Mitodepressive and Genotoxic Potential of Crude Body Wall and Cuvierian Tubule Extracts of Manimani (*Pearsonothuria graeffei* Semper) on Root Meristems of Onion (*Allium cepa* L.)

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Abstract

The need to discover novel natural products has resulted in several explorations in the marine environment. The biological compounds from aquatic resources can boost effort on drug development and may promote species conservation. In this study, the mitodepressive and genotoxic properties of the extracts obtained from Pearsonothuria graeffei – a widely distributed and poorly explored sea cucumber species – were evaluated using the Allium test. The sea cucumber body wall and cuvierian tubule crude methanol, ethyl acetate and n-hexane extracts were found to have potential genotoxic properties at concentrations of 500, 1,000 and 1,500 μg mL^{-1}. A significant ($p < 0.05$) growth inhibition in both length and the number of roots was exhibited at higher concentrations (1,000 and 1,500 μg mL^{-1}) of the crude extracts similar to the control mutagen. The 1,000 μg mL^{-1} of cuvierian tubule methanol extract resulted in higher general toxicity in terms of root length inhibition than the control mutagen. A significantly lower mitotic index was exhibited in the crude body wall methanol extract at 500 μg mL^{-1} concentration. Also, a higher percent chromosomal aberration ($p < 0.05$) was observed in onions treated with all concentrations of crude body wall ethyl acetate and n-hexane extracts, and 500 μg mL^{-1} of the crude cuvierian tubule methanol and n-hexane extracts. In conclusion, the crude extracts of P. graeffei have mitodepressive and genotoxic properties, which are potential sources of bioactive compounds necessary for the development of treatment for some serious human diseases like cancer.

Keywords*: Allium* test, genotoxicity, mitodepressive, *Pearsonothuria graeffei*, sea cucumber

1. Introduction

The sea cucumbers are marine organisms with more than 1,400 species belonging to the phylum Echinodermata (Tehranifard *et al*., 2011). These organisms perform essential functions in the marine ecosystem (Purcell, 2010). Some species are known as source of food in the processed state (Akamine, 2010; Tehranifard *et al*., 2011). In the Philippines, there are about 100 species of sea cucumbers and 25 of them are commonly exploited for trade (Gamboa *et al*., 2007). In fact, the country is already recognized as one of the largest exporters of the known valuable sea cucumber species (Uthicke and Conand, 2005; Gamboa *et al*., 2007). Southeast Asian countries like Indonesia and the Philippines are two of the biggest contributors to sea cucumber production and trade (Southeast Asian Fisheries Development Center, 2012). Research undertakings on sea cucumber conservation and management in five regions, including Asia, identified the Philippines as one of the 'hot spots' owing to the country's active participation in sea cucumber trade in the global market (Choo, 2008; Toral-Granda *et al*., 2008; Tehranifard *et al*., 2011).

In a report by Lawrence *et al*. (2009), *Pearsonothuria graeffei*, a sea cucumber locally known as "mani-mani", "bulaklak", "trompa" or "piña" (Brown *et al*., 2010), is among the third class resource, which is of lowest commercial importance. It is known as the black-spotted sea cucumber as described by the Food and Agriculture Organization (FAO); orange fish or 'Shoab' in Egypt; flowerfish in India, Papua New Guinea and Vietnam; and 'Nool attai' in India (Purcell *et al*., 2012). Presently, there is no existing conservation concern status of this species in the international market except for Papua New Guinea with its regulated fishing season (Toral-Granda, 2006). Also, there is limited information on its biology (Purcell *et al*., 2012) and potential bioactivities (Torres, 2017). From a global standpoint, there is great concern on conservation of *P. graeffei* for conservationists despite its wide distribution worldwide based on the International Union for Conservation of Nature and Natural Resources (IUCN) red list of threatened species report (Conand *et al*., 2013; IUCN, 2013).

