

Identification of Immunity-Inducing Biological Compounds from Coelomic Fluid of *Eudrilus eugeniae*

Dr. S. Murugan^{1*}, Dr. S. Umamaheswari²

1*. Assistant Professor, Department of Microbiology, Kamaraj College (Autonomous), Thoothukudi-628003, Tamil Nadu, India, Affiliated to Manonmaniam Sundaranar University, Abishekapatti, Tirunelveli – 627012, Tamil Nadu, India.

Email: svkm.muns@gmail.com

2. Professor, Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli- 627012, Tamil Nadu, India.

Email: umamsu@gmail.com

Corresponding author: Dr. S. Murugan, svkm.muns@gmail.com

DOI: [https://doi.org/10.63001/tbs.2026.v21.i03.S.I\(3\).pp1507-1516](https://doi.org/10.63001/tbs.2026.v21.i03.S.I(3).pp1507-1516)

KEYWORDS

Earthworm; Coelomic fluid; *Pseudomonas aeruginosa*; HPLC; Erythrolytic activity

Article Info

Received:07.07.2026

Accepted:21.08.2026

Published: 04.09.2026

Abstract

The coelomic fluid of Annelids exerts numerous biological activities that are involved in effective defense mechanisms against invaders. The coelomic fluid of the earthworm contains more than 40 proteins and exhibits several biological activities. About 20µl of *Pseudomonas aeruginosa* culture inoculated broth were injected into earthworm and disturbed at 24 hours to obtain the coelomic fluid. The uninoculated broth alone served as the control. *P. aeruginosa* injected coelomic fluid exerted higher antibacterial, proteolytic and erythrolytic activity along with a newly expressed protein in HPLC. This concludes that when the appearance of newly expressed protein in the body cavity of earthworm automatically kills various foreign intruders.

Introduction

Invertebrates evince different immune mechanisms against environmental pathogens. Earthworms are relatively long living invertebrate organisms which persist of rich micro-organisms, fungi, and other potential pathogens. The success of the earthworms' survival depends on the conditions supported by efficient innate immune mechanisms based on both the components of the earthworm coelomic

fluid such as cellular activity and humoral activity of coelomocytes. The coelomic fluid is very important to carry immune properties to affected parts in vertebrates. The coelomocytes present in the coelomic fluid plays a significant role in the active functioning of the humoral and cell-mediated immune response. They are also responsible for eliminating foreign materials by proteolytic, hemolytic and cytolytic properties. Lysozyme is the factor responsible for the

destruction of the antibacterial activity of the coelomic fluid checked against Gram positive and Gram negative bacteria [1]. This function is mediated by two proteins namely fetidin and lysine of presumed molecular mass 40 kDa and 45 kDa respectively that has a similarity of 35% when compared with the immunoglobulins[2]. The major component of the coelomic fluid peptides was found to be present in both the above-mentioned proteins (20% of total CF proteins) and are released from the chloragogue cells[3]. Similar cytolytic activities are also elucidated in bacteria [4], [5] and in some eukaryotes such as Molluscs, Echinodermata, Polychaeta, Oligochaeta, Arthropoda [6],[7] and vertebrates[8],[9],[10],[11]. Lysenin a consequence of its specific binding to sphingomyelin (SM), lysenin induced the smooth muscle contraction[12], red blood cells were lysed [13] and destruction of vertebrate spermatozoa[14],[15]. The coelomic fluid had strong hemolytic activity against various mammalian erythrocytes. The hemolytic proteins of molecular weight 38 kDa induced eiseniapore that was also isolated from the coelomic fluid of the same species[16]. The coelomocytes were immunocompetent cells that are amoebocyte, chloragocytes, and eleocytes. The induced coelomic cytolytic factor 1 (CCF-1) 42-kDa protein triggered the prophenoloxidase cascade[17]. A novel protein of 297 amino acids with a molecular mass of 33 kDa from the CF, This protein induced the

contraction of smooth muscle and was named lysenin[12, 18]; [19].

