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Cellular enzymatic anti-oxidants of fractionated mucus proteins from *Eudrilus eugeniae* (African night crawler) and *Perionyx excavatus* (Blue worm) in MC3T3

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Abstract. – OBJECTIVE: To investigate SOD-liked, GPx-liked and cellular anti-oxidation activities of fractionated mucus proteins from *Perionyx excavatus* (Pe) and *Eudrilus eugeniae* (Ee) in the MC3T3 osteoblast precursor cell line.

MATERIALS AND METHODS: The crude mucus proteins were extracted from Pe and Ee and fractionated using the anion exchange chromatography method with FPLC. The fractionated proteins were studied to determine their SOD-liked and GPx-liked activities. The most efficient fractions were studied for cellular anti-oxidation activities in the MC3T3 cell line including scavenging, protecting and repairing conditions.

RESULTS: The results showed that the highest SOD-liked activities of EeANX1 were found at 4.17 μg/ml and GPx-liked activities of EeANX4 were found at 5.26 μg/mL. EeANX1 had no cytotoxic effect on MC3T3 at 500 μg/mL, and the IC50 of EeANX4 was over 300 μg/mL. When both EeANX1 and EeANX4 were investigated for cellular anti-oxidation activity in MC3T3 cells at 20 μg/mL, the cellular superoxide and total ROS production of were significantly ($p<0.05$) lower than those of 250 μM pyocyanin (ROS inducer) in ROS scavenging, protecting and repairing conditions. The 20 μg/mL of EeANX1 and EeANX4 demonstrated increased SOD and GPx activities in MC3T3. Investigation of the scavenging, protection and repairing conditions of MC3T3 treated with 20 μg/mL each of EaANX1, EaANX4 and 250 μM pyocyanin demonstrated that the proteins had significantly lower SOD activities and higher GPx activities than the 250 μM pyocyanin.

CONCLUSIONS: The EeANX1 and EeANX4 fractions demonstrate SOD-liked and GPx-liked activities, as well as cellular anti-oxidation activities. These fractions could be developed as a natural anti-oxidant. This research could provide benefit to the study of cellular anticancer.

Key Words:
Earthworm, *Perionyx excavatus, Eudrilus eugeniae,* African night crawler, Anti-oxidation, Superoxide dismutase, Glutathione peroxidase.

Abbreviations

Pe = *Perionyx excavates*; Ee = *Eudrilus eugeniae;* SOD = superoxide dismutase; GPx = glutathione peroxidase; MC3T3 = osteoblast precursor cell line; ANX = HiTrap ANX FF anion exchange column; DEAE = HiTrap DEAE FF anion exchange column; IC50 = 50% of inhibition concentration; μg = microgram; mL = milliliter; mg = milligram; μM = micromolar; ROS = reactive oxygen species; $OH^{\bullet}$) = hydroxyl radical; NO = nitric oxide; $O_2^{\bullet-}$ = superoxide anion radical; $^1O_2$ = oxygen singlet; PBS = phosphate-buffered saline; α-MEM = Minimum Essential Medium Alpha; EDTA = Ethylenediaminetetraacetic acid; DPBS = Dulbecco's phosphate buffer saline; PMSF = phenylmethylsulfonyl fluoride; rpm = round per min; DDW = deionized distilled water; XO = xanthine oxidase; WST = water soluble tetrazolium salt; nm = nanometer; nmol = nanomole; μL = microliter; ddH_2O = double distilled water; NAC = N-acetylcysteine; SD = standard deviation; EeANX = fractionated Ee with HiTrap ANX FF; EeDEAE = fractionated Ee with HiTrap DEAE FF; PeDEAE = fractionated Pe with HiTrap DEAE FF; RFU = relative fluorescent unit; CAT = catalase; GSH = reduced glutathione; NAD(P)H = nicotinamide adenine dinucleotide; H_2O_2 = hydrogen peroxide.

Introduction

Mucus of the earthworm is a primary humoral immune defense mechanism. The earthworm's mucus is a crucial factor for earthworm survival and protecting the earthworm

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from the environment. The respiration, stress reduction and detoxification mechanisms of the earthworm are related to the mucus. The earthworm mucus is secreted from glandular cells located in the epidermis layer. The three types of glandular cells secrete different biomolecules. The orthochromatic cells secrete a neutral mucopolysaccharides complex as well as protein, pheromones for mating, and a lubricant for motion[1]. Carboxylated, slightly sulfated mucus is produced from the metachromatic cells that provide a respiratory film. Small granular cells, which are protein-rich, secrete an acidic mucus film that adjusts water retention[2]. It has been identified and reported that the mucus contains antimicrobial peptide, hemolytic protein, fibrinolytic enzymes, anti-cancer and anti-oxidant proteins[3-5]. This evidence shows that earthworm mucus consists of many active molecules. Most previous research of active earthworm proteins was studied in *Eisenia fetida* and *Lumbricus rubellus*.

Free radicals or reactive oxygen species (ROS) are a group of unstable molecules that easily react with cellular substances. ROS are generally generated during cellular respiration or another metabolism[6]. The dramatic increase of cellular ROS can accelerate cell structure damage. The general targets of ROS are active macromolecules such as lipid, proteins, DNA and RNA[7,8]. The causes of many diseases are ROS, such as hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), oxygen singlet ($^1O_2$), hypochlorite, nitric oxide (NO) radical, and peroxynitrite ($ONOO^{\bullet-}$) radical[9]. Most ROS are generated by mitochondria and peroxisome[10]. ROS causes the oxidative stress that contributes to many groups of diseases such as inflammation, ischemia, hemochromatosis, acquired immunodeficiency syndrome, emphysema, gastric ulcers, hypertension, preeclampsia, and neurological disorders[11]. The free radicals need to be balanced by anti-oxidant molecules by donating an electron to neutralize those free radicals[12].

ROS can be detoxified by enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase) and non-enzymatic anti-oxidants (ascorbic acid, glutathione, melatonin, and tocopherols)[13]. Superoxide dismutases (SODs) are generally found in an extracellular matrix of aerobic cells[14,15]. The major families of SOD previously reported are copper and zinc binding SOD (CuZn-SOD), iron binding SOD (Fe-SOD) and manganese binding SOD (Mn-SOD)[16]. These three SODs were presented in different locations within cells. Mn-SOD is presented in mitochondria. Fe-SOD is found in chloroplasts and peroxisomes while CuZn-SOD is found in cytosol, chloroplasts, peroxisomes, and apoplasts[17,18]. The enzymatic anti-oxidation mechanism of SOD is that it recognizes and neutralizes $O_2^{\bullet-}$ and generates H_2O_2. Hydrogen peroxide is then neutralized by catalase (CAT) in peroxisomes or glutathione peroxidase (GPx) with glutathione in mitochondria. The product of this reaction is water and oxygen gas[19,20].

In this study, we investigated the anti-oxidation activities of mucus proteins from *Perionyx excavatus* (Pe) and *Eudrilus eugeniae* (Ee). Pe and Ee mucus was extracted and fractionated by anion exchange chromatography. The fractions demonstrating high SOD and GPx-liked activities were selected and were further investigated for their cellular anti-oxidation activity including ROS scavenging, ROS protecting, and repairing of ROS damaged cells in the MC3T7 cell line. Moreover, the cellular SOD and GPx activities of MC3T3 were treated and evaluated with the similar conditions (ROS scavenging, ROS protecting, and repairing of ROS damaged cells).

Materials and Methods

Mucus Extraction

Earthworms were provided by Professor Dr. Somchai Chantsavang, Department of Animal Science, Faculty of Agriculture, Kasetsart University (Bangkok, Thailand). This study was approved in the Ethical treatment of earthworms by the Institutional Committee under the ID ACKU59-SCI-017. Prior to mucus extraction, the earthworms were isolated from their bedding and washed by 0.01 M phosphate-buffered saline (PBS) (Sigma-Aldrich®, St. Louis, MO, USA) pH 6.5 followed by the addition of PBS at a ratio of 1:1 (g of fresh weight/mL of PBS). The extraction of mucus from the earthworms was achieved by electricity at 10 mA for 2 min and then cleansed of bedding by centrifugation. 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 min. The supernatants containing mucus proteins were collected and stored at -20ºC until utilization.

Mucus Proteins Precipitation

The mucus proteins in the supernatant of Ee and Pe were precipitated by 85% ammonium sulfate at 4ºC overnight with stirring, then centrifuged at 10,000 rpm at 4ºC for 45 min. The obtained pellets were dissolved in deionized distilled water (DDW). The sample solutions were desalted with DDW at 3 times the sample solution volume and changed to 0.01 M PBS with pH 7.5 by Vivaspin 20 with 3KDa molecular weight cut-off (GE Healthcare, Marlborough, MA, USA). The desalted samples were stored at -20ºC.