Sea cucumbers are believed to possess secondary metabolites with unique chemical properties that are different from those found in terrestrial environments indicating a wide range of possibilities for their unique mode of action (Montaser and Leusch, 2011). The marine-derived bioactive compounds are also often unique and promising novel scaffolds for druggable natural products (Li *et al*., 2008a, 2008b). In a comparative study by Kong *et*

al. (2010), marine natural products were described to be superior to their terrestrial counterparts in terms of chemical novelty. Consequently, marine organisms become potential sources of natural products that can contribute to the development of biological and medical armamentaria. However, research works about the biological properties of the bioactive compounds from sea cucumber are scant, especially in the Philippines, despite the heavy overexploitation of few known valuable species as reported by the Bureau of Fisheries and Aquatic Resources (BFAR) (Brown *et al*., 2010). Available references or publications about sea cucumber species composition in the Philippines are mostly limited to local description (Gamboa *et al*., 2007). The limited pieces of information on the local marine resources like the sea cucumbers emphasize the need for more researches on the species biology and ecology (Choo, 2008). One way to address this issue is through bioprospecting, which adds value (option values) to the species of sea cucumber, which in turn, will open an opportunity for the exploration of new natural product sources with potential bioactivities. Hence, biological properties will provide more evidence for proving its importance, which is essential for future management, protection and conservation efforts.

An important biological property to discover is the potential of a compound from a sea cucumber as a source of new drugs. Toxicity tests hold an important role in the in vitro early screening of potential drugs and proper formulation of effective doses of drugs. Toxicity assays are seen as important reliable indicators for biological evaluation (Bácskay *et al*., 2018). The key concept in discovering the genotoxic property of the crude extracts from *P. graeffei* is not solely geared towards the demonstration of the potential of *P. graeffei* for human consumption as food but also for pharmaceutical purposes intended for human use. Genotoxicity is the ability of any natural or synthetic compound to cause damage to the cell's genetic information. The effect of a genotoxic compound may vary from the intervention in the various proteins that are involved in the replication process and maintenance of chromosomal activity. A genotoxic compound may also possess a property that causes mutations and breaks in the deoxyribonucleic acid (DNA) structure (Ranganatha *et al*., 2016). Many of the discovered drugs used to treat cancer (Gwozdzinski and Lichota, 2018; Swift and Golsteyn, 2014), microbial infection (Brambilla *et al*., 2012; Galdiero *et al*., 2016), wound (Mattana *et al*., 2014) and inflammation (Brambilla and Martelli, 2009) have demonstrated genotoxic properties.

Thus, this study aimed to evaluate the mitodepressive and the genotoxic properties of the crude methanol, ethyl acetate and n-hexane extracts from the body wall and the cuvierian tubule of *P. graeffei* Semper as potential sources of bioactive compounds.

2. Methodology

2.1 Securing of Certification, Clearance and Gratuitous Permit

Certification was obtained from the Department of Environment and Natural Resources (DENR) particularly from the office of Protected Areas and Wildlife and Coastal Management Service (PAWCZMS). The authors also got a clearance to collect samples from the Office of the Mayor of the City of San Fernando, La Union, Philippines and a certification from the City Agriculturist Office. In accordance with the Philippine national law (Section 15 of Republic Act No. 9147 [Wildlife Conservation and Protection Act] and Executive Order No. 247 [Bioprospective Law of 1995]) and BFAR's Fisheries Administrative Order No. 233, the researchers secured the final clearance with the Gratuitous Permit No. 01-14 from the BFAR Regional Office No. 1.

2.2 Sample Collection and Dissection

The sea cucumbers were collected from Poro Point, City of San Fernando, La Union around the coordinates of 16 °35' 34.17" N and 120° 14' 59.75" E. The samples were harvested with the help of the local fishermen along the intertidal and deeper portions ranging from regions within 15-20 ft. The sea cucumbers were temporarily stored in coolers with ice and transported to the Natural Products Laboratory, Don Mariano Marcos Memorial State University South La Union Campus. Upon arrival, they were immediately washed with distilled water to remove sand and other foreign particles from the body walls. *P. graeffei* Semper specimens with the length of 20-50 cm and weight of 30-180 g were collected. Taken using a digital camera (WB350F, Samsung, United States), Figure 1 shows the photographs of the collected sea cucumber samples before the dissection and preservation methods.

Figure 1. Photographs of the collected *P. graeffei* Semper

Dissection was done by separating the body wall and the cuvierian tubules from other sea cucumber body parts. The cuvierian tubules were obtained by disturbing the sea cucumber forcing secretion and by cutting through the tentacle portion of the body. Bulk weighing of the fresh samples and dissected body parts was done using a digital electronic weighing scale (ACS TCS System 3208, Dahongying, China).

