

# Bioactivities of crude mucus proteins from *Eudrilus eugeniae* (African night crawler) and *Perionyx excavatus* (Blue worm)

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**Abstract. – OBJECTIVE:** This study aimed to investigate the enzymatic anti-oxidative, hemolytic and cytotoxic activities of crude mucus proteins from the two earthworms including *Eudrilus eugeniae* (African night crawler) and *Perionyx excavatus* (Blue worm).

**MATERIALS AND METHODS:** The bioactivities were determined by hemolytic activity, cytotoxic activity using HepG2 human hepatocellular carcinoma cells, L-929 mouse fibroblast and TK6 human lymphoblast cell lines and antioxidant activity.

**RESULTS:** The results indicated that the hemolytic activity of mucus proteins of *P. excavatus* was higher than that of *E. eugeniae*. The cytotoxic activity of the mucus proteins of *E. eugeniae* could inhibit the growth of HepG2 with the IC<sub>50</sub> at 144.2 ± 0.18 µg/mL but showed no effect on L-929 and TK6. On the other hand, the crude proteins of *P. excavatus* decreased cell viability of both L-929 and HepG2 with their IC<sub>50</sub> respectively were 6.87 ± 0.16 and 174.3 ± 0.19 µg/mL but they did not reduce the growth of TK6 cell line. The SOD-like activity of *P. excavatus* and *E. eugeniae* crude proteins were found with the IC<sub>50</sub> at 149 µg/mL and 386.2 µg/mL, respectively. For GPx-like activity, crude proteins of *P. excavatus* exhibited significant ( $p \leq 0.05$ ) greater activity than those of *E. eugeniae* when tested at the concentration 100 µg/mL.

**CONCLUSIONS:** This study revealed the bioactivities of crude mucus of earthworms which served as alternative natural proteins for the prophylaxis or treatment of free radical-related diseases as well as development of dietary supplements and cosmetics.

*Key Words:*

Crude mucus proteins, *Eudrilus eugeniae*, *Perionyx excavatus*, Enzymatic-like Antioxidant, Cytotoxicity.

## Introduction

Earthworm's immunity is mediated by cellular and humoral immune system. The cellular immunity of earthworm is mainly based on phagocytosis, leucocyte and cell to cell recognition<sup>1</sup>. The humoral system of earthworm includes coelomic fluid was investigated on proteolytic, hemolytic, cytolytic and antimicrobial activities. The coelomic fluid or mucus of *Eisenia fetida* was already identified. The 42 kDa coelomic cytolytic factor-1 (CCF-1) was discovered as an immune molecule and EFAF (*E. foetida andrei factor*), named fetidin, could exhibit antibacterial and hemolytic activities<sup>2</sup>. The CCF-1 had homology with the putative catalytic region of  $\beta$ -1,3- and  $\beta$ -1,3-1,4-glucanase that was involved in the immune mechanism. CCF-1 binds to microbe  $\beta$ -1,3-glucan and lipopolysaccharide that triggered the prophenoloxidase cascade which is a major invertebrate immune defense mechanism<sup>3,4</sup>. CCF-1 also recruits opsonizing for phagocytosis<sup>5</sup>. There was a report that the source of CCF is the coelomocyte of the earthworm<sup>6</sup>. Moreover, fetidin was found containing two molecular masses of 40-kDa isoform and 45-kDa monofom<sup>7-9</sup>. The previous works studied on earthworms, *Eudrilus eugeniae* (African night crawler: Af) and *Perionyx excavatus* (Blue worm: Blu). Recently, the cell-free coelomic fluid of Af was reported to induce apoptosis and necrosis in SiHa cell (human cervical cancer cell line). The coelomic fluid at 100 µg/mL concentration inhibited the growth of SiHa cells *in vitro* by up to 78.52% in 48 hours and induced necrotic cell death. The apoptotic rate of SiHa cells increased from 38.58 % to 59.21 % when the coelomic fluid concentrations were increased from 40 to 50 µg/mL in 48 hours<sup>10</sup>.