Protein Fractionation by FPLC

The crude Ee and Pe mucus proteins were fractionated by ion exchange chromatography with fast protein liquid chromatography (FPLC, GE Healthcare, Marlborough, MA, USA). The ion exchange chromatography medium was HiTrap DEAE FF and HiTrap ANX FF (GE Healthcare, Marlborough, MA, USA). The mucus protein solutions were dialyzed in 20 mM Tris-HCl pH 7.5. The elution buffer was 20 mM Tris-HCl with pH 7.5 and 1 M NaCl. The proteins were eluted by the gradient from 0-100% elution buffer at 4ºC. 200 µl of 40 mg/mL clear crude mucus protein were loaded into each of 2 equilibrated purification columns. In the HiTrap ANX FF column, flow through was collected for 3 min. The gradient of elution buffer was then increased from 0 to 100% over 19 min. In the HiTrap DEAE FF column, flow through was collected for 5 min. The gradient of elution buffer was then increased from 0 to 100% over 10 min. The Ee and Pe protein fractions were collected and verified using 15% glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Enzymatic Anti-oxidation Activity Superoxide dismutase (SOD) assay

The SOD-liked activity of the Ee and Pe fractionated mucus proteins was determined by colorimetric method using a SOD determination kit (Sigma-Aldrich®, St. Louis, MO, USA). The assay is based on the reduction rate of superoxide anion ($O_2^{\cdot-}$) by SOD. The $O_2^{\cdot-}$ was 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 reduction rate of $O_2^{\cdot-}$ is linearly related to the XO activity, and is inhibited by SOD. Therefore, inhibition activity of SOD or SOD-liked materials can be determined by a colorimetric method. The Ee and Pe fractions were tested for their SOD-liked activity at 4.17 µg/mL final concentration.

The WST working solution was prepared by dilution of 1 mL of WST solution with 19 mL of 1XPBS buffer solution. Then, the enzyme solution was prepared by centrifugation of the enzyme tube for 5 s and mixed well using a pipette. Then, 15 µL of SOD enzyme solution was added to 2.5 mL of dilution buffer. Ee and Pe sample solutions (20 µL) were added to each well sample and blank of a 96 well microplate. A solution comprised of 200 µL of WST working solution plus 20 µL of enzyme working solution was added to each sample and blank. There were 3 blank conditions in this assay. Blank 1 included DW (20 µL), WST working solution (200 µL) and dilution (20 µL). Blank 2 was a mixture of sample solution (20 µL), WST working solution (200 µL) and dilution buffer (20 µL). Blank 3 included DW (20 µL), WST working solution (200 µL) and dilution buffer (20 µL). The wells were mixed thoroughly and incubated at 37°C for 20 min. After incubation, the absorbance was determined at 450 nm. The SOD-liked activity (% inhibition rate) levels of each fraction were calculated by using the following equation.

$$\text{SOD activity (\% inhibition rate)} = \frac{[(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})]}{(A_{blamk1}-A_{blank3})} \times 100$$

Glutathione Peroxidase (GPx) Assay

GPx-liked activity levels of fractionated Ee and Pe were clarified using Glutathione Peroxidase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The concentrations of the Ee and Pe fractionated proteins were tested in a final concentration of 5.26 µg/mL to compare levels of the GPx-liked activity. The absorbency of NADPH (ΔA340), was calculated at the starting point and at 20 min, and calculated as in equation (I). The GPx reaction rate at 340 nm was determined using the NADH extinction coefficient of 0.00373 µM-1. 1 unit was defined as the amount of enzyme that would cause oxidation of 1 nmol of NA-

DPH to NADP+ per min at 25ºC calculated using the following equation (II).

$$\Delta A_{340}/\text{min} = \frac{(A_{340}(\text{second point}) - A_{340}(\text{first point}))}{\Delta t} \quad \text{(I)}$$

$$\text{GPx activity} = \frac{\frac{\Delta A340}{\text{min}} \times 0.19 \text{ ml} \times \text{sample dilution}}{0.00373\ \mu M^{-1} \times 0.02 \text{ mL}} \quad \text{(II)}$$

Cell Culture and Cytotoxic Assay

MC3T3 subclone 4 (ATCC® CRL-2593™) is an adherent preosteoblast cell of *Mus musculus* (mouse). MC3T3 was cultured in α-MEM medium (GIBCO® BRL, Gaithersburg, MD, USA), 1% penicillin and streptomycin mix and 10% fetal bovine serum (FBS) (Gibco® BRL, Gaithersburg, MD, USA). The cells were propagated in a tissue culture flask (Corning®, Corning, NY, USA) at 37ºC in a humidified atmosphere incubator with 5% CO_2. Sub-culturing of cells was carried out every 2-3 days by trypsinization with 0.25% trypsin-EDTA to allow detachment of cells. Detached cells were aspirated and dispended into new culture flasks. Fresh culture medium was added. Cells were maintained in the culture conditions described above.

The MC3T3 was selected to study cellular anti-oxidation activity of Ee ANX1 and Ee ANX4. Prior to the anti-oxidation experiment, cytotoxicity of Ee and Pe protein fractions was evaluated in the cells. The overnight culture of MC3T3 cells was trypsinized using 0.025% trypsin-EDTA. Cells were seeded into a micro-plate at a concentration of 5×10^5 cells/mL by 200 µL and left to grow overnight under standard culture condition. The medium was discarded and the cells were separately treated with EeANX1 and EeANX4 at five concentrations (5, 10, 15, 20, and 30 µg/mL) prepared by dilution with fresh α-MEM medium without fetal bovine serum. The treated cells were incubated at 37ºC in 5% carbon dioxide incubator for 24 hours. The medium was discarded and the cytotoxicity was detected using the Cell Proliferation Reagent WST-1 (Roche, Branchburg, NJ, USA) at 1:10 of the corresponding media. The cells were further incubated at 37ºC in 5% CO_2 for 30 min and protected from light. The reactions were measured using a microplate reader (Tecan infinite®, Morrisville, NC, USA) at the absorbance of 430 nm. The obtained optical density (OD) values were used to calculate percentages of cell viability.

Determination of Enzymatic Anti-oxidation induction Activity of EeANX1 and EeANX4

MC3T3 cell suspension at 5×10^5 cells/mL concentration was seeded at 500 µL into a 24-well plate and cultured overnight at 37ºC in 5% CO_2 incubator. The culture medium was discarded and the cells were treated with 500 µL of 20 µg/mL EeANX1 and EeANX4 for 24 hours. At the end of the treatment time, cells were washed 3 times using 200 µL DPBS to remove the sample residue. The treated cells were trypsinized with 200 µL of 0.025% trypsin-EDTA. 200 µL of fresh α-MEM was added into each well to stop trypsin activity. The cells were then washed using DPBS for 3 times. 500 µL of 20 mM Tris-HCl pH 7.5 and 4 µL of 100 mM PMSF were added. The cell lysis was conducted on ice with sonication (amplitude 50% with pulse alternating between on for 40 s and off for 10 s for a total of 3 min). The proteins from the cell lysates were collected with centrifugation at 10,000 rpm for 30 min.

Cellular SOD Activity Induction by EeANX1 and EeANX4

The cellular anti-oxidant capacity was determined by measuring SOD activity on EeANX1 and EeANX4 from treated MC3T3 lysates using the 19160 SOD determination kit (Sigma-Aldrich®, St. Louis, MO, USA). EeANX1 and EeANX4 samples (20 µL each) were added into each sample well and blank 2 wells. 20 µL of ddH_2O (double distilled water) was added into blank 1 and blank 3 wells. The 200 µL of WST working solution was mixed into each well. Then, the 20 µL of dilution buffer was added into the blank 2 and blank 3 wells. 20 µL of enzyme working solution was mixed in the sample and blank 1 well. All reactions were incubated at 37ºC for 20 min. The absorbencies were then read at 450 nm using a microplate reader (Tecan infinite®, Morrisville, NC, USA). The SOD-liked activity (% inhibition rate) levels of each fraction were calculated using the following equation.

$$\text{SOD activity (\% inhibition rate)} = \frac{[(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})]}{(A_{\text{blamk1}} - A_{\text{blank3}})} \times 100$$

Cellular GPx Activity induction by EeANX1 and EeANX4

The cellular GPx activity of the treated MC3T3 lysates was determined using a Glutathione Pe-

roxidase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The GPx activity was evaluated from the level of NADPH measured absorbance reading at 340 nm using a plate reader at time zero, then 5-min intervals for a total of 5-time points (20 min total).