2.3 Extraction of the Active Principle for Allium Test

The steps in the preparation of crude extracts were based on the procedure patented by Collin (1999) with few modifications. The present method did not involve maceration using 10% Alcalase after separation of the needed body parts; instead of just utilizing distilled water, various solvents were used. The drying of the isolated body parts of *P. graeffei* was done under 45 °C instead of 40 °C.

The present study also utilized vacuo concentration by rotary evaporator (RE300/MS, Stuart, United Kingdom) instead of the boiling process in an aqueous solution to obtain the crude extracts or fraction. The freshly separated body wall and cuvierian tubules of *P. graeffei* were heated for 30 min in

deionized freshwater at 140 °F. The heated body parts were cooled, later on, cut into smaller pieces and ground using a blender (4172-074, PN30598-074, Oster, Mexico) for 15 to 20 min. After which, each of the ground body parts was dried at 60 °C under an electric oven (HEO-17R, Hanabishi, China) for 24 h.

Drying of the cuvierian tubules was carried out for only 4 h. The dried raw materials were further used for the isolation of active components by soaking 1:10 w/v separately in each of the analytical solvents, namely methanol, ethyl acetate and n-hexane for seven days (Azwinida, 2015; Zhang *et al*., 2018). The samples were stored in the dark at room temperature to prevent possible photolytic action and thermo degradation of metabolites. The soaked samples were then filtered using sterile cheesecloth. To concentrate and remove the solvents, filtrates were evaporated under vacuum at 45 °C through a rotary evaporator with a digital water bath (RE300DB, Stuart, United Kingdom) under 337 psi (methanol), 240 psi (ethyl acetate), 335 psi (n-hexane) and 72 psi (water) for effective evaporation of the remaining water content. The resulting substance for each solvent served as the crude extracts. To avoid confusion in handling the six crude extracts, the body wall extracts were labeled with body wall-methanol (BM), body wall-ethyl acetate (BEA) and body wall-hexane (BH) while extracts from cuvierian tubules were each labeled with cuvierian tubule-methanol (CM), cuvierian tubule-ethyl acetate (CEA) and cuvierian tubule-hexane (CH).

2.4 Genotoxicity Test using Onion (Allium cepa) Assay

Devising from the protocols suggested by Tripathy *et al*. (2013), Tedesco and Laughinghouse (2012), Firbas (2011) and Firbas and Amon (2013, 2014), a modified *Allium* test was applied in this study. The three different concentrations (500, 1,000 and 1,500 μg mL^{-1}) of the methanol, ethyl acetate, and n-hexane extracts from the body wall and cuvierian tubules were used as test liquids (TLs). Aqueous hydrogen peroxide (H_2O_2) (300 mM) (RCI Labscan Limited, Bangkok Thailand) and distilled water were utilized as control mutagen and negative control, respectively.

2.4.1 Setup for *Allium* test

Allium test was applied with the integration of temperature monitoring and humidity using an air thermometer (HTC-1, Hygrometer, China) as well as the equal exposure of the growing onions to the moderated intensity of lighting.

The setup made use of artificial lights from incandescent bulbs (E27, GE, China) and daylight fluorescent lights (FLT5/CE1X14, Firefly, Philippines) to simulate a moderate lighting intensity. Incandescent bulbs served as the source of red light while the fluorescent (daylight lighting) provided the blue light. This means that the onions were not allowed to receive sunlight – windows or other points of entry of other light sources in the room were covered with black paper and curtains.

To avoid the possible effect of small space container (small or medium-sized test tubes) on the bending or coiling of the growing roots, wider glass jars with increasing diameter from top to bottom was used in the bioassay. Moreover, to stimulate the planting of onion and prevent photolytic degradation of metabolites from the crude extracts used, the glass jars from which the onions were grown, were covered with black paper around the perimeter. Meanwhile, the top portion of the container was also covered allowing only small bottom portions of onion to fit – just enough for the growth of the roots at the base of each utilized onion bulb.

The humidity and temperature fluctuation ranges were closely monitored and recorded during daytime (6:01 AM to 6:00 PM) and at night (6:01 PM to 6:00 AM) using a temperature and humidity meter with a built-in timer and alarm (HTC-1, Hygrometer, China) placed inside the main setup area in the experiment room. The highest temperature (35 °C) and lowest humidity (47%) were usually observed from 12:00 PM to 2:00 PM, while the lowest temperature (25 °C) and highest humidity (79%) were noticed during 4:00 AM to 5:00 AM. The temperature fluctuated within the range of 30-34 °C and the humidity recorded was mostly stable at 65% during the day. In addition, lighting was synchronized with the regular standard time – that is, artificial sources of light were switched on and off during the regular daytime and nighttime, respectively.