Material Methods

2.1 Extraction of coelomic fluid

The coelomic fluid was extracted by using the cold shock method. In this method, gut cleared worms were washed with distilled water and injected with 50µl of *Pseudomonas aeruginosa*. After 24 hours of injecting microorganisms, the worms were placed in a sterile Petri plate to which 1ml of 1X PBS was added. Then it was kept between the ice packs for 5 minutes. The fluid was collected in a sterile tube and stored at 4°C.

2.2 Preparation of Tissue Extract (G90 Protein)

The earthworms were washed with running tap water. It was separated into two groups. One group of earthworms was injected with the microorganism *Pseudomonas aeruginosa*, another one group was used normally. Both the normal and injected worms were kept in 0.65% NaCl at room temperature for 1 hour. Later the worms were cut into small pieces. Three gram of the earthworm tissue was homogenized in 40ml of chloroform: methanol 1:1 (v/v) and kept at 4°C for overnight. Next day, about 16 ml of distilled water was added to the homogenate and centrifuged at 3000 rpm for 10 minutes. Three clearly visible layers were

obtained. Bottom and middle layer was chloroform and tissue layer. Upper layer contained water and methanol. The methanol was evaporated in a laminar airflow chamber for one day. Remaining water extract of both the normal and injected worms tissue extract (G90) was collected separately and stored at 4°C.

2.3 TLC

The slurry was prepared by mixing silica gel with water in the ratio 3:2 the slurry was coated onto the glass plate at a thickness of about 0.25mm and the plates were allowed to dry at room temperature for 15-30 minutes. Then the plates were kept in a hot air oven at 100-200°C for 2 hours. The samples were resolved using the solvent system Butanol: Acidic acid: Water (60:15:25) and then visualized in gel documentation.

2.4 Antibacterial Activity

Antibacterial activity of compound 1 and compound 2 from TLC were tested against selected Gram-positive (*B.subtilis*, *S.faecalis*, *M.luteus*) and Gram-negative bacteria (*S.abony*, *P.aeruginosa*, *E.coli*) using well diffusion method. The bacteria were grown on nutrient broth. Mueller-Hinton agar medium was poured into a sterile Petri plate and allowed to solidify. The 24 hours bacterial culture was swabbed on the medium and wells were bored. About 50µl of the samples were added into the well and the plates were incubated at 37°C for 24 hours. After incubation, the inhibition zone was

observed around the well and recorded.

2.5 Proteolytic Activity

The proteolytic activity of the compound 1 and compound 2 from TLC were determined by using Gelatin lysis activity. Gelatin is the denatured product of collagen and is an abundant protein. About 20 ml of gelatin-agarose gel was prepared and poured into the Petri plate and allowed to solidify. About 20µl of each sample was added to the well created in the agar plate. It was incubated for 4-6 hours at 37°C. The gelatin-agarose gel was precipitated with 5ml of a solution containing 15g of HgCl₂ and 20ml of 12N HCl in 80 ml distilled water. The diameter of the clear circle around each well was measured.

2.6 Erythrocytic Activity

About 1.5 % of agarose was prepared. 20 ml of sheep blood was collected and subjected to centrifuge action at 10000 rpm for 10 minutes. RBC was collected. 9ml of 0.9% freshly prepared NaCl was added to 1ml of collected RBC. 20 ml of prepared RBC was added to sterilized 1.5 % agarose and poured on plates, and cooled. Samples of compound 1 and compound 2 from TLC were loaded on the wells cut on the plate. It was incubated for 24 hours at 37°C. After incubation, the clear zone was recorded.

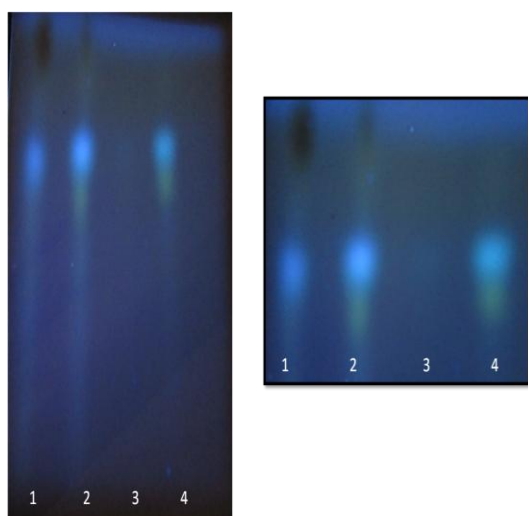
2.7 HPLC for active compounds

HPLC is an analytical chemistry technique that combines the physical separation capabilities of liquid Chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. Two mg of the sample (active compound 1) was prepared by dissolving in 2ml methanol in a volumetric flask. The solution was then filtered. Stock solution 1mg/ml was kept in the fridge at 4°C. HPLC for various layers were performed.