Determination of Cellular ROS Scavenging Activity of EeANX1 and EeANX4

200 µL of 5×10^5 cells/ml of MC3T3 cell suspension were prepared in microcentrifuge tubes and the culture medium was then discarded. The positive control was mixed with 250 µM pyocyanin (Sigma-Aldrich®, St. Louis, MO, USA) and a staining reagent (2:2500) (Sigma-Aldrich®, St. Louis, MO, USA) in 200 µL. The negative control was mixed with 250 µM pyocyanin, a staining reagent and 10 mM NAC in 200 µL. Both the positive and negative controls were compared to the treatment and non-treatment conditions. The activities of the treatment conditions with EeANX1 and EeANX4 were investigated using 20 µg/mL EeANX1 and EeANX4 solutions mixed with 250 µM pyocyanin and diluted staining reagent (2:2500). These reactions were incubated for an hour at 37°C. Cells were then collected by centrifugation at 400×g for 5 min. 200 µL of 3 µg/mL Hoechst (Sigma-Aldrich®, St. Louis, MO, USA) was added to each reaction and incubated at 37°C for 20 min for nuclei staining. After incubation, the cells were washed with 200 µL of washing buffer for 3 times. The levels of cellular total ROS and $O_2^{\cdot-}$ were detected using an InCell Analyzer 2200 (GE Healthcare, Marlborough, MA, USA).

Determination of Cellular ROS Protecting Activity of EeANX1 and EeANX4

500 µL of 5×10^5 cells/mL of MC3T3 cell suspension was seeded into a 24 well plate and incubated at 37°C overnight. The culture medium of the positive control and non-treatment reactions was then discarded and 500 µL of fresh medium culture was added to both the positive control and non-treatment reactions and incubated at 37°C overnight. Also, MC3T3 cells were treated with 500 µL of 10 mM NAC and incubated at 37°C overnight as a negative control. Additionally, MC3T3 cells were treated with 500 µL of 20 µg/mL EeANX1 and EeANX4 and incubated at 37°C overnight. Next, the medium was discarded and the treated cells were washed twice with DPBS (Gibco® BRL, Gaithersburg, MD, USA). These treated cells were trypsinized with 200 µL 0.025% trypsin-EDTA. Cells were collected by centrifugation at 400×g for 5 min. Then, 200 µL of α-MEM were added to stop the trypsin activity. Non-treated cells were stained with green and orange fluorescent dyes for an hour. 250 µM pyocyanin was mixed with green and orange fluorescent dyes and 200 µL of the mixture was added to the treated samples, positive and negative controls for an hour at 37°C. The cells in each reaction were further treated with 200 µL of 3 µg/mL Hoechst (Sigma-Aldrich®, St. Louis, MO, USA) for 20 min to stain the nuclei. The cells were then washed 3 times with 200 µL washing buffer and 200 µL of DPBS were added to accommodate visualization with the InCell Analyzer 2200.

Determination of Repairing Activity Against ROS-Induced Damage of EeANX1 and EeANX4

500 µL of MC3T3 cells (5×10^5 cells/mL) were seeded into a 24 well plate and incubated at 37°C overnight. The culture medium of all groups (treatment, positive and negative control reactions) was then discarded and the cells were further incubated with 500 µL of 250 µM pyocyanin for 1 h at 37°C. The culture medium of the non-treated cells was discarded and replaced with 500 µL fresh α-MEM and incubated for 1 h at 37°C. Negative reactions were mixed with 500 µL of 10 mM NAC and incubated overnight at 37°C. Treatment reactions were incubated overnight at 37°C with 500 µL of 20 µg/mL EeANX1 and EeANX4. Positive control and non-treated were incubated with 500 µL of the culture medium overnight at 37°C. The culture medium in all reactions was then discarded and the cells were washed 3 times with 200 µL of DPBS followed by trypsinization with 0.025% trypsin-EDTA. The trypsin was discarded and replaced with 200 µL of α-MEM to stop trypsin activity. The trypsinized cells were stained with green and orange fluorescence dye for 1 hour. The cells were further treated with 200 µL of 3 µg/mL of Hoechst for 20 min to detect cellular ROS level using the InCell Analyzer 2200.

Cellular SOD and GPx Activities of EeANX1 and EeANX4 on ROS Scavenging, Protecting and Repairing conditions

500 µL of 5×10^5 cells/mL cultured MC3T3 were seeded into a 24 well plate and cultured overnight in an incubator at 37°C. For positive control reactions, the culture medium was discarded and replaced with 500 µL of fresh medium and incubated for one hour at 37°C. The cells collected from this reaction were used as a

negative control. For negative control reactions, the culture medium was discarded and replaced with 500 µL of 250 µM pyocyanin and incubated for 1 h at 37°C. For ROS scavenging condition the seeded cells were treated with a 500 µL mixture of 250 µM pyocyanin with 20 µg/mL EeANX1 and a 500 µL mixture of 250 µM pyocyanin with 20 µg/mL EeANX4 1 h as ROS scavenging. For repairing of ROS damaged cell condition, the seeded cells were treated with 250 µM pyocyanin for 1 h. After that, the pyocyanin was discarded and the cells were treated with 500 µL of 20 µg/mL of EeANX1 and EeANX4 and incubated overnight at 37°C. For the protection of ROS damaged cells condition, the cells were treated with 500 µL of 20 µg/mL of EeANX1 and EeANX4 and incubated overnight at 37°C. The EeANX1 and EeANX4 were discarded. The cells were then treated 500 µL of 250 µM pyocyanin for 1 h at 37°C. The treated cells from all conditions were washed with 500 µL of DPBS 3 times. Cells from the ROS repairing and protection conditions were trypsinized with 200 µL of 0.025% trypsin-EDTA. The trypsin was then discarded and replaced with 200 µL of α-MEM to stop trypsin activity, then the medium was discarded and replaced with 500 µL of 20 mM Tris-HCl pH 7.5 and 4 µL of 100 mM PMSF. Cell lysis was conducted on ice with sonication (amplitude 50% with pulse alternating between on for 40 s and off for 10 s for a total of 3 min). The proteins from the cell lysates were collected with centrifugation at 10,000 rpm for 30 min. The extracted proteins from cell lysates were evaluated for levels of cellular SOD activities with the 19160 SOD determination kit and cellular GPx activities with the Glutathione Peroxidase Assay Kit.

Statistical Analysis

The results are shown as the mean ± standard deviation (SD). The significant difference between means was determined using the one-way ANOVA with Tukey's multiple comparison test. The $p \leq 0.05$ was considered statistically significant. The non-linear curve fit was generated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Protein Fractionation by FPLC

The fractionation of both crude mucus proteins was performed by HiTrap ANX FF and HiTrap DEAE FF columns. The protein fractions of Ee separated by HiTrap ANX FF yielded four fractions, namely EeANX 1, 2, 3, and 4 based on their different retention times. Each fraction was compared with the crude mucus protein of *E. eugeniae* (Ee) by using 15% glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein patterns of these four fractions were different from crude Ee while patterns of EeANX1 resembled EeANX4. Moreover, EeANX1 and 4 exhibited higher purity than those of EeANX2 and 3. The Ee fractions separated by HiTrap DEAE FF columns were collected for two fractions as EeDEAE 1 and 2. The protein patterns of these two fractions were different. The fractionation of *P. excavatus* (Pe) crude mucus proteins was also performed by HiTrap ANX FF and HiTrap DEAE FF columns. The fractionation of Pe by HiTrap ANX FF was done but no protein was detected in the elution fraction (data not shown). Pe was further fractionated by HiTrap DEAE FF columns. The fractions were collected for three fractions named PeDEAE 1, 2 and 3 in which their protein patterns were similar. The results showed that 45 kDa protein appeared in the only PeDEAE2. The 60 kDa protein was shown in PeDEAE1 and 2. The detection of 28 kDa protein was found in PeDEAE1 and 2 but was not detected in PeDEAE3 (Figure 1).

SOD-liked Activity of Ee and Pe Fractions

Each protein fraction of Ee and Pe exhibited different levels of SOD-liked activity. The highest SOD-liked activity was found in EeANX1 while EeDEAE1 and PeDEAE1 exhibited a lower level. The others fractions demonstrated lower levels. PeDEAE1 showed significant higher SOD-liked activity than those of Ee, Pe, PeDEAE2 and PeDEAE3 (Figure 2).