Furthermore, the *Allium* test in the present study did not include the typical pre-rooting or germination phase. The typical rooting process for three to four days in distilled water may occasionally result in unequal numbers of emerging roots in the onion bulbs. In this way, the correlation of the number of emerging roots to the cytotoxic effect of the evaluated TLs or *P. graeffei* crude extracts and controls becomes less reliable. Thus, to fully evaluate the genotoxic property of crude extracts, the red creole variety of *A. cepa* (Super Pinoy, East-West, Philippines) bulbs were directly allowed to germinate for five days in each experimental concentration/test liquids before macroscopic

evaluation. With this procedure, possible inaccuracies in the correlation of the emerging roots and their lengths to the genotoxic effect of the evaluated TLs can be eliminated.

2.4.2 Preparation of Onion and Research Design

A total of 180 mature and sun-dried red creole variety of *A. cepa* L. bought from a local distributor were utilized in the bioassay. Onions around 10-18 mm were selected using a digital caliper (B07VSVMWTJ, Sangabery, China) and weighed within the range of 25-30 g using an electronic balance (GM-300P E.61863, Lutron Electronic, Taiwan). Onions were further sorted out by the removal of outer scales and brownish bottom plates including dried old roots (Firbas, 2011). Each of the onion bulbs was allowed to freely germinate directly in glass jars with the different concentrations (500, 1,000 and 1,500 µg mL^{-1}) of TLs and in the controls at 33±1 °C for four days. This means that bulbs of *A. cepa* were grown in the glass jars containing distilled water (negative control) (TL_0), 300 mM control mutagen aqueous H_2O_2 (control mutagen) (TL_1) and varying concentrations at 500 µg mL^{-1} (TL_{2-8}), 1,000 µg mL^{-1} (TL_{9-14}) and 1,500 µg mL^{-1} (TL_{15-20}) each of BM, BEA, BH, CM, CEA and CH.

On the fifth day, which was equivalent to 120 h exposure to the TL, macroscopic observation (i.e., discoloration, turgidity, bending or form, length and number of emerging roots) started. Meanwhile, macroscopic observations for 144 and 168 h were also done on the following sixth and seventh days, respectively. Onions were exposed within 12-h light and 12-h dark under moderate light intensity and general temperature of 33 °C±1 and general humidity 65%±1. After seven days of subjecting the onions in the different TLs, cytological observation was followed wherein onion root tips in each replicate were evaluated for the chromosomal aberrations and nuclear abnormalities.

2.5 Macroscopic Observation and General Toxicity Evaluation

Assessment of general toxicity was done by macroscopic evaluation on the roots of each onion bulb within the period of 168 h. Root lengths of all onions were measured prior (fourth day) to their exposure to the tested concentrations of the different crude *P. graeffei* extracts. The root lengths of the onions were measured using a digital caliper after the periods of 120, 144 and 168 h. The mean root length for every test concentration of the different extracts and controls was calculated by dividing the total root length per bulb by the total

number of measured roots. Other parameters like the character of roots such as coiling and branching, color and turgescence were also assessed.

2.6 Cytological Observations and Genotoxicity Evaluation

Immediately after macroscopic observation (i.e., after 168 h), each of the apical portions (3-15 mm) of the onion roots was cut off. The roots were then washed for 20-25 min using distilled water. The cut 15-mm portion of the roots including the tip was initially soaked in Carnoy's solution I or Farmer's solution for 24 h at 4 °C. Afterward, rootlets were transferred to a new container that had 70% ethanol and refrigerated at 4 °C until use. Root tips of approximately 1-2 mm were used in the microscopic observation in the squashing procedure. Onion root tips were cleansed using distilled water. After which, the roots were hydrolyzed in one part 1 N HCl at 60 °C for 1 min and washed with distilled water three times. Staining was done using 2% acetocarmine solution chromosome stains at the dark for 1 h followed by squashing using 45% glacial acetic acid (v/v).