*E. eugeniae* extracts inhibited plant pathogens<sup>11</sup> and *E. eugeniae* paste had antimicrobial activity inhibited *Escherichia coli*, *Salmonella abony*, *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella pneumonia*. Also, the earthworm paste had antifungal activity against *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium notatum* and *Trichophyllum rubrum*<sup>12</sup>. *P. excavatus* is an earthworm that is commonly found in tropical South Asia<sup>13</sup>. The biomass of *P. excavatus* was discovered six fibrinolytic protease fractions namely, FI, FII, FIII-1, FIII-2, FIII-3 and FIV were fractionated by ion exchange and hydrophobic interaction chromatography. These fractions were fractionated from acetone precipitated earthworm biomass. The tandem amino acid sequence of FII-1 and FIII-2 shared homology with the fibrinolytic enzyme of *Lumbricus rubellus* and *Eisenia fetida* by 16.9% and 13.2, respectively. From the protease activity indicated that these proteases were classified as serine protease that has antithrombotic activity<sup>14</sup>.

In this study, the mucus proteins of *E. eugeniae* and *P. excavatus* were extracted and determined on their hemolytic activity in human red blood cells, cytotoxic property against various cell lines including L-929 (mouse fibroblast cells), TK-6 (human lymphoblast cells) and HepG-2 (human hepatocellular carcinoma cells) and enzymatic antioxidant activity.

## Materials and Methods

### Mucus Extraction

Living earthworms of *E. eugeniae* and *P. excavatus* were cultured and maintained in the laboratory condition. The healthy earthworms of the two species were isolated from their bedding and washed by 0.01 M phosphate buffer saline (PBS) (Sigma-Aldrich®, St. Louis, MO, USA) pH 6.5 followed by adding of PBS at ratio 1:1 (g of fresh weight /mL of PBS). For the mucus extraction, earthworms were activated by electricity at 10 mA for 2 minutes and then separated from the fluid. The samples of extracted fluid were mixed with 100 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich®, St. Louis, MO, USA) and centrifuged at 3,000 rpm for 15 minutes. The supernatants were collected and kept at -20 °C.

### Mucus Protein Precipitation and Characterization

The proteins in the supernatant were precipitated by 85% ammonium sulfate at 4°C overnight

with stirring and centrifuged at 10,000 rpm 4°C for 45 minutes and dissolved in distilled water. The proteins pattern was analyzed by glycine and tricine SDS-Polyacrylamide Gel Electrophoresis in 15% acrylamide resolving gels. The glycoprotein in the crude mucus was checked by Schiff's reagent staining.

### Cell Lines and Culturing Condition

Three cell lines including mouse fibroblasts (L929, ATCC® CCL-1), human lymphoblastoid (TK6, ATCC-CRL 8015) and the transformed or cancer cell line human hepatocellular carcinoma (HepG2, ATCC®HB-8065) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The L-929 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated (56°C for 30 minutes) horse serum and 1% penicillin and streptomycin (P&S). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% PS. TK6 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% heat-inactivated horse serum and 1% PS. The cells were propagated in tissue culture flask (Corning®, NY, USA) at 37°C in humidified atmosphere incubator with 5% CO<sub>2</sub>, sub-culturing every 2-3 days by trypsinization with 0.25% trypsin-EDTA to allow detachment of cells and add fresh culture medium, aspirate and dispense into new culture flasks. The L-929 and HepG2 cell lines were grown as adherent whereas the TK6 cell line was grown as suspension. All culture media, serums and antibiotics were purchased from GIBCO® BRL (Grand Island, NY, USA).

### Determination of Hemolytic Activity

The mucus proteins of *E. eugeniae* (Ee) and *P. excavatus* (Pe) were evaluated for their hemolytic activity using human erythrocytes (prepared at 2% concentration). The red blood cells were freshly collected from a volunteer. They were gently washed with 0.01 M PBS, pH 7.4 and centrifuged at 3,000 rpm at 4°C for 5 minutes to remove the supernatant. The washing step was performed for 3 times. Then the cells were prepared at 2% erythrocytes concentration in 0.01M PBS buffer. The mucus proteins were diluted in the various concentrations by PBS pH 7.4 and mixed with 2% of RBC by 1:1 (v/v). The mixtures were incubated at room temperature for 1 hour and

centrifuged using the condition described above to collect the supernatant. The hemoglobin releasing level was monitored by the spectrophotometer at 420 nm.

