive bacteria (Table 1).

Table 1. List of test organisms

Bacteria species
Citrobacter freundii
Pantoea spp.
Enterobacter cloacae
Klebsiella terrigena
Klebsiella pneumophila
Bacillus pumilus
Acinetobacter calcoaceticus
Bacillus megaterium
Bacillus cereus
Chryseomonas luteola
Stenotrophomonas maltophilia
Serratia marcescens

Non-diluted coelomic fluid of (V) displayed antibacterial activity against a broad spectrum of bacteria, including *Citrobacter freundii*, *Pantoea spp.*, *Enterobacter cloacae*, *Klebsiella terrigena*, *K. pneumophila*, *Bacillus pumilus*, *B. megaterium*, *B. cereus*, *Chryseomonas luteola*, *Stenotrophomonas maltophilia* and *Serratia marcescens* (Table 2). Although, diluted (V) fluids was not effective as non-diluted (V) fluid, just *B. megaterium* strain was inhibited at least 50% ratio by all diluted (V) fluids.

The antibacterial activity of (S) was less effective than (V) (Table 2). Non-diluted (S) inhibited also nine bacteria species (*C. freundii*, *Pantoea spp.*, *E. cloacae*, *K. pneumophila*, *B. pumilus*, *B. megaterium*, *B. cereus*, *C. luteola* and *S. maltophilia*). Additionally, Non-diluted (B) was effective on 8 bacteria species (*C. freundii*, *E. cloacae*, *K. terrigena*, *K. pneumophila*, *B. pumilus*, *B. megaterium*, *S. maltophilia* and *S. marcescens*). Nevertheless, all diluted (1:10, 1:100, 1:250 and 1:500) and non-diluted (B) suspensions were effective on *C. freundii*, *B. megaterium* and *S. marcescens*, whereas all dilutions of (V) and (S) only inhibited *B. megaterium.* In these experiments, growth of *Acinetobacter calcoaceticus* was not inhibited by any coelomic fluid suspensions. The antibacterial activity of coelomic fluids of three earthworms groups could not be detected with diffusion method (data not shown).

Evaluation of hemolytic activity

To evaluate the hemolytic activity of coelomic fluids, sheep, human and rabbit erythrocytes were used. Coelomic fluid was evaluated as efficient when hemolysis was more than 50%. In these experiments, human, rabbit and sheep erythrocyte types showed different sensitivities to coelomic fluids (77.78, 66.67 and 14.81%, respectively). Rabbit and human erythrocytes were hemolyzed by all suspensions of S, whereas sheep erythrocytes were hemolyzed by only non-diluted (S) fluid at least 50% (Fig. 2). In addition to this, all suspensions of (B) were able to hemolysis only rabbit erythrocytes. Additionally, any of these vertebrates' erythrocytes were effectively hemolyzed by all (V) suspensions. Non-diluted and 1/2 to 1/128 (V) suspensions demolished human erythrocytes, whereas rabbit and sheep erythrocytes were hemolyzed only in non-diluted (V). According to these results, (S) had the strongest hemolytic activity, followed by (B) and (V).

Table 2. Efficacy of coelomic fluid of earthworms collected from 3 different localities on bacterial count ($1x10^7$ cfu/ml) using spread-plate technique.