The cytogenetic investigation was conducted with the aid of compound electric microscopes (G015045945, G015059588, and G015059596, LaboMed, United States). The microscopic analysis was performed by looking at the mitotic index and describing non-dividing cells: interphase (emergence of micronuclei), prophase (numerical alteration and polar deviations) chromosomal aberrations in metaphase (e.g., numerical alteration, single break chromatid, double break chromatid, gap break and centromere break), anaphase (e.g., the appearance of the aphasic bridge, loss of chromosome and decompressed chromosomal bridge), and telophase stage of cells (sticky chromosomes and numerical alteration). Microphotography of some represented stages and observed aberrations was done with the aid of a cellular phone camera (Flare, Cherry Mobile, Philippines). Typical magnifications used were 400 and 1,000x. Mitotic index (MI) was evaluated by counting all stages of mitotic cells (dividing cells) in 1,000 cells multiplied by 100 (Equation 1). This means that 333 or 334 cells were counted for each bulb totaling 1,000 cells per replicate or 3,000 per treatment group. Chromosomal aberrations were estimated only whenever the MI result is above 10 per 1,000 (Equation 2). On the other hand, the chromosomal aberration was scored in the first 100 cells which were done while slides were scanned from right to left, up and down.

$$Percent\ Mitotic\ Index\ (MI) = \frac{No.\ of\ dividing\ cells}{Total\ no.\ of\ cells\ scored} \times 100 \qquad (1)$$

$$Percent\ Chromosomal\ Aberration\ (PCA) = \frac{No.\ of\ aberrant\ cells}{Total\ no.\ of\ mitotic\ cells\ scored} \times 100 \quad (2)$$

2.7 Data Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 21 (IBM Corp., United States). One-way analysis of variance (ANOVA) was utilized to determine the differences of the means in the measured root number and root length when compared with the controls. Correlation analysis was done using Pearson correlation.

3. Results and Discussion

3.1 Cytotoxic and Mutagenic Properties of H_2O_2

Based on the study of Egito *et al.* (2007), the chemical (H_2O_2) utilized in this study was a control mutagen. This chemical, being naturally produced by the cells, is a common genotoxin or a form of reactive oxygen species (ROS) produced by oxidative physiologic stress of cells. It is then considered a natural cause of damage in cells by producing hydroxyl radicals (OH^-) leading to tissue injury (Benhusein *et al*., 2010; Sarker and Oba, 2018a, 2018b, 2020). It has long been described that H_2O_2 causes cytotoxic activity even at low concentrations in mammalian cells. It can affect Ca^{2+} balance which is a result of promoting activation of Ca^{2+}-dependent nucleases destroying a cell's DNA. It can also activate the cell's apoptotic and necrotic effector Caspase-3 (Cantoni *et al*., 1989). ROS like H_2O_2 easily causes cytotoxic effect by passing through plasma membrane via aquaporin channels and interfering in normal cell processes by producing radical OH^-, which attacks at the sugar residue of the DNA backbone leading to chromosome breaks. It can also alter the purine and pyrimidine bases to their hydroxyl derivatives causing damage to cells' normal mitotic process (Benhusein *et al*., 2010).

3.2 Macroscopic Observations for Evaluation of General Toxicity

Abnormalities in the morphology of onion roots can be a result of the changes in physiological processes of cells which are linked to the nuclear-regulatory and/or DNA-damaging effects of certain compounds. This means that morphological and cytological changes in *A. cepa* root meristem cells can be used as toxicity indicators. The initial macroscopic observations of the present

study revealed the appearance of unusual root growth characteristics in *A. cepa* induced by the different concentrations of the TLs. This could suggest that the evaluated crude extracts have some genotoxic components that promote the malformations in the developing roots of *A. cepa*. The higher concentration of the crude extracts resulted in the production of swollen roots, which in turn, caused their wavy appearance. Also, the onions exhibited shorter roots, reduced root emergence and occasional slight brown discoloration. However, most of the emerged roots appeared to still have a firm structure in all doses of each treatment.

At the highest dose of the crude BEA, an unusual branching rootlet outgrowth was observed from a single lengthened rootlet (Figure 5f).