GPx-liked Activity of Ee and Pe Fractions

The GPx-liked activity of crude Ee, Pe and their fractions were found at different levels (Figure 3). It was noticed that Ee exhibited a greater GPX activity than Pe. Among all 11 samples, the highest level of GPx-liked activity was discovered in EeANX4 which was significantly greater than those of Ee, EeANX1, EeANX2 as well as Pe and all their fractions. Most fractions including EeANX1, EeDEAE1 and EeDEAE2 exhibited their high GPx-liked activity in the rage of 10 to 15 units. The lower GPx-liked activity was

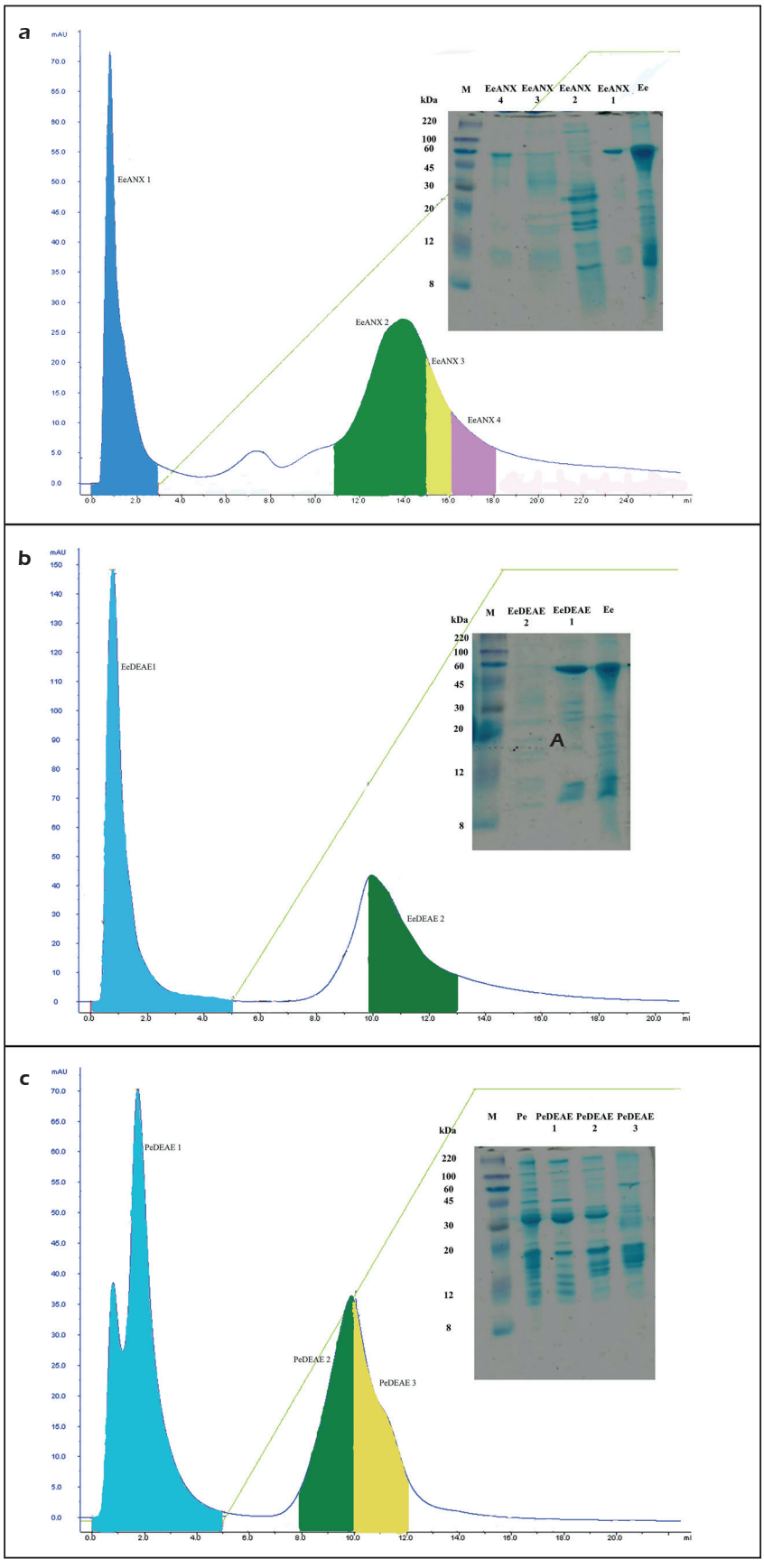

Figure 1. Purification of *E. eugeniae* and *P. excavatus* crude mucus proteins by FPLC. The crude mucus proteins of *E. eugeniae* was fractionated in different columns with HiTrap ANX FF and HiTrap DEAE FF. These fractions were collected and the protein patterns were verified with 15% glycine SDS-PAGE as shown in Figures 1a and 1b. *P. excavatus* crude mucus protein was fractionated in the HiTrap DEAE FF column. The fractions were collected and the protein patterns were verified with 15% glycine SDS-PAGE illustrated in Figure 1c.

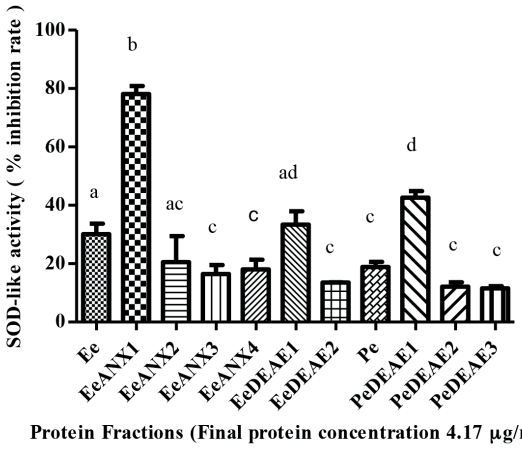

Figure 2. SOD-liked activity of Ee and Pe fractions using FPLC. These nine fractions of Ee and Pe were evaluated for their SOD-liked activity levels comparing between Ee and Pe at the same final protein concentration of 4.17 µg/mL. The lower case letters located at the top of the bars indicate the results of Tukey's multiple comparison test with $p<0.05$ indicating significantly different levels of SOD-liked activity.

found at less than 8 units in Pe, PeDEAE1, PeDEAE2 and PeDEAE3 fractions.

Cytotoxicity of EeANX1 and EeANX4 against MC3T3

EeANX1 at a final protein concentration of 5-500 µg/mL did not produce a cytotoxic effect on MC3T3 cells indicated by a viability rate of greater than 100%. The percentage of MC3T3 viability remained stable following treatment with EeANX4 (at 5-25 µg/mL final protein concentration) and was over 100%. A slight decrease of MC3T3 cell viability was observed when EeANX4 was tested up to 25-300 µg/mL final protein concentrations.

Cellular ROS Scavenging Activity of EeANX1 and EeANX4

EeANX1 and EeANX4 revealed their cellular ROS scavenging activity against superoxide ($O_2^{\cdot-}$) radicals in MC3T3 (Figures 4 and 5). The $O_2^{\cdot-}$ production of each condition was significantly different with $p<0.05$. The highest $O_2^{\cdot-}$ production was found in MC3T3 cells treated with 250 µM pyocyanin (ROS inducer). The $O_2^{\cdot-}$ production in non-treatment (control) and 10 mM NAC conditions was higher than that of 20 µg/mL EeANX1 and EeANX4. Treatment at a similar dose (20 µg/mL), EeANX1 produced lower levels of $O_2^{\cdot-}$ than that of EeANX4. Therefore, the EeANX1 and EeANX4 fractions showed better $O_2^{\cdot-}$ scavenging activity than NAC (positive control). The total ROS production of pyocyanin treatment was significantly higher while the total ROS production level of non-treatment and EeANX1 was similar and lowest. The total ROS production of 10 mM NAC and EeANX4 were also similar. The EeANX1 demonstrated the best total ROS scavenging and EeANX4 efficiently scavenged total ROS as well as NAC (anti-oxidant).

Protecting Activity against Cellular ROS of EeANX1 and EeANX4

The highest significant $O_2^{\cdot-}$ and total ROS production levels were found in 250 µM pyocyanin. The level of $O_2^{\cdot-}$ production in non-treated cells was similar to that of 10 mM NAC and 20 µg/mL EeANX4 treatments. The $O_2^{\cdot-}$ production of MC3T3 treated with EeANX1 showed significantly higher than that of EeANX4 (when treated at the same dose of 20 µg/mL). The similar total ROS production levels were found in 10 mM NAC and 20 µg/mL EeANX4 that were significantly lower than that of 20 µg/mL EeANX1. Non-treatment condition showed significantly lower total ROS production. EeANX1 clearly demonstrated the highest reduction in $O_2^{\cdot-}$ and total ROS production (Figures 6 and 7).