Bacteria	Localities	Coelomic Fluids					Control
		Non-diluted	1:10	1:100	1:250	1:500	
Citrobacter freundii	Vezneciler	10[a]	57	52	143	166	36
	Süleymaniye	1.5[a]	7[a]	40	37	46	55
	Beykoz	12[a]	126[a]	113[a]	136[a]	147[a]	295
Pantoea spp.	Vezneciler	6[a]	17	17	19	15	28
	Süleymaniye	1.7[a]	20[a]	21[a]	21[a]	40	46
	Beykoz	5,5	6	6.7	8.8	13.8	7.4
Enterobacter cloacae	Vezneciler	0.9[a]	29	24	31	32	16.1
	Süleymaniye	0.1[a]	11[a]	21	22	24	30
	Beykoz	12.8[a]	25.5	25.5	25.7	25	34
Klebsiella terrigena	Vezneciler	5.5[a]	12[a]	15[a]	16[a]	27	43
	Süleymaniye	20	22	28	29	29	26
	Beykoz	40[a]	96	103.5	105	133	137
Klebsiella pneumophila	Vezneciler	7[a]	19[a]	22[a]	37[a]	59	80
	Süleymaniye	2.7[a]	20[a]	28	28	28	48
	Beykoz	0.4[a]	0.8[a]	7.9[a]	13.9	19.6	22.4
Bacillus pumilus	Vezneciler	0.02[a]	0.9	0.8	0.8	0.9	0.52
	Süleymaniye	0.2[a]	3[a]	13[a]	15[a]	24	36
	Beykoz	0.2[a]	0.3[a]	1	1	1	1.2
Acinetobacter calcoaceticus	Vezneciler	29	45	48	49	55.9	57.1
	Süleymaniye	33	34	51	51	79	63
	Beykoz	22	24.4	26.2	28.2	32	26.2
Bacillus megaterium	Vezneciler	0[a]	0.33[a]	0.27[a]	0.9[a]	0.9[a]	2.3
	Süleymaniye	0[a]	0,1[a]	6.7[a]	6[a]	7[a]	37
	Beykoz	0[a]	0,3[a]	0,2[a]	0.9[a]	3[a]	6.2
Bacillus cereus	Vezneciler	6.5[a]	18	20	25	26.9	33
	Süleymaniye	0.9[a]	16.4	16	18	18	31
	Beykoz	23.4	24.9	25.9	24.4	25.9	26.2
Chryseomonas luteola	Vezneciler	14[a]	16[a]	16[a]	22[a]	28	51
	Süleymaniye	17[a]	27.7	28	30	36	44.2
	Beykoz	19	20.9	20.9	23.7	24	24
Stenotrophomonas maltophila	Vezneciler	0[a]	3.8[a]	52	60	63.9	67
	Süleymaniye	0,1[a]	10.7[a]	11.5[a]	18	19.5	23
	Beykoz	1[a]	13[a]	28	30	27	32.5
Serratia marcescens	Vezneciler	1[a]	67[a]	153	164	167	178
	Süleymaniye	50	65	55	92	92	98
	Beykoz	82[a]	144[a]	145[a]	148[a]	169[a]	425

Repetition (n): 4
cfu : colony forming unit
a: activity is more than 50%
($P<0.05$)