#### **Determination of Cytotoxic Activity**

The cell lines were maintained in the suitable media and under conditions as described above. The cytotoxic activity of the mucus proteins prepared from Ee and Pe earthworms was measured by water-soluble tetrazolium salt (WST) (Roche, Basel, Switzerland) assay. The overnight L-929 and HepG2 cell cultures were harvested by trypsinization as explained before in Hank's balanced salt solution (HBSS, Gibco®) at 0.05% and 0.25% concentrations, respectively). The cells were seeded in the growth medium into a 96-well microtiter plate ( $1 \times 10^5$  cells/well) and then incubated at 37°C in 5% carbon dioxide incubator overnight. The Ee and Pe crude proteins were diluted with EMEM for L-929, DMEM for HepG2 and RPMI for TK6 cells at 5 concentrations (0.1, 1, 10, 100 and 1,000 µg/mL). At the end of incubation time, the culture media of the seeded L-929 and HepG2 cell lines were discarded and the cells were treated with the diluted crude proteins. The maintained TK6 cells were centrifuged at 3,200 rpm for 3 minutes to discard medium and the cells were mixed with the diluted crude proteins. The treated cells were incubated at 37°C in 5% carbon dioxide for 24 hours before the results' analysis. To detect cell viability, the treated cells of L929, HepG2 and TK6 were mixed with diluted WST (1:10 of their corresponding media). The cells were further incubated at 37°C in 5% carbon dioxide incubator for 30 minutes and protected from light. The reactions were measured at the absorbance 430 nm to calculate percentages of cell viability of each cell line.

#### **Determination of Antioxidant Enzyme-like Activity**

##### **Superoxide Dismutase (SOD) Activity**

The SOD-like activity of the Ee and Pe crude proteins was determined by a colorimetric method using SOD determination kit (Sigma-Aldrich Chemie GmbH, Basel, Switzerland). The assay is based on the reduction rate of superoxide anion ( $O_2^{\cdot-}$ ) by SOD. The  $O_2^{\cdot-}$  molecules were generated in the system by xanthine oxidase (XO) activity. The water-soluble tetrazolium (WST) salt produced a water-soluble formazan

dye upon reduction with a superoxide anion. The rate of the reduction with  $O_2^{\cdot-}$  is linearly related to the XO activity, and is inhibited by SOD. Therefore, inhibition activity of SOD or SOD-like materials can be determined by a colorimetric method. The Ee and Pe protein samples were tested in 5 final concentrations (0.00833, 0.0833, 0.833, 8.33 and 83.3 µg/mL) prepared by dissolving in kit dilution buffer. The wells were mixed thoroughly and incubated at 37°C for 20 minutes. After incubation, the absorbance was determined at 430 nm. The SOD-like activity (% inhibition rate) levels of Ee and Pe protein samples were calculated.

##### **Glutathione Peroxidase (GPx) Activity**

The GPx activity was determined by using glutathione peroxidase (GPx) assay kit (Abnova<sup>R</sup>, Singapore). The principle of the kit assay is based on GPx enzyme that will catalyze the reduction of hydrogen peroxide ( $H_2O_2$ ) and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent. In this assay, GPx reduced cumene hydroperoxide while GSH was converted to GSSG. The 40 mM NADPH solution was prepared. GR and GSH were diluted with 0.22 mL assay buffer. Then, cumene hydroperoxide and GPx were reconstituted with 1.25 mL and 100 µL assay buffer respectively. The NADPH standard curve was generated. The amount of NADPH was calculated to obtain the GPx activity. In this experiment, the crude proteins were treated and compared GPx-like activity of 5 final concentrations (0.05, 0.5, 5, 50 and 500 µg/ml) of both species.

##### **Statistical Analysis**

The results were shown as the mean  $\pm$  standard deviation (SD). The significance test was analyzed by the *t*-test with GraphPad Prism 6. The significant level was at  $p < 0.05$ .