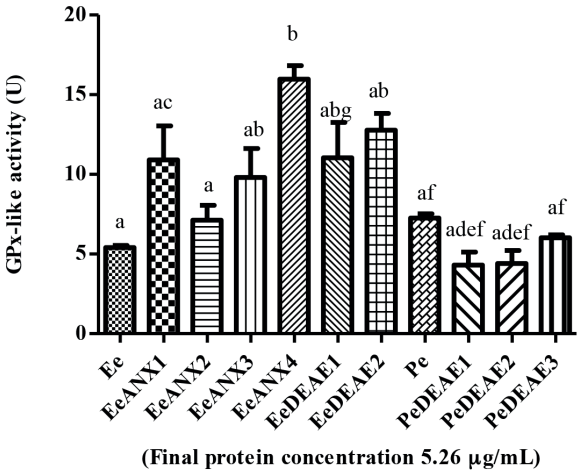

Figure 3. Evaluation of GPx-liked activity levels of Ee and Pe fractions using FPLC. The GPx-liked activity of Ee and Pe fractions were tested and compared between Ee and Pe at the same final protein concentration of 5.26 µg/mL. The lower case letters located at the top of the bars indicate the results of Tukey's multiple comparison test with $p<0.05$ indicating significantly different levels. of GPx-liked activity.

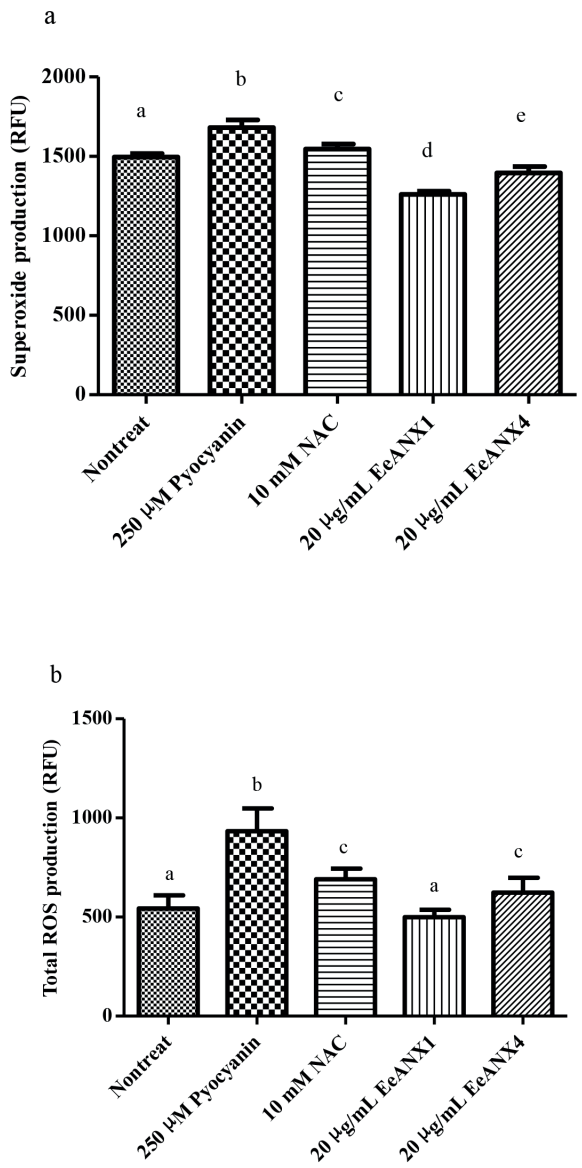

Figure 4. EeANX1 and EeANX4 scavenged cellular superoxide and total ROS. MC3T3 was treated with 20 μg/mL EeANX1 and EeANX4, 250 μg/mL pyocyanin and 10 mM NAC and the cellular superoxide (a) and total ROS production (b) levels were compared with the non-treated condition. Cellular superoxide and total ROS levels were detected using a wide-field fluorescence microscope with a wavelength of 550/620 and 490/525 nm. The cellular superoxide and total ROS levels were tested using the homogeneity test with Tukey's multiple comparison test at $p<0.05$ significant level. The lower case letters located at the top of the bars indicate significantly different superoxide and total ROS levels.

Repairing Activity of Oxidative Damage of EeANX1 and EeANX4

The EeANX1 and EeANX4 repairing activity of ROS damaging MC3T3 were indicated by cellular $O_2^{\cdot-}$ and total ROS production. These $O_2^{\cdot-}$ and total ROS production levels were significantly different with $p<0.05$ between conditions. The results showed that 250 μM pyocyanin treatment induced the highest $O_2^{\cdot-}$ and total ROS production in MC3T3 cells. In the presence of EeANX1 and EeANX4, lower $O_2^{\cdot-}$ production (1,057.18 ± 5.95 and 1,226.95 ± 12.80 RFU) was found when compared to pyocyanin treatment (1,472.76 ± 17.47 RFU). The lowest total ROS production (405.61 ± 3.05 RFU) was found in the non-treatment condition. The total ROS production levels of EeANX1 (502.80 ± 4.25 RFU) were lower than that of EeANX4 (605.02 ± 8.18 RFU), and both conditions showed lower levels of total ROS production from 10 mM NAC condition (701.44 ± 8.79 RFU) (Figures 8 and 9).

EeANX1 and EeANX4 Induced SOD Activity in MC3T3

The SOD activity of each treatment was significantly different with $p<0.05$. The obtained results indicated that EeANX1 fraction (42.13 ± 0.71%) significantly exhibited the highest induction of SOD activity in MC3T3 while cellular SOD activity of EeANX4 (34.69 ± 0.85%) was significantly higher than that of non-treatment (23.45 ± 1.70%) (Figure 10).

EeANX1 and EeANX4 Induced GPx Activity in MC3T3

Cellular GPx inducing activity of EeANX1 and EeANX4 was detected in MC3T3. The GPx activity of non-treatment condition was significantly lower than that of EeANX1 and EeANX4 with $p<0.05$ while the cellular GPx induction of EeANX1 was similar to EeANX4 (Figure 11).

Antioxidation Property of EeANX1 and EeANX4 on SOD Activity in MC3T3

The cellular SOD scavenging, protecting and repairing activities of MC3T3 treated with EeANX1 and EeANX4 was determined (Figure 12). The SOD activity of conditions containing pyocyanin (250 μM) was significantly higher than other conditions in scavenging, protecting and repairing studies. In the ROS scavenging study, we found that cellular SOD activity in MC3T3 was induced by EeANX4 and was significantly higher than that of EeANX1 and non-treated cells. The protection of ROS damaged cell study found that SOD activity of each condition was significantly different with the lowest SOD activity in non-treatment. Moreover, EeANX4 showed significantly higher SOD

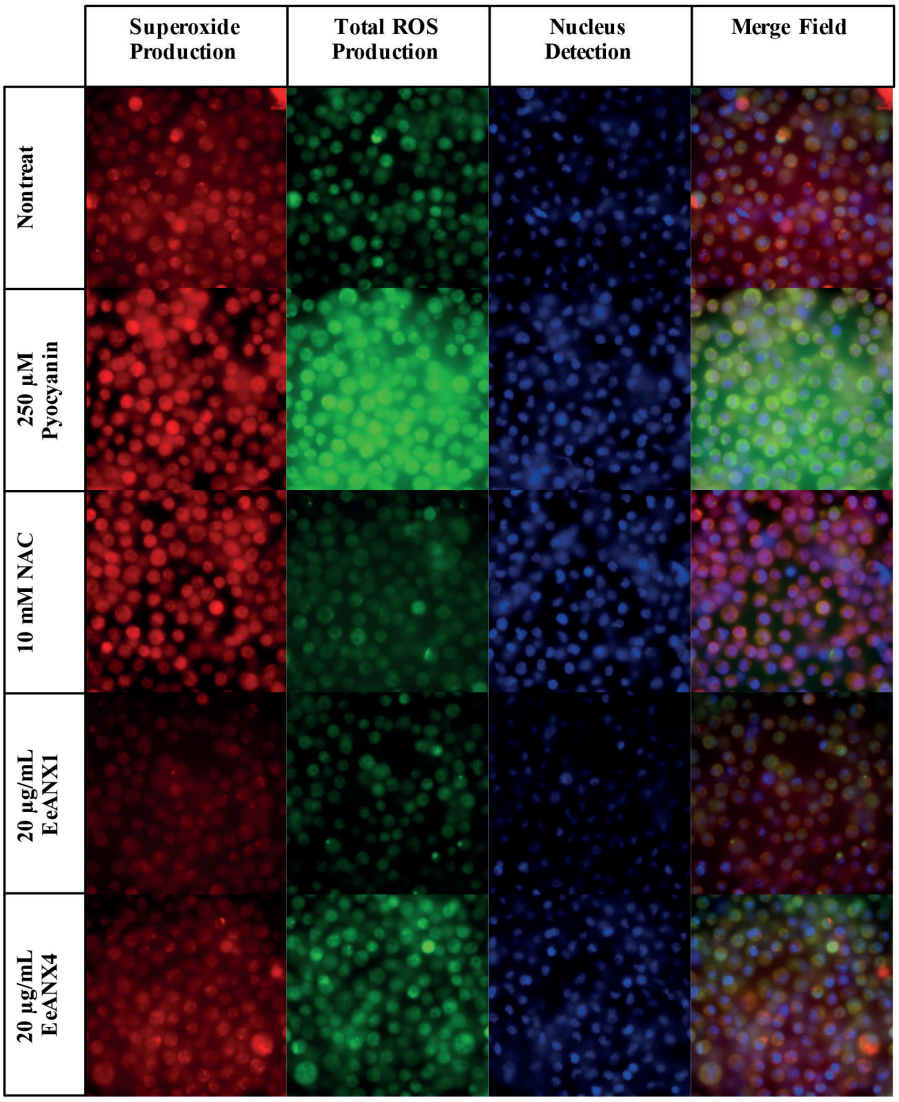

Figure 5. Cellular ROS detection of ROS scavenging condition. Cellular superoxide and total ROS were detected by superoxide detection reagent (orange) and oxidative stress detection reagent (green), which were visualized using a wide-field fluorescence microscope with standard orange (550/620 nm) and green (490/525 nm). The cell number was detected with Hoechst nuclear staining and was visualized with standard cyan filter (358/461 nm).

activity than EeANX1 in the ROS protection study. Repairing of ROS damaged cells was performed and it was found that SOD activity of EeANX1 was significantly higher than that of non-treatment and EeANX4. The SOD activity of EeANX4 resembled to that of non-treatment (Figure 12).