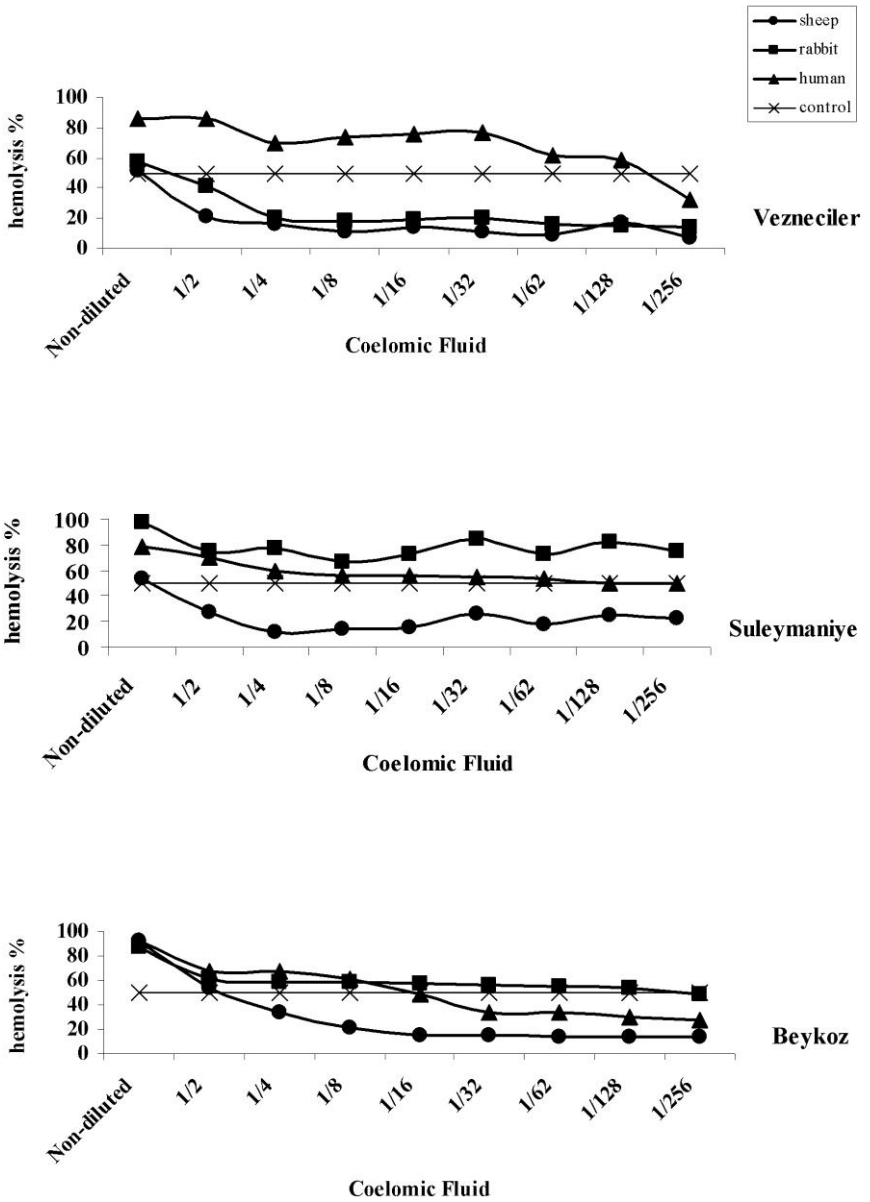

Figure 2. Hemolysis percentages of different vertebrate erythrocytes by coelomic fluids (up to the 50 line is effective doses)

Similar to diffusion assay which was carried on determination of antibacterial activity, these assays didn't show sufficient results with respect to dilution assays, except one result, which were carried out with (B) on the rabbit erythrocytes (Fig. 3).

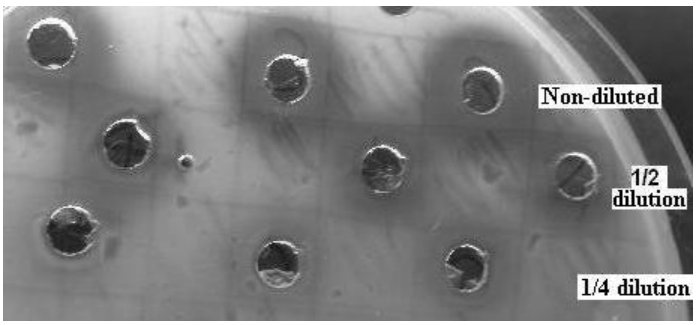

Figure 3. Hemolytic activity of (B) on Blood agar which contains 1% rabbit erythrocyte. Non-diluted, ½ and ¼ (B) dilutions produced hemolytic zones of different sizes.

Discussion

Earthworms' immunity consists of humoral and cellular components and works as specific and non-specific mechanisms which occur in coelomic fluid (Roch and Cooper 1991). The coelomic fluid of earthworms is known to contain a variety of humoral factors to combat potential pathogens that may migrate from environment into the body (Cho et al. 1998; Cooper 2002; Cooper and Roch 2003). The studies showed that some toxic agents such as Aroclor 1254 and cadmium were suppressive for earthworms' immunity (Roch and Cooper 1991; Sauve and Fournier 2003). When the relevant literatures were examined, it was found that there was no report focusing on immune properties of earthworms which live in different localities. Considering these, hemolytic and antibacterial differences of coelomic fluid of *D. veneta* which was collected from three different regions were studied.

When the antibacterial activities of non-diluted coelomic fluids were compared, (V) was the most effective on bacteria with 91.67%, while (B) was the least with 66.67%. However, when we compared the efficacy of all diluted fluids, (B) was the most effective against 3 bacteria (*C. freundii*, *B. megaterium* and *S. marcescens*) among 12 bacteria; on the other hand (V) and (S) inhibited only *B. megaterium*. Additionally, antibacterial activities of these three groups showed different results for each bacterium. For example, all dilutions of (B) were bactericidal for *S. marcescens*, while any (S) suspensions were effective on this bacterium. In contrast to this, all dilutions of (S) (except 1:500 dilution) were effective against *Pantoea spp.*, when (B) did not inhibit it. These results showed that earthworms which lived in Beykoz were different immunologically from other earthworms living in Vezneciler and Süleymaniye. However, earthworms which were collected from Vezneciler and Süleymaniye did not showed prominent differences from each other. In this regard, it can be say that earthworm populations in Vezneciler and Süleymaniye do not completely differentiate taxonomically.