RESULT

3.1 Thin layer chromatography

Coelomic fluids and skin extracts of *Eudrilus eugeniae* were prepared for thin layer chromatography. Two metabolites (blue (compound 1) and yellow (compound 2)) were observed (Figure 1) in coelomic fluids and *P. aeruginosa*-injected coelomic fluid and skin extract and there is no spot was observed in normal skin extract.



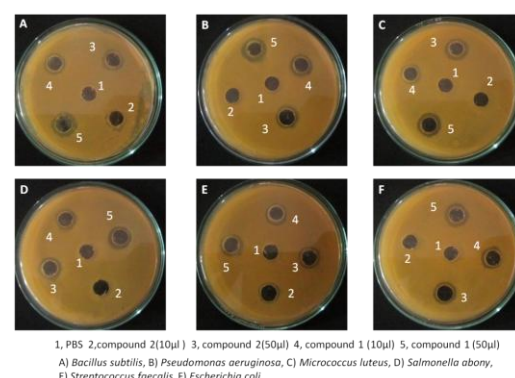
1, normal coelomic Fluid 2, *P.aeruginosa* injected coelomic Fluid
 3, normal G90 4, *P.aeruginosa* injected G90

Figure 1: Thin layer chromatography of earthworm samples

Two metabolites were synthesized in coelomic fluids and *P.aeruginosa* injected skin extract but there is no metabolites were noticed in skin extract.

3.2 Antimicrobial activity

TLC compound 1 and compound 2 extract from *Eudrilus eugeniae* were used to examine the antibacterial activity against gram-positive (*B.subtilis*, *S.faecalis*, *M.luteus*) and gram-negative (*S.abony*, *P.aeruginosa*, *E.coli*) bacterial pathogens. compound 1(50µl), (10µl) and compound 2 (10µl) showed high antibacterial activity against the pathogens. Compound 1(50µl) rendered the maximum zone of inhibition against *P.aeruginosa* whereas Compound 2 (50µl) (Figure 2). Compound 2 showed less antibacterial activity against all pathogens when compared to Compound 1.



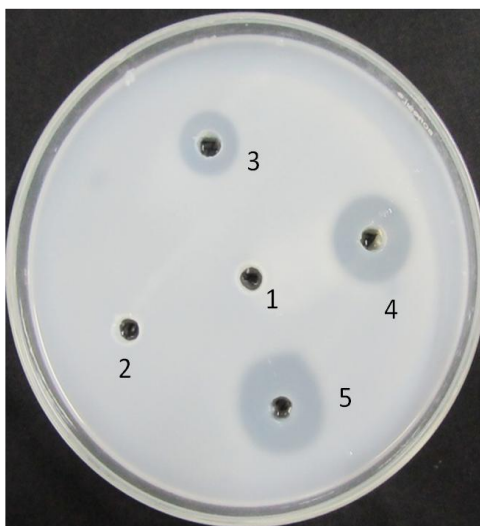
1, PBS 2, compound 2(10µl) 3, compound 2(50µl) 4, compound 1 (10µl) 5, compound 1 (50µl)
 A) *Bacillus subtilis*, B) *Pseudomonas aeruginosa*, C) *Micrococcus luteus*, D) *Salmonella abony*,
 E) *Streptococcus faecalis*, F) *Escherichia coli*.

Figure 2: Antimicrobial activity of earthworm samples

3.3 Proteolytic activity

A simple agar diffusion assay

incorporating gelatin as substrate was used to demonstrate the characteristics of TLC compounds extracts from *Eudrilus eugeniae*. After 4-6 hours of incubation of 30 μ l different sample with agarose medium, the clear zone was viewed around the well which was due to the degradation of gelatin. The clear zone was observed in the Compound 1(50,10 μ l) and Compound 2 (50 μ l), and there is no zone observed in Compound 2 (10 μ l). High proteolytic activity was observed in the Compound 1 (figure 3).