Antioxidation Properties of EeANX1 and EeANX4 on GPx Activity in MC3T3

The effects of EeANX1 and EeANX4 antioxidation activity on cellular GPx activity were evaluated. The cellular GPx activities were studied for scavenging, protection and repairing. The lowest GPx activity was found in pyocyanin treatment of these three experiments. The significant difference of GPx activities was detected in all conditions in the scavenging experiments (Figure 13). The highest GPx activity in the scavenging experiment was found when cells treated with EeANX4. The level of GPx activity of EeANX1-treated cells was significantly higher than non-treated cells. In the ROS protection study, GPx activity of EeANX4 showed the highest level while the GPx activities of non-treatment and EeANX1 were similar and both higher than

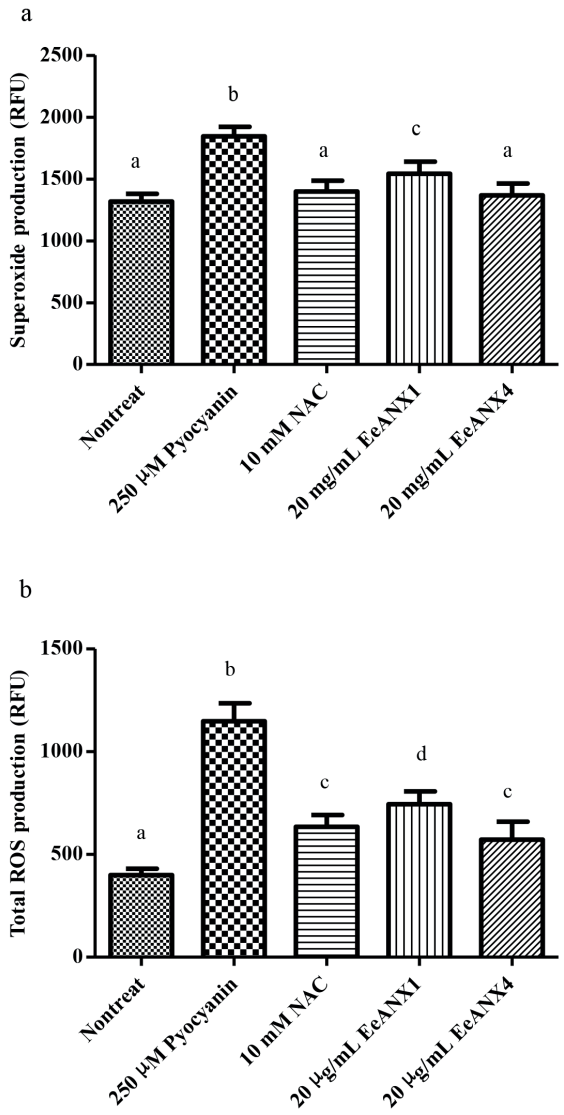

Figure 6. Protecting activity of EeANX1 and EeANX4 against cellular superoxide and total ROS. MC3T3 cells were treated with 20 μg/mL EeANX1and EeANX4, 250 μg/mL pyocyanin and 10 mM NAC and the cellular superoxide (a) and total ROS production (b) levels were compared with the non-treated condition. Cellular superoxide and total ROS levels were detected using a wide-field fluorescence microscope with a wavelength 550/620 and 490/525 nm. The cellular superoxide and total ROS levels were tested using the homogeneity test with Tukey's multiple comparison test at $p<0.05$ significant level. The lower case letters located at the top of the bars indicate significantly different superoxide and total ROS levels.

pyocyanin treatment. The significantly highest level of GPx activity in the repairing experiment was found in EeANX1 and EeANX4 treatments. Non-treatment condition represented higher GPx activity than the pyocyanin treatment (Figure 13).

Discussion

The enzymatic anti-oxidants are a natural protective mechanism against cellular peroxidation reaction. SOD, CAT, and GPx are efficient cellular superoxide scavengers to prevent hydroxyl radical[21]. The previous study investigated the SOD-liked and GPx-liked activities in both of *P. excavatus* (Pe) and *E. eugeniae* (Ee) crude mucus protein. In this research, the IC50 values of SOD-liked activity of Pe and Ee crude mucus proteins were 30.67 ± 0.22 and 12.46 ± 0.19 μg/mL, respectively. It indicated that crude mucus proteins of Ee possessed higher SOD-liked activity than that of Pe. However, it was previously reported that Pe had a higher GPx-liked activity than that of Ee[5]. The potent SOD-liked and GPx-liked activities were discovered in two Ee mucus protein fractions namely EeANX1 and EeANX4, respectively. Therefore, these two fractions were selected for further studies on their biological properties including cytotoxicity and various cellular antioxidation capacities against ROS.

The cellular anti-oxidation capacity of EeANX1 and EeANX4 was studied in the MC3T3 osteoblasts cell line. This cell type was selected according to the previous evidence showing that bone formation and osteoporosis relates to cellular ROS level in osteoblasts[22-24]. Some agents such as ghrelin, actein, and kaempferol, were found in the protective activities of MC3T3[25-27]. Regarding the WST-1 assay, our results suggested that 500 μg/mL of EeANX1 was non-cytotoxic to MC3T3, and a very low cytotoxicity was observed when tested at 300 μg/mL EeANX4.

Pyocyanin toxin is one of the virulence factors of *Pseudomonas aeruginosa*[28]. This toxin was reported for its antagonist effects, including pro-inflammatory and production of cellular ROS[29-32]. Superoxide and hydrogen peroxide are formed by pyocyanin when accepting electrons from NAD(P)H[33]. The anti-oxidant enzymes, SOD, CAT, and GPx exhibited effects on the cellular protective mechanism from ROS induction of pyocyanin[34]. Cellular oxidative stress induction of pyocyanin could increase SOD and CAT activities in order to balance cellular ROS[35]. Corresponding to our study, pyocyanin induced the highest SOD activity. Pyocyanin indicated that it directly oxidized glutathione (GSH) to increase cellular oxidative stress[33]. In our current study, we found that