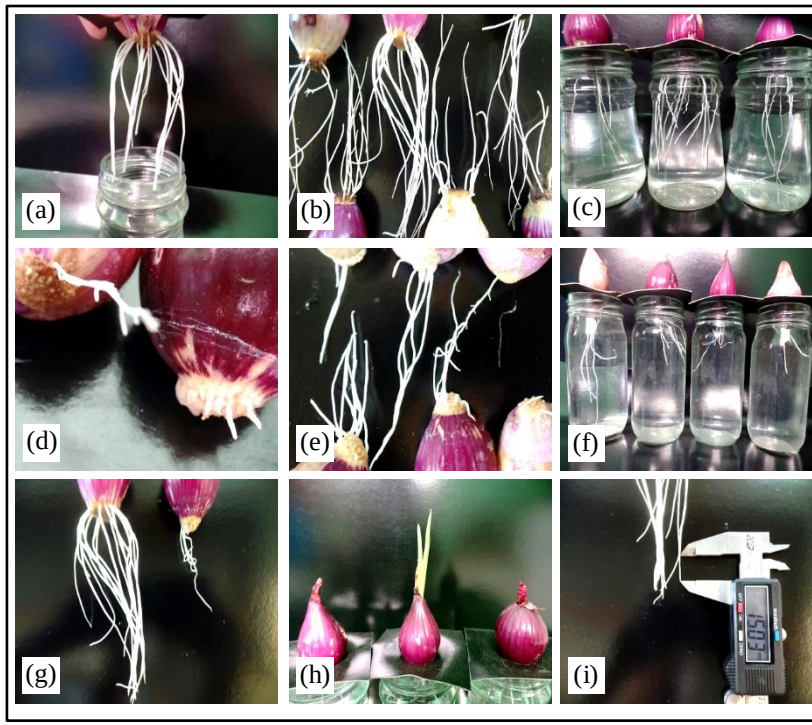

Figure 2. Macroscopic observations of onions grown in control groups – root growth in distilled H_2O (negative control) (a-c); root growth in 300 mM of H_2O_2 (positive control showing lateral outgrowths) (d-f); root growth of onion in distilled H_2O (left) and root growth of onion in 300 mM H_2O_2 (right) (g); young leaves of onion grown in distilled H_2O (h); and use of digital caliper in measuring the roots (i)

This root morphology was also observed in the *A. cepa* grown in the control liquid cytotoxicant (H_2O_2) (Figure 2d and 2e). The usual root growth form in the onions was commonly exemplified by the emergence of rootlets directly from the primordial root region at the base of the bulb (Figure 2a to Figure 2c). The root growth of onions grown in H_2O_2 was much different. Most of the emerged roots were thin and less rigid or more slacken. Removal of the onion in the TL would definitely show the inability of the emerged roots to maintain their original form. The roots were also highly coiled or bent, shorter and less in number as illustrated in Figures 2e and 2g and in Figures 3, 4 and 5. From the total of nine onions subjected to the TL control, only four bulbs successfully produced roots with at least 3 mm in length. The other five bulbs only managed to create small rootlets similar to what is shown in Figure 2d. In addition to the macroscopic root growth comparison for genotoxicity evaluation, one bulb grown in distilled H_2O developed young leaves demonstrating the existence of a favorable condition or non-toxic growing environment (Figure 2h). The inhibition and shoot growth are shreds of evidence of general toxicity in the evaluated crude extracts and control mutagen (Gajalakshmi and Ruban, 2014).

Turgescence is illustrated by the enlarged portion of the onion roots. This is usually characterized by swollen regions which may result in bending or coiling of the roots. Turgescence ensues from the unusual accumulation of fluids inside the cells. The appearance of turgescence on the developing onion root may be viewed as a result of damaged root tip cells or compromised normal mitotic process due to genotoxic compounds. Extreme bending or coiling of roots (high toxicity) was observed in the onions grown in the 300 mM H_2O_2, which is a known as cytotoxicant (Figures 2d to 2f).

Fiskesjö (1985) reported that in the presence of a highly toxic chemical, roots are observed to slacken and may die. However, this extreme loss of turgidity is only observed in the roots from onions grown in the control mutagen H_2O_2. A slight loss of turgidity is characterized by roots exhibiting some degree of discoloration. Subsequently, discoloration may be observed on portions where onion root cells are damaged. Root tips with a brownish discoloration may be interpreted as a result of toxic effects causing cell death (Fiskesjö, 1985). In the present study, discoloration in some emerging roots was observed in 1,000 μg mL^{-1} concentration of BM, BEA, BH, CM and CEA. A more pronounced discoloration was noticed in a higher dose (1,500 μg mL^{-1}) of BEA (Figure 5d).