## **Results**

### **Mucus Protein Patterns and Glycoprotein Distribution**

The patterns of crude mucus proteins from *P. excavatus* and *E. eugeniae* were verified by SDS-PAGE of both glycine and tricine systems. The differences between crude proteins from *P. excavatus* and *E. eugeniae* were clearly seen. The

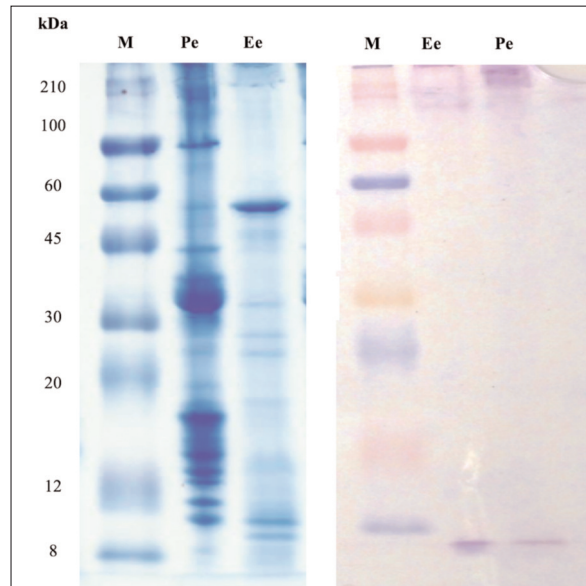
mucus proteins of *E. eugeniae* contain the majority of a protein band in rang of 45-60 kDa together with 3 bands of 20-30 kDa and 2 bands at of 8-12 kDa. The *P. excavatus* mucus proteins revealed more proteins bands than those of *E. eugeniae*. The *P. excavatus* mucus proteins size was found at 100 kDa, 4 bands in the range of 30-45 kDa and 6 bands of 12-20 kDa. The highest intensity bands were approximately 32 kDa and 18 kDa (Figure 1A). There was no glycoprotein observed in the crude mucus proteins of both earthworm species detected by Schiff's staining (Figure 1B).

### Hemolytic Activity

The hemolytic activity of mucus proteins obtained from *P. excavatus* was higher than that of *E. eugeniae* (Figures 2A and 2B). The inhibitory concentration (IC<sub>50</sub>) values of the mucus proteins of *P. excavatus* ( $0.76 \pm 0.14 \mu\text{g/mL}$ ) was significantly lower than that of *E. eugeniae* ( $14.33 \pm 0.12 \mu\text{g/mL}$ ) (Table I).

### Cytotoxic Activity

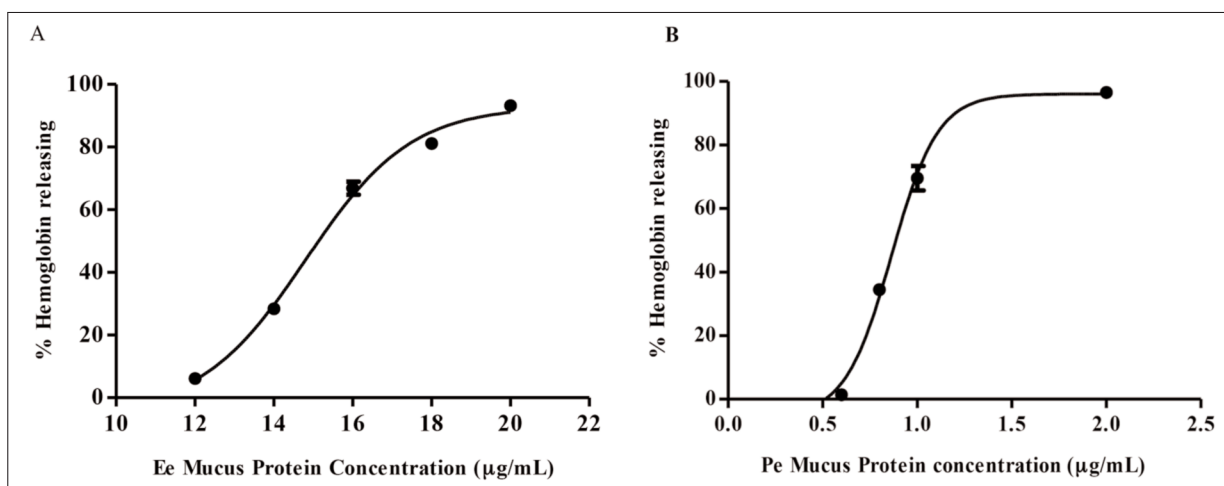
*E. eugeniae* mucus proteins exhibited effective inhibition against HepG2 cells with IC<sub>50</sub> at  $144.2 \pm 0.18 \mu\text{g/mL}$  whereas *P. excavatus* mucus proteins were highly toxic to L-929 (IC<sub>50</sub> =  $6.87 \pm 0.16 \mu\text{g/mL}$ ) but slightly toxic to HepG2 (IC<sub>50</sub> =  $174.3 \pm 0.19 \mu\text{g/mL}$ ) as demonstrated in Table II. On the other hand, the crude proteins of both earthworm species showed no effective inhibition against human TK6 cells. It was