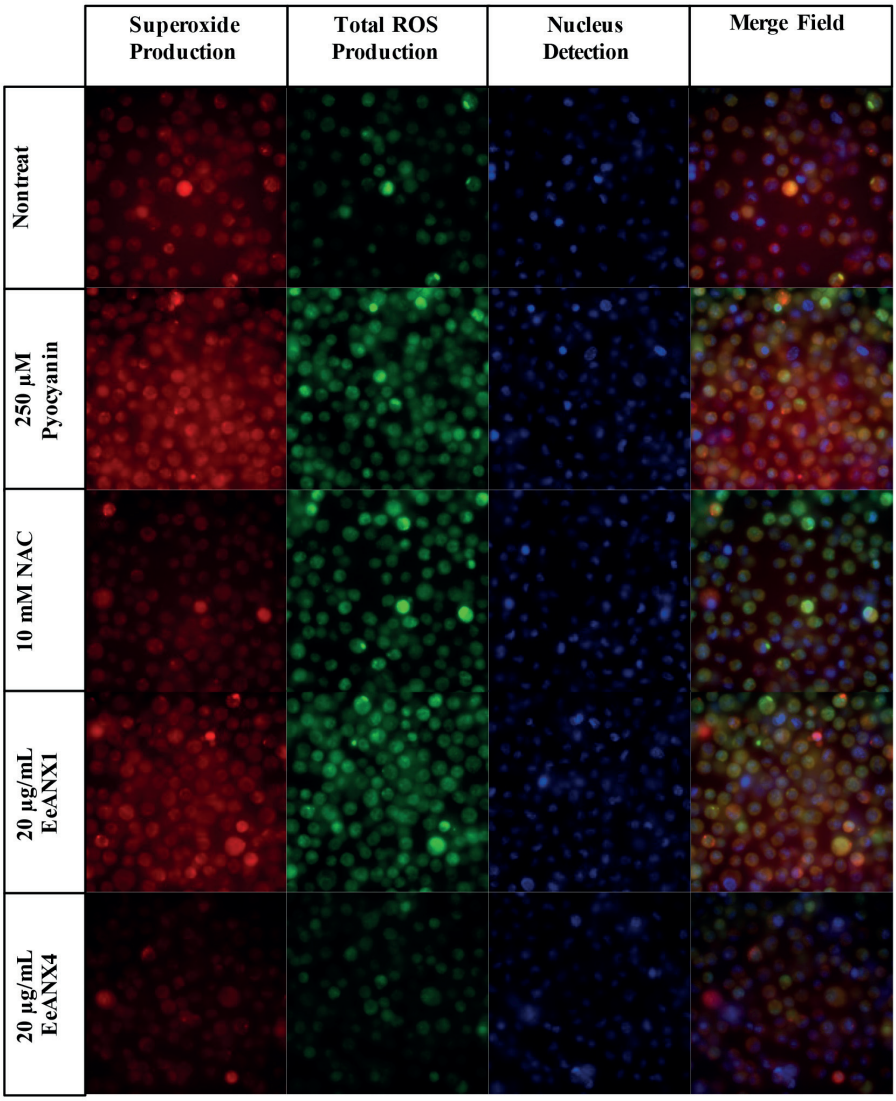

Figure 7. Cellular ROS detection of ROS protection condition. Cellular superoxide and total ROS were detected by superoxide detection reagent (orange) and oxidative stress detection reagent (green) that were visualized using a wide-field fluorescence microscope with standard orange (550/620 nm) and green (490/525 nm). The cell number was detected with Hoechst nuclear staining and was visualized with standard cyan filter (358/461 nm).

pyocyanin decreased GPx activity in non-treated cells.

The antioxidation activities of earthworm powder were studied in some species. Hepatoprotective and antioxidation activities of *Lampito mauritii* earthworm extract were investigated with paracetamol induced hepatotoxicity in rats (Rattus norvegicus). The results indicated that the earthworm extract caused a significant increase of SOD, CAT and GPx activities. The increase in these enzymatic antioxidation activities revealed that the earthworm extract could scavenge and prevent the oxidative damage[36]. It was reported that alcohol-induced hepatotoxicity in rats had been neutralized by *P. excavatus* earthworm powder. This earthworm powder could recover SOD, CAT and GPx activities in the liver and kidneys of rats as much as non-treated rats[37]. We demonstrated in this study the SOD-liked and GPx-liked activities of the crude mucus proteins of Pe and Ee earthworms. The IC50 of Pe and Ee SOD-liked activities was previously reported at 149 and 386.2 µg/mL respectively. The GPx-liked activity was found in

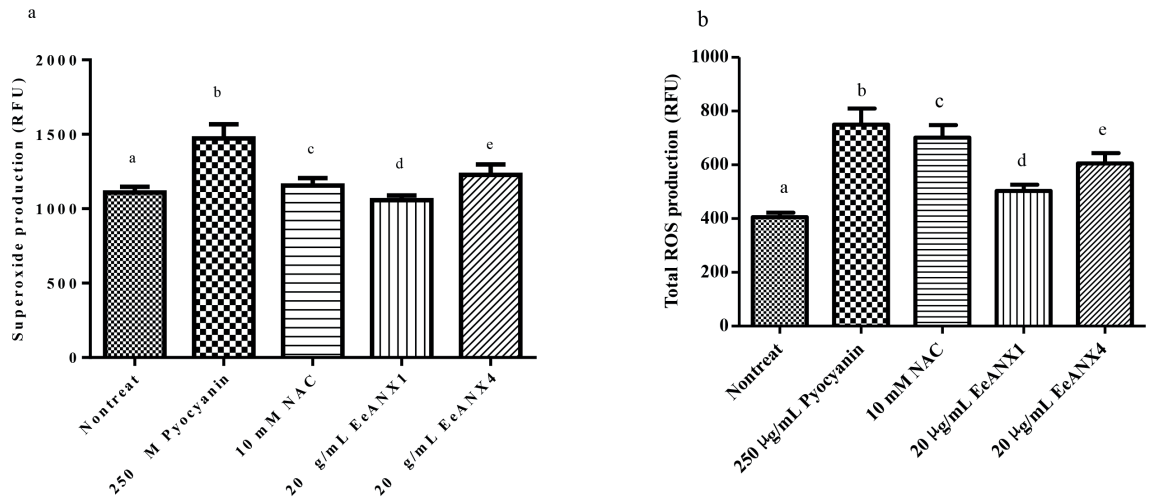

Figure 8. Repairing activity of EeANX1 and EeANX4 in superoxide and total ROS damaged cells. MC3T3 cells were treated with 20 μg/mL EeANX1 and EeANX4, 250 μg/mL pyocyanin and 10 mM NAC and the cellular superoxide (a) and total ROS production (b) levels were compared with the non-treated condition. Cellular superoxide and total ROS levels were detected using a wide-field fluorescence microscope with a wavelength 550/620 and 490/525 nm. The cellular superoxide and total ROS levels were tested using the homogeneity test with Tukey's multiple comparison test at $p<0.05$ significant level. The lower case letters located at the top of the bars indicate significantly different superoxide and total ROS levels.

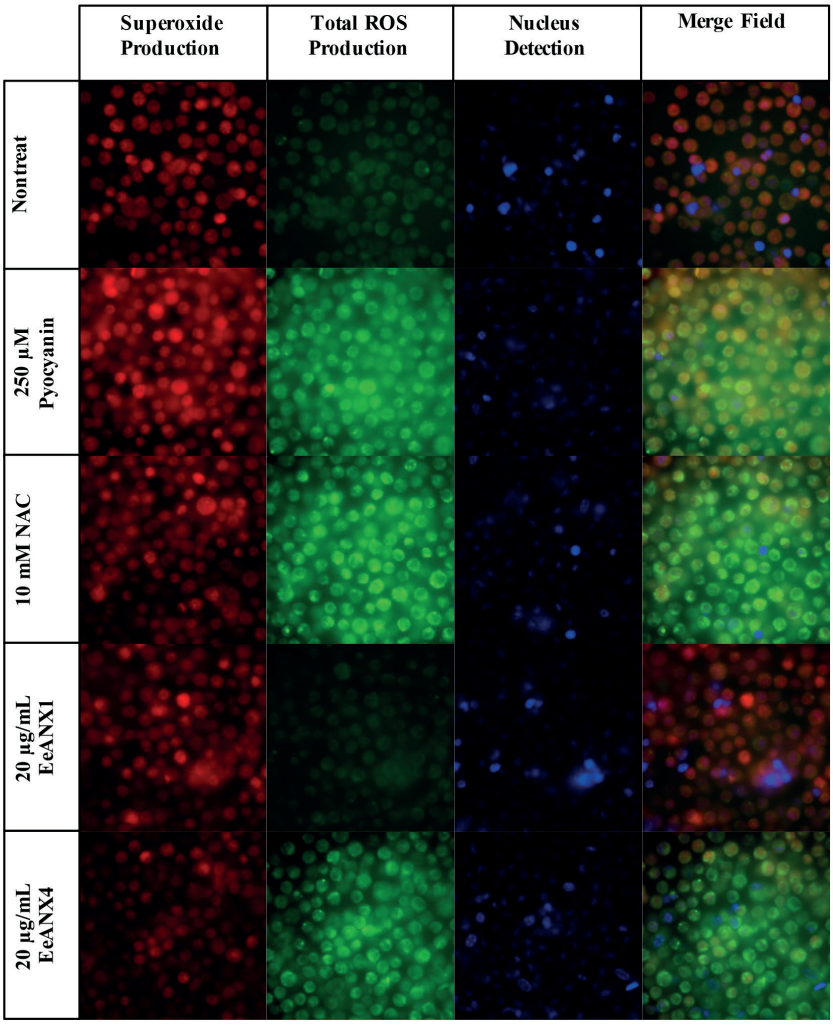

Figure 9. Detection of cellular ROS following repairing of damaged cell condition. Cellular superoxide and total ROS were detected by superoxide detection reagent (orange) and oxidative stress detection reagent (green), which were visualized using a wide-field fluorescence microscope with standard orange (550/620 nm) and green (490/525 nm). The cell number was detected with Hoechst nuclear staining and was visualized with standard cyan filter (358/461 nm).