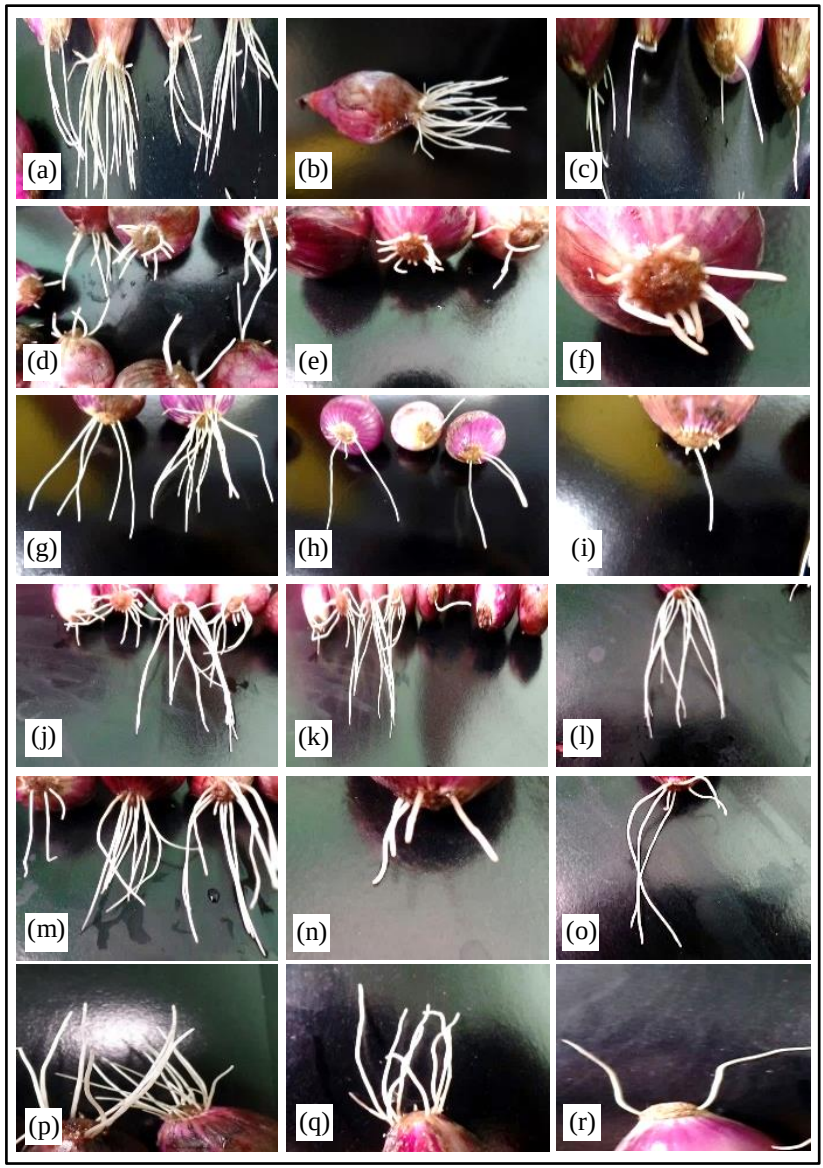

Figure 3. Macroscopic observations on root growth under 500 µg mL^{-1} dose of crude extracts: BM (a-c); BEA (d-f); BH (g-i); CM (j-l); CEA (m-o); and CH (p-r)

It was observed that the general toxicity effects of *P. graeffei* crude extracts were dose-dependent. The indicators of toxicity in terms of turgescence, discoloration and bending/coiling of onion root, which were observed in the control mutagen treatment, were more noticeable when the concentration of

the crude extract was increased and followed the order – 500 µg mL^{-1} < 1,000 µg mL^{-1} < 1,500 µg mL^{-1}. It also means that abnormalities in the emerged roots of onions appeared pronounced at a higher dose of the crude extracts.