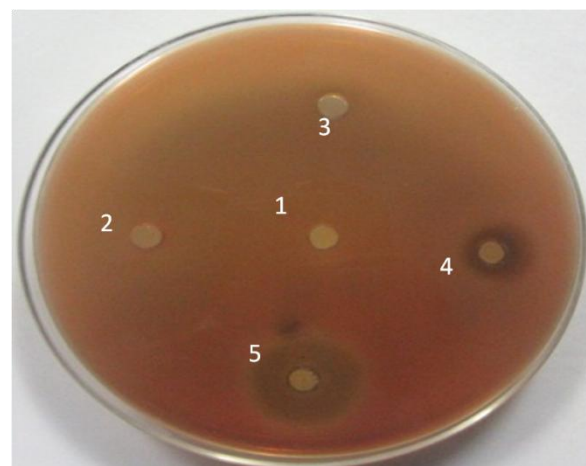
1, PBS 2, compound 2(10 μ l) 3, compound 2(50 μ l)
 4, compound 1 (10 μ l) 5, compound 1 (50 μ l)

Figure 3: Proteolytic Activity of earthworm samples

3.4 Erythrolytic activity

Compound 1 and Compound 2 were examined against Red Blood Cells (RBC) for erythrolytic activity. After incubation of 30 μ l different sample with blood agar media, the clear zone was viewed around the well. Degradation of red blood cells was observed in

the Compound 1, whereas there is no lytic activity was observed in Compound 2 (figure 4).



1, PBS 2, compound 2(10 μ l) 3, compound 2(50 μ l)
 4, compound 1 (10 μ l) 5, compound 1 (50 μ l)

Figure 4: Erythrolytic activity of earthworm samples

3.5 HPLC Analysis

HPLC is an analytical chemistry technique that combines the physical separation capabilities of liquid Chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. The HPLC was performed to confirm the presence of active constituents in compound 1. Purified fractions of the compound 1 were analyzed for HPLC.

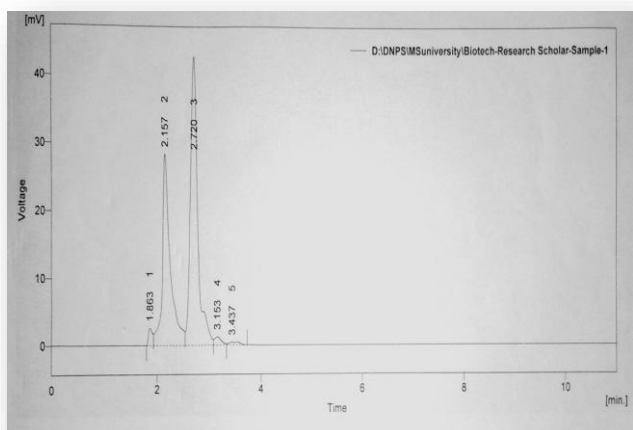


Figure 5: HPLC Analysis of specific spots (yellow and brown color spots)

The HPLC profile compound 1 represents characteristic peaks at retention time 2.1, 4.3, 6.0, 14.8, 19.2, 23.2, 23.5 and 24.7 min at the wavelength 220 nm (figure 5). Herein, the intense peaks were attained at 4.3 and 6.0 min with the area 38688882 and 85811534, respectively.