**Figure 1.** Crude mucus proteins of *P. excavatus* and *E. eugeniae* stained with Coomassie Blue R-250 (A) and glycoprotein staining (B). M: protein marker, Pe: crude mucus protein of *P. excavatus*, Ee: crude mucus protein of *E. eugeniae*.

**Table I.** The inhibitory effect is presented as giving 50% inhibition concentration (IC<sub>50</sub>) of hemolytic activity of the crude proteins from *Eudrilus eugeniae* (Ee) and *Perionyx excavatus* (Pe) (mean  $\pm$  SEM).

Sample	IC <sub>50</sub> ( $\mu\text{g/ml}$ )
Ee	$14.33 \pm 0.12$
Pe	$0.76 \pm 0.14$



**Figure 2.** Dose-response curve of hemolytic activity of crude protein from *Eudrilus eugeniae* (Ee) (A) and Dose-response curve of hemolytic activity of crude protein from *Perionyx excavatus* (Pe) (B).



**Table II.** The level of cytotoxicity of the crude proteins of coelomic fluid from *Eudrilus eugeniae* (Ee) and *Perionyx excavatus* (Pe) were shown in term of 50% inhibition concentration (IC<sub>50</sub>) in µg/ml. The cytotoxic effects were compared between three types of cell lines (L-929, TK6 and HepG2). (mean ± SEM).

Sample	IC <sub>50</sub> (µg/ml)		
	L-929	TK6	HepG2
Ee	> 1000	> 1000	144.2 ± 0.18
Pe	6.87 ± 0.16	>1000	174.3 ± 0.19

found that the cytotoxic effect of *E. eugeniae* and *P. excavatus* mucus proteins on L-929 and HepG2 cell lines was a dose-dependent response. It was noticed that *E. eugeniae* and *P. excavatus* mucus proteins were less toxic to TK6 cell line when tested at the concentration from 0.1 to 100 µg/mL (Figure 3).