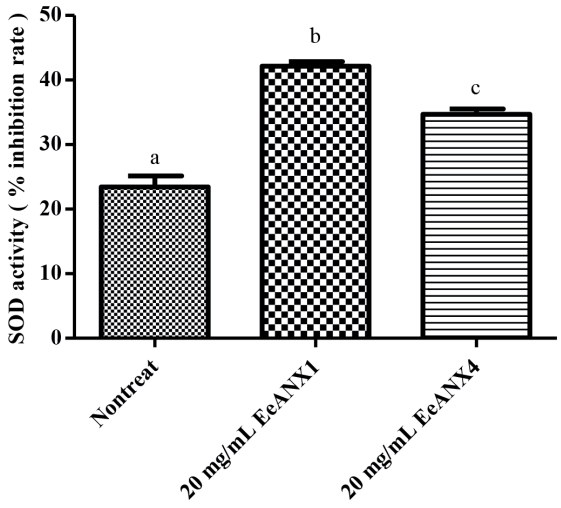

Figure 10. The effects of EeANX1 and EeANX4 induced cellular SOD activity. MC3T3 cells were treated with 20 μg/mL EeANX1 and EeANX4 for 24 hours. In the 4.17 μg/mL final concentration protein lysates from the treated cells SOD activities were detected that were compared with that of the non-treated condition. The significantly different cellular SOD activities were tested using Tukey's multiple comparison test at $p<0.05$ significant level. The lower case letters at the top of each bar indicate significantly different cellular SOD activities.

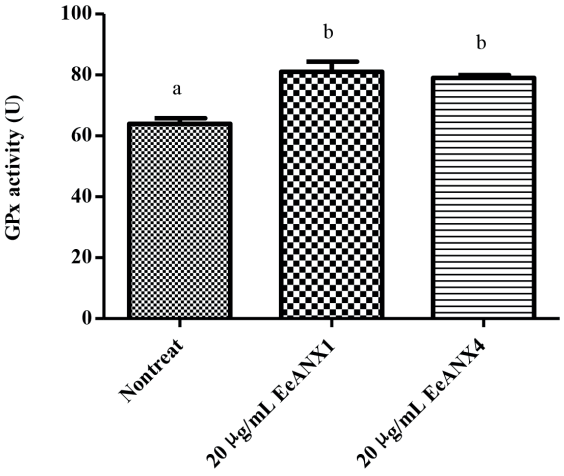

Figure 11. The effects of EeANX1 and EeANX4 induced on cellular GPx activity. MC3T3 cells were treated with 20 μg/mL EeANX1 and EeANX4 for 24 hours. In the 5.26 μg/mL final concentration protein lysates from the treated cells, GPx activities were detected and compared with that of the non-treated condition. The significantly different cellular GPx activities were tested using Tukey's multiple comparison test at $p<0.05$ significant level. The lower case letters at the top of each bar indicate significantly different cellular GPx activities.

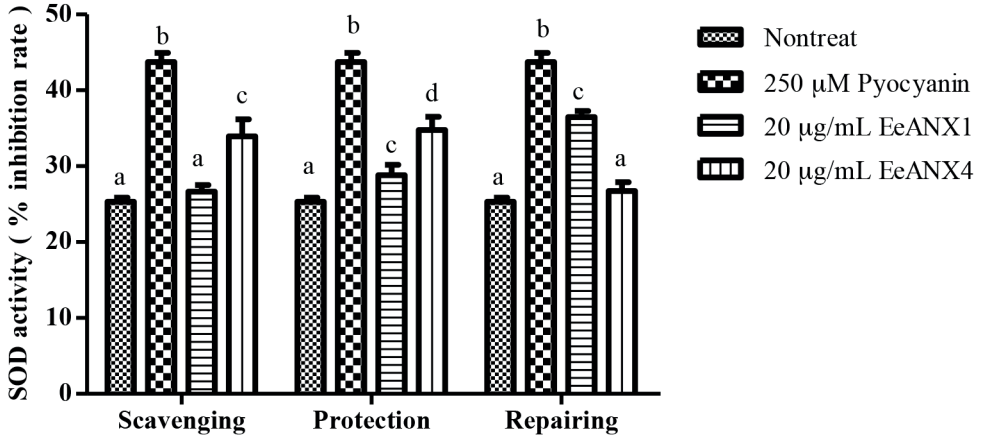

Figure 12. Anti-oxidant property of EeANX1 and EeANX4 investigated on cellular SOD activity on scavenging, protecting and repairing conditions. MC3T3 cells were treated with EeANX1 and EeANX4 at 20 μg/mL following the scavenging, protecting and repairing conditions. MC3T3 were treated with 250 μM pyocyanin as a positive control. The cellular SOD activity was investigated with a 4.17 μg/mL final concentration of MC3T3 lysate. The mean cellular SOD activity levels were analyzed using Tukey's multiple comparison test at $p<0.05$ significant level within each condition. The lower case letters at the top of each bar indicate significantly different levels of cellular SOD activity in each condition.

both Pe and Ee mucus proteins. We previously reported that Pe and Ee illustrated their superoxide and mitochondrial hydrogen peroxide scavenging capability with SOD-liked and GPx-liked activities[5] In the present study, our results indicated that EeANX1 and EeANX4 could elevate SOD activities to be normal or slightly higher than normal levels, while their

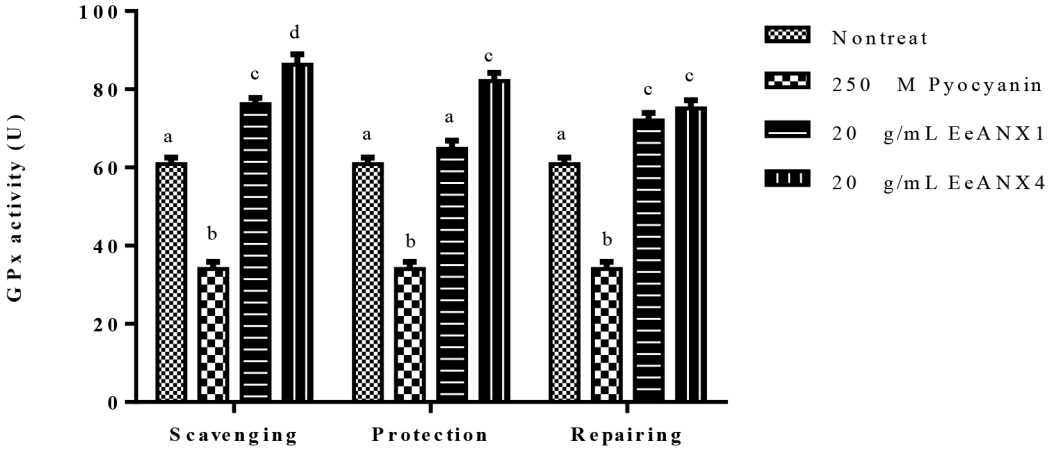

Figure 13. Anti-oxidant property of EeANX1 and EeANX4 investigated on cellular GPx activity on scavenging, protecting and repairing conditions. MC3T3 cells were treated with 20 μg/mL EeANX1 and EeANX4 following the scavenging, protection and repairing conditions. MC3T3 were treated with 250 μM pyocyanin as a positive control. The cellular GPx activity was investigated with a 5.26 μg/mL final concentration of MC3T3 lysate. The mean cellular GPx activity levels were analyzed using Tukey's multiple comparison test at $p<0.05$ significant level within each condition. The lower case letters at the top of each bar indicate significantly different levels of cellular GPx activity in each condition.

activities were lower than that of pyocyanin in scavenging, protecting and repairing conditions. These two fractions already demonstrated ROS scavenging, protecting and repairing of cellular ROS damage in MC3T3 cells.

In the previous reports[36], earthworm extract from *L. mauriti* recovered GPx activity of paracetamol-induced hepatotoxic rats to be normal. Moreover, GPx activity of the liver and kidneys were recovered in alcohol-induced hepatotoxic rats by administration of earthworm powder from *P. excavatus*[37]. In the same way, we discovered that EeANX1 and EeANX4 fractions of *Eudrilus eugeniae* could recover GPx activities to be at normal levels in the scavenging, protection and repairing experiments. The EeANX1 and EeANX4 demonstrated ROS scavenging, protecting, and repairing of cellular ROS damage cells as clearly demonstrated in our present study.

Conclusions

Results of our current study suggested that EeANX1 and EeANX4 fractions from *Eudrilus eugeniae* earthworm possess various biological activities including SOD-liked, GPx-liked, cellular ROS scavenging, and protecting and repairing of cellular oxidative damage activities in MC3T3 osteoblasts. Pyocyanin increased cellular ROS and SOD activity, but reduced cellular GPx activities. In contrast, EeANX1 and EeANX4 induce cellular SOD and GPx activities without cellular ROS induction when tested in MC3T3 cells.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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