Discussion

{Wang, 2007 #1} Reported that the coelomic fluid of *Dichogaster bolau* showed antibacterial activity against pathogenic bacteria like *E.coli*, *S.aureus*, *Pseudomonas*, *Proteus* and *Bacillus* sp. The best inhibitory effect of coelomic fluid of *Dichogaster bolau* on the growth of *Proteus* is 16 mm and that on *Pseudomonas* 8mm, whereas the zone of inhibition is only 7mm against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus*. In that *Eudrilus eugeniae* injected coelomic fluid had high zone against in gram-positive and negative

bacteria better than to normal coelomic fluid. Earthworm paste of *Eudrilus eugeniae* was tested for their antibacterial effect against five strains of bacteria viz., *E.coli*, *S.abony*, *S.aureus*, *K.pneumoniae*, and *B.subtilis*. The earthworm pastes highly inhibit the growth of *S.aureus* and inhibit the growth of *S.abony* in minimum level [20]. [21] Reported that the ethanol extracts of dried earthworm powder obtained from *Lampito mauritti* were a potent antibacterial agent against *Aeromonas hydrophila* and antifungal agent against *Candida albicans*. In that result compound 1 have high zone better than to compound 2 in gram positive and gram negative pathogens.

In earthworms, proteolytic enzymes have been found in the coelomic fluid [22], [23], [24] reported that the wounding and injection of foreign material into the coelomic cavity increase coelomocyte numbers and the activity of easily measurable humoral immune factors like agglutinins, lysins and protease. An increase in the number of proteases after stimulation has been shown before, but it is still unclear whether this increased number as demonstrated after native PAGE separation is due to newly synthesized proteins or whether it results from limited proteolysis. Compound 1 and Compound 2 possessed proteolytic activity. Compound 2 (50 μ) was able to lyse the protein but the Compound 2 (10 μ) showed no activity on Gelatin Agarose medium. Among the two

samples, Compound 1(50 μ) exhibited higher proteolytic activity. Compound 1(10 μ) possessed low activity Normal coelomic fluid had lower activity in protein degradation.

Roch et al. and Milochau both found proteins with molecular weights of 45 kDa and 40 kDa possessing several activities, such as antibacterial, hemolysis, and hemagglutination from *E. fetida*. Some studies about the hemolytic and antibacterial effect of coelomic fluids have exhibited that antigenic determinants on the surface of sheep erythrocytes and bacteria which are inhibited by coelomic fluid are similar [25]; [24]. In this experiment Compound 1 possess highly erythrolytic activity and Compound 2 shows no activity.

The peak at retention time 4.3 exemplifies the presence of lysenin protein in the coelomic fluid of *Perionyx escavatus*. Lysenin is one of the toxin proteins present in the coelomic fluid of earthworm might be responsible for pore formation in the bacterial cell membranes with specifically bind to sphingomyelin and resulted in the death of bacterium cell [26]; [27]. Similar the *Eudrilus eugeniae* also exhibits major peaks at 2.1 and 2.7 min with an area 299.074 and 420.248, respectively (fig). The peak at retention time 2.1 and 2.7 min indicates the presence of biological active compound in the coelomic fluid of *Eudrilus eugeniae*.

Conclusions

This work is concluded normal coelomic fluid possess some biological activity without skin extract. Any substance disturbed from outside the body cavity suddenly the immunological compound are secreted within 24 hours. The secreted the compounds are involved in the immunological function and destroy the all entry the foreign particles. So this protein compound is highly applicable and used in pharmaceuticals for inducing immunity in animals and humans.

Conflict of Interest

The authors confirm that the manuscript has no conflict of interest.

Reference

1. Lassalle F, Lassegues M, Roch P: Protein analysis of earthworm coelomic fluid IV. Evidence, activity induction and purification of *Eisenia fetida andrei* lysozyme (Annelidae). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 1988, 91(1):187-192.
2. Roch P, Valembois P, Davant N, Lassegues MJCB, Biochemistry PPBC: Protein analysis of earthworm coelomic fluid—II. Isolation and biochemical characterization of the *Eisenia fetida andrei* factor (EFAF). 1981, 69(4):829-836.