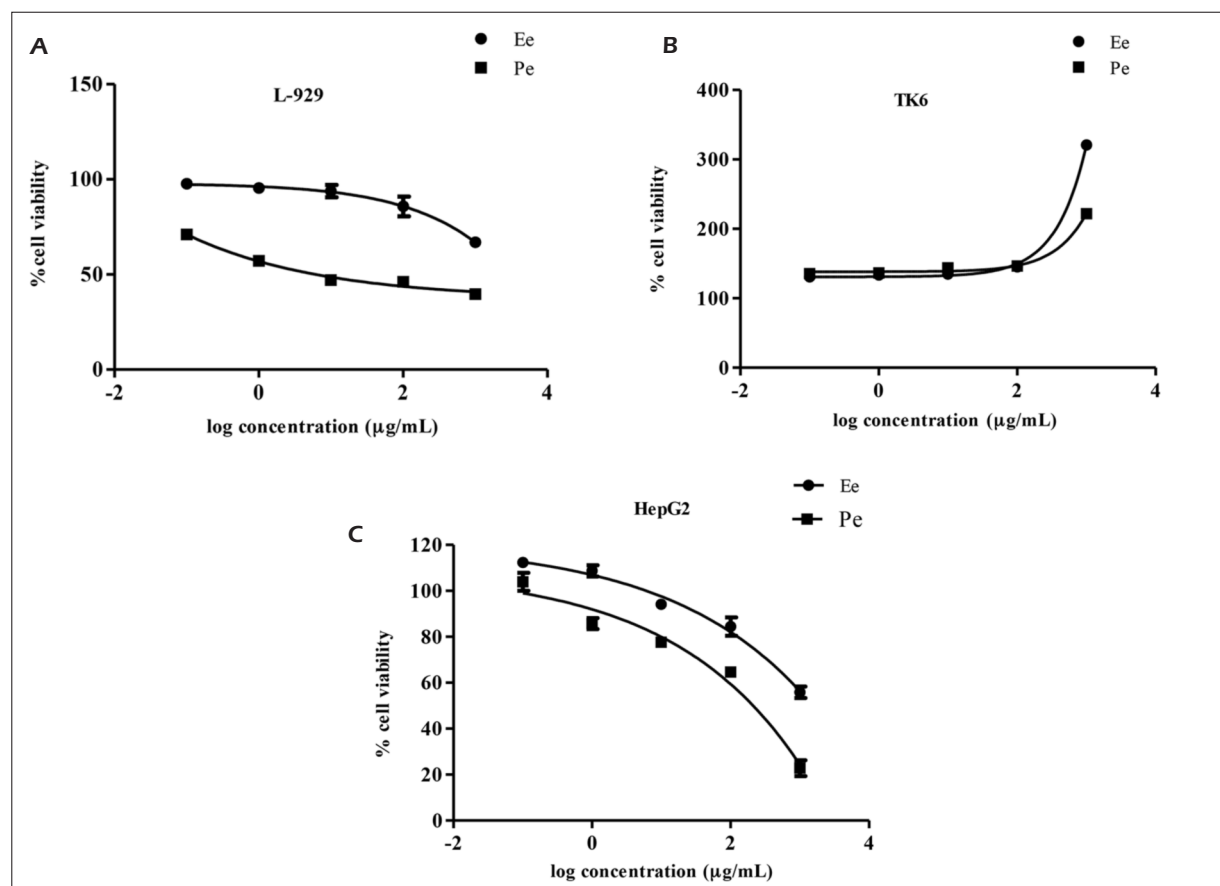
## Enzymatic Antioxidant like Activities

### SOD-Like Activity

This activity was investigated to obtain the superoxide scavenging efficiency of the mucus proteins of the two earthworms. The results showed the dose-dependent response of *E. eugeniae* and *P. excavatus* mucus proteins on SOD-like activity (Figure 4A). The O<sub>2</sub><sup>-</sup> inhibition rate of mucus proteins of *E. eugeniae* (IC<sub>50</sub> = 12.46 ± 0.19 µg/mL) was significantly different ( $p < 0.05$ ) from that of *P. excavatus* (IC<sub>50</sub> = 30.67 ± 0.22 µg/mL). The results indicated that mucus proteins of both earthworms possessing superoxide scavenging activity.

### GPx-Like Activity

The GPx-like assay was carried out to confirm the enzymatic anti-oxidation activity of the mucus proteins of coelomic fluid of *E. eugeniae* and *P. excavatus*. It was found that *P. excavatus*



**Figure 3.** Percentage of cell viability indicated level of cytotoxic effects of the crude proteins from *Eudrilus eugeniae* (Ee) and *Perionyx excavatus* (Pe) on L-929 (A), TK6 (B) and HepG2 (C) cell lines.

**Table III.** The SOD-like activity level was compared by 50% superoxide inhibition concentration (IC<sub>50</sub>) of the mucus protein from *Eudrilus eugeniae* (Ee) and *Perionyx excavatus* (Pe) in µg/ml. (mean ± SEM).

Sample	IC <sub>50</sub> (µg/ml)
Ee	12.46 ± 0.19
Pe	30.67 ± 0.22

demonstrated higher GPx activity than that of *E. eugeniae*. The GPx activities of mucus proteins of *P. excavatus* tested at 5 and 50 µg/mL concentrations were significantly ( $p \leq 0.05$ ) higher than those of *E. eugeniae*. (Figure 4B). These results illustrated that the *E. eugeniae* and *P. excavatus*. Mucus proteins had GPx-like activity as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenger. The H<sub>2</sub>O<sub>2</sub> is the major final oxidant in oxidative stress pathway.