Besides, while all coelomic fluid suspensions were effective on *B. megaterium*, none of them were effective on *A. calcoaceticus*. Valembois et al. (1983) found that the coelomic fluid of *E. foetida* was effective against *B. megaterium* like us. On the other hand, Çotuk and Dales (1984) showed that coelomic fluid of the *E. foetida* was ineffective against this bacterium. These differences in results support to our opinion about the differences of immune system of earthworms which leave different regions. Furthermore, these differences can also be originated because of different antibacterial substances in coelomic fluids. Moreover, the studies which focused on different earthworm species indicated that more than one antibacterial agent in coelomic fluid existed and their activities were different from each other (Dales and Kalaç 1992; Milochau et al. 1997; Cho et al. 1998).

Interestingly, it was also found that some coelomic fluids were stimulator for some bacteria. For example, *Pantoea spp.* growth was more than control in 1:250 and 1:500 (B) dilutions with 74.32 and 186.49%, respectively. Furthermore, some (S) (1:500) and (B) (1:250 and 1:500) dilutions supported the growth of *A. calcoaceticus*. Actually this result is quite reasonable, earthworms ingest large amounts of microorganisms with soil and some of them, which are not pathogenic for these animals (such as *Acinetobacter*), are inserted in excretion system except to kill and in fact these animals may stimulate growth of these bacteria (Dublier et al. 1995; Daane et al. 1999; Salanitro et al. 1997; Furlong et al. 2002; Horn et al. 2003; Hubalek et al. 2007; Andreoni and Gianfreda 2007). *B. pumilis*, which is suggested by U.S. Environmental Protection Agency (EPA) as a biological pesticide, showed different sensitivities to V, (S) and B. Growth of these bacteria was stimulated by (V) dilutions, while non-diluted V, all suspensions of (S) and (B) inhibited it. This result is important, because the quantity of these bacteria may be reduced or induced by earthworms in the application area. Therefore, before using these bacteria in environmental treatments, preliminary studies must be made to avoid unexpected results.

When hemolytic activities of coelomic fluids of three groups are investigated, regional differentiations come into prominence. (B) has the strongest activity on sheep erythrocytes, while (V) and (S) showed same effect. Hemolytic activity were in the order of (S) > (B) > (V) for rabbit erythrocytes, whilst (S) > (V) > (B) for human erythrocytes.

Interestingly, in evaluation of hemolytic activity with diffusion assays, only (B) showed hemolytic activity on rabbit erythrocytes. Moreover, no antibacterial activity of (V), (S) or (B) was detected in diffusion assays. From these results, it can be concluded that some antibacterial and hemolytic matters are not able to diffuse.

Some studies about hemolytic and antibacterial effect of coelomic fluids have exhibited that antigenic determinants on surface of sheep erythrocytes and bacteria which are inhibited by coelomic fluid are similar (Valembois et al. 1982; Eue et al. 1998). In our study, the weakest effect was observed against sheep erythrocytes for all coelomic fluids. Therefore, to understand the relationship between antibacterial and hemolytic activity, antigenic determinants of erythrocytes and bacteria should be investigated together.

As a conclusion, if low concentrations of coelomic fluid are effective against bacteria but not on vertebrate erythrocytes, it can be used as alternative drug. Moreover, earthworms have been used to treat upper respiratory tract infections, typhoid, and diarrheal pathogenic bacteria as a natural drug in Indonesia more than 50 years (Bakti et al. 2003). When all results of the present study are evaluated, (B) exhibits the best result. Because, (B) has high antibacterial activity with low concentrations while shows weak hemolytic activity on human erythrocytes with non-diluted and 1:2, 1:4 and 1:8 diluted suspensions. As a result of this, (B) can be used as an alternative drug for the treatment of *C. freundii, K. pneumophila, B. megaterium, S. maltophila* and *S. marcescens*, which are opportunistic pathogen for humans, while (V) can be used against only *B. megaterium* and (S) is not an alternative treatment agent against any of these bacteria.

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