3. Valembois P, Cazaux M: Radioautographic study of the trophic role of the chloragogen cells of earth worms. *Comptes rendus des seances de la Societe de biologie et de ses filiales* 1970, 164(5):1015.
4. Bernheimer AW, Rudy BJB:BA-RoB: Interactions between membranes and cytolytic peptides. In., vol. 864; 1986: 123-141.
5. Kini RM, Evans HJ: A model to explain the pharmacological effects of snake venom phospholipases A2. *Toxicon* 1989, 27(6):613-635.
6. Canicatti C, Roch P: Erythrocyte membrane structural features that are critical for the lytic reaction of *Spirographis spallanzani* coelomic fluid hemolysin. *Comparative biochemistry and physiology C, Comparative pharmacology and toxicology* 1993, 105(3):401-407.
7. Roch P: A definition of cytolytic responses in invertebrates. In: *Invertebrate Immune Responses*. Springer; 1996: 115-150.
8. Bernheimer AW, Rudy B: Interactions between membranes and cytolytic peptides. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes* 1986, 864(1):123-141.
9. Condrea E: Membrane-active polypeptides from snake venom: cardiotoxins and haemocytotoxins. *Experientia* 1974, 30(2):121-129.
10. Kini RM, EVANS HJ: A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *International journal of peptide and protein research* 1989, 34(4):277-286.
11. Maloy WL, Kari UP: Structure-activity studies on magainins and other host defense peptides. *Biopolymers: Original Research on Biomolecules* 1995, 37(2):105-122.
12. SEKIZAWA Y, HAGIWARA K, NAKAJIMA T, KOBAYASHI H: A novel protein, lysenin, that causes contraction of the isolated rat aorta: its purification from the coelomic fluid of the earthworm, *Eisenia foetida*. *Biomedical Research* 1996, 17(3):197-203.
13. Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda M: Lysenin, a novel sphingomyelin-specific binding protein. *Journal of Biological Chemistry* 1998, 273(9):5300-5306.
14. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I: An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements.

- Biochemical and biophysical research communications* 1997, 236(2):313-322.
15. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinoshita M, Yamaguchi K, Shima N: Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. *Journal of Experimental Medicine* 2000, 191(2):275-286.
 16. Lange S, Nüßler F, Kauschke E, Lutsch G, Cooper EL, Herrmann A: Interaction of earthworm hemolysin with lipid membranes requires sphingolipids. *Journal of Biological Chemistry* 1997, 272(33):20884-20892.
 17. Beschin A, Bilej M, Hanssens F, Raymakers J, Van Dyck E, Revets H, Brys L, Gomez J, De Baetselier P, Timmermans M: Identification and Cloning of a Glucan-and Lipopolysaccharide-binding Protein from *Eisenia foetida* Earthworm Involved in the Activation of Prophenoloxidase Cascade. *Journal of Biological Chemistry* 1998, 273(38):24948-24954.
 18. Sekizawa Y, Kubo T, Kobayashi H, Nakajima T, Natori S: Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle. *Gene* 1997, 191(1):97-102.
 19. Kobayashi H, Sekizawa Y, Shioda S, Natori S, Nakajima T, Umeda M: Lysenin, a novel bioactive protein isolated from coelomic fluid of the earthworm *Eisenia foetida*-structure, secretion and biological activity. In: *Neuroendocrinology*. Springer; 1997: 255-269.
 20. Vasanthi K, Chairman K, Singh ARJAJoES, Technology: Antimicrobial activity of earthworm (*Eudrilus eugeniae*) paste. 2013, 7(8):789-783.
 21. Bhorgin A, Uma KJIJCMAS: Antimicrobial activity of earthworm powder (*Lampito mauritii*). 2014, 3(1):437-443.
 22. Kauschke E, Mohrig WJJJoCPB: Cytotoxic activity in the coelomic fluid of the annelid *Eisenia foetida* Sav. 1987, 157(1):77-83.
 23. Roch P, Stabili L, Pagliara PJCB, Biochemistry PPBC: Purification of three serine proteases from the coelomic cells of earthworms (*Eisenia fetida*). 1991, 98(4):597-602.
 24. Eue I, Kauschke E, Mohrig W, Cooper ELJD, Immunology C: Isolation and characterization of earthworm hemolysins and agglutinins. 1998, 22(1):13-25.

25. Valembois P, Roch P, Lassegues M, Cassand PJJoiP: Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida andrei*. 1982, 40(1):21-27.
26. Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda MJJoBC: Lysenin, a novel sphingomyelin-specific binding protein. 1998, 273(9):5300-5306.
27. Shogomori H, Kobayashi TJBeBA-GS: Lysenin: a sphingomyelin specific pore-forming toxin. 2008, 1780(3):612-618.