### Discussion

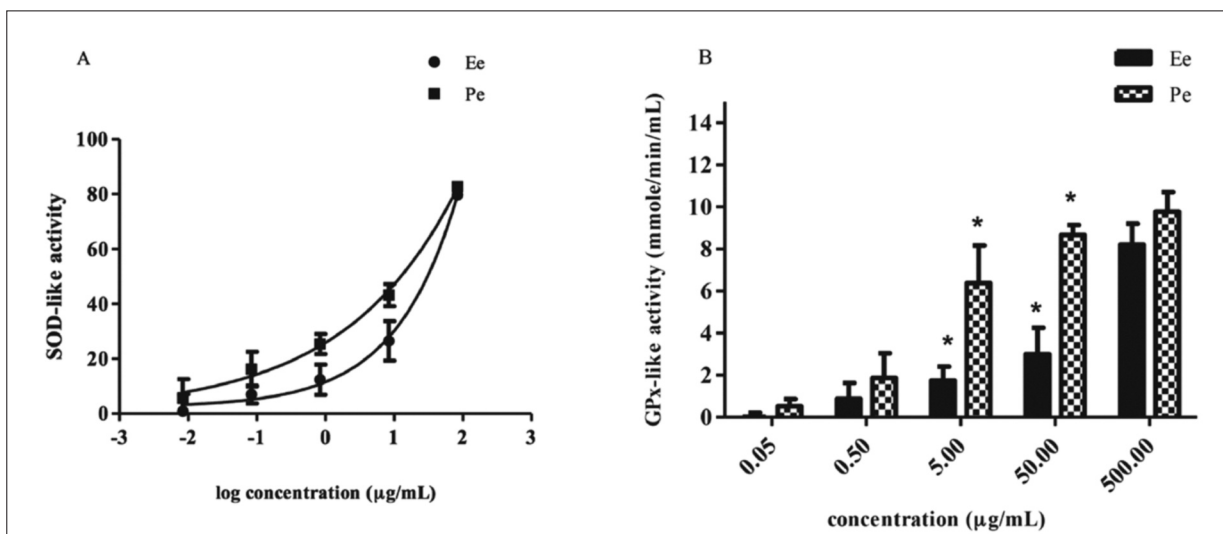
The components of earthworm mucus were identified in various species that showed there is a hemolytic factor. The hemolytic proteins of *E. fetida* were characterized from coelomocyte lysate described by Koenig et al<sup>15</sup>. The mass spectrometry showed the identities of fetidin and lysenin. In this work, the hemolytic activity was discovered in the mucus proteins of *E. eugeniae* and *P. excavatus* whereas the hemolytic factor in the mucus proteins was not reported in the previ-

ous studies. We also found that the hemolytic level of mucus proteins of *P. excavatus* was higher than that of *E. eugeniae*.

The mucus of the earthworm contains cytolytic factor, hemagglutinating molecules, coelomocytes and bioactive proteins. The previous reports indicated the presence of hemolytic proteins in earthworm coelomic fluid. Fetidin was found in coelomic fluid of *Eisenia Andrei*. It was the hemolytic protein that had 45-kDa mono-form and 40-kDa isoform<sup>16</sup>. The coelomic cytolytic factor 1(CCF-1) was discovered in the coelomic fluid of *E. fetida*. The molecular weight of CCF-1 was found at 42-kDa and showed hemolytic activity<sup>3</sup>. In this work, we found the hemolytic activity of the mucus proteins from the *E. eugeniae* and *P. excavatus* that related to the previous works. We herewith reported the protein bands whose molecular weights are in the range of 40-50 kDa. These hemolytic proteins will be verified in the future studies.

The cytotoxic effect of the earthworm coelomic fluid peptides, earthworm coelomic fluid and earthworm leukocyte were reported. In 2004, coelomocyte lysate of *E. fetida* was studied. In particular, its cytotoxicity on HeLa, HEp-2, PC-12 and PA317 cell lines *in vitro*. The results showed the coelomocyte lysate killed those cell lines and they suggested that the coelomocyte lysate was responsible for generating the cytotoxic components<sup>17</sup>.

The apoptotic induction of *E. fetida* coelomic fluid on HeLa cell line was investigated by MTT



**Figure 4.** SOD-liked activity of coelomic fluid of *Eudrilus eugeniae* (Ee) and *Perionyx cavatus* (Pe) (A) and GPx-liked activity of the crude proteins of coelomic fluid of Ee and Pe (B) The data were represented as mean ± SEM.

assay, acridine orange/ethidium bromide (AO/EB) fluorescent staining methods. At 48 h, coelomic fluid treatment showed HeLa cell lysis activity and the inhibition rate at 84.22%, 10.24% and 2.99% by the concentrations at 1, 0.1 and 0.01 mg/mL, respectively. The apoptotic rates were found at 79.1% and 22.2% with the concentrations of coelomic fluid at 0.1 and 0.01 mg/mL, respectively<sup>18</sup>. The coelomic fluid of *E. eugeniae* was reported that it could inhibit HeLa cells, WBC malignant tumor cells and brain tumor cells. The cell lines were treated with the coelomic fluid of *E. eugeniae* that was extracted by heat and cold shock method and the protein was precipitated by various percentage of ammonium sulfate fractionation (20%, 30%, 45% and 60%). They reported that at 200 µg/mL of *E. eugeniae* coelomic fluid protein inhibited HeLa cell, colon cancer tumor cells, WBC malignant and brain tumor cells with 19%, 14.25%, 13.87% and 9.7%, respectively without cytotoxicity data of normal cell lines<sup>19</sup>.

In our study, the *E. eugeniae* and *P. excavatus* mucus proteins were prepared by electricity and they were precipitated with 85% ammonium sulfate. The precipitated proteins were then dissolved in PBS buffer. Results of WST-1 assay suggested that *E. eugeniae* mucus proteins had no cytotoxic effect on L-929 and TK6 cell lines but were toxic to HepG2 cell line with the IC<sub>50</sub> at 144.2 µg/mL. Though *P. excavatus* mucus proteins did not express cytotoxic effect on TK6 cell line but they represented cytotoxicity on L-929 and HepG2 cell line with IC<sub>50</sub> at 6.87 and 174.3 µg/mL, respectively. The cytotoxicity (WST-1) results indicated that the mucus protein extraction method used in our current work was effective. It may be the suitable method to recover the active proteins for anti-cancer activity. However, the cancer cell inhibition level might depend on cancer cell types. The *E. eugeniae* mucus proteins were more appropriate to develop in vermiceuticals than that of *P. excavatus* because they showed cytotoxicity on L-929 which is normal cell line derived from mouse connective tissue.

To determine the enzymatic antioxidant capacity of the *E. eugeniae* and *P. excavatus* mucus proteins, the SOD- and GPx-like activities were performed. There were previous reports on the investigation of antioxidant activities of earthworm powder. In 2008, the *P. excavatus* earthworm powder was investigated for its enzymatic antioxidant in alcohol hepatotoxic rats. This earthworm powder represented the recovery of

SOD and GPx activities in liver and kidney of the induced rats those were fed 500 mg of the earthworm powder per kg of body weight<sup>20</sup>. The *E. eugeniae* earthworm powder was investigated for antioxidant activity on isopropanol induced myocardial infarction in rats. The *E. eugeniae* earthworm powder could recover SOD and GPx activities in serum and heart of these induced rats<sup>21</sup>.

We reported here the enzymatic antioxidant with SOD and GPx activities of the *E. eugeniae* and *P. excavatus* mucus proteins. Results obtained in our study suggested that these mucus proteins might incorporate superoxide and mitochondrial hydrogen peroxide scavengers. Superoxide anions (O<sub>2</sub><sup>-</sup>) are major free radicals produced in cells and induce cellular oxidative stress at the first step. They can be converted into other free radicals such hydroxyl (OH<sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Therefore, the superoxide scavenger is very crucial for cell protection from oxidative stress. The GPx neutralizes hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced by oxidative damage in mitochondria that always generates reactive oxygen species (ROS) in electron transport chain of cellular aerobic respiration. The H<sub>2</sub>O<sub>2</sub> is the oxidant molecule that induces oxidative stress in the final step. The mucus proteins from the both *E. eugeniae* and *P. excavatus* could scavenge and neutralize oxidative stress at the first and final steps.

## Conclusions

This study revealed the bioactivities of crude mucus of earthworms. Consequently, these mucus proteins should be identified for their protein components and amino acid sequences. It may lead to a discovery of a new alternative source of natural proteins from earthworms for the prophylaxis or treatment of free radical-related diseases as well as development of dietary supplements and cosmetics.

## Acknowledgements

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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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