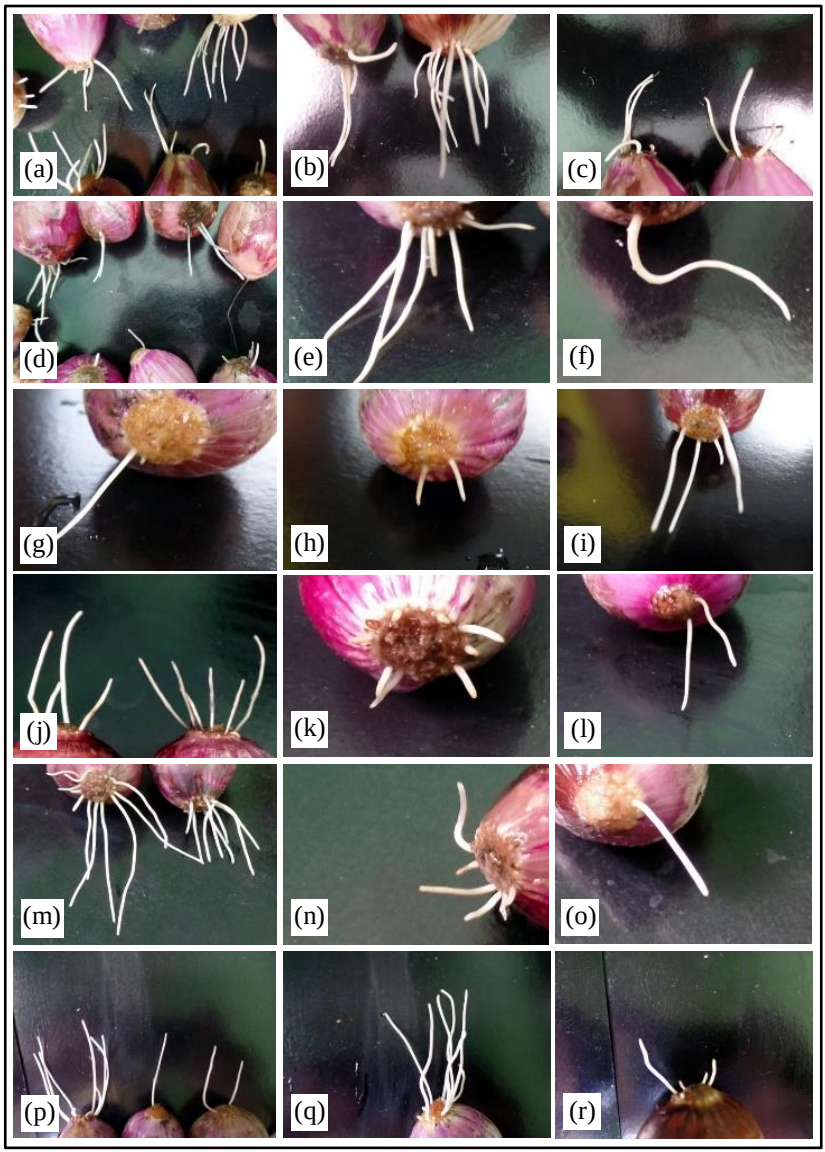

Figure 4. Macroscopic observations on root growth under 1,000 µg mL^{-1} dose of crude extracts: BM (a-c); BEA (d-f); BH (g-i); CM (j-l); CEA (m-o); and CH (p-r)

Figure 5. Macroscopic observations on root growth under 1,500 µg mL^{-1} dose of crude extracts: BM (a-c); BEA (d-f); BH (g-i); CM (j-l); CEA (m-o); and CH (p-r)

3.3 Evaluation of the Root Number and Lengths

The visible effect of toxicity, considered as a good indicator of the toxic properties of the crude extracts, is the stunted growth of onion roots causing differences in root length. Similarly, genotoxicity is also evaluated by the

ability of the extract to inhibit root meristem cells' mitotic process or promote apoptotic activity (Hamid *et al.*, 2013; Ridzwan *et al*., 2014; Khodayar *et al*., 2015) which may lead to a lesser number of roots to develop. Consequently, macroscopic parameters such as the number of emerging roots and root lengths were also considered indicators for evaluating the general toxicity of *P. graeffei*'s crude methanol, ethyl acetate, and n-hexane body wall and cuvierian tubule extracts. Table 1 presents the mean emerged roots and root lengths of *A. cepa* (2n = 16) grown in the different treatment groups within the periods of 120, 144 and 168 h.

A trend in the emerged number of roots was noted when the concentration of the crude extract. It appears that at higher concentrations of the *P. graeffei* crude extracts (1,000 and 1,500 μg mL^{-1}), a significant difference ($p < 0.05$) was revealed when data is compared with the negative control group and significantly similar to the inhibitory effect of the cytotoxicant (H_2O_2) (Table 1). The higher inhibition of emerged root length was exhibited by onions treated with 1,000 μg mL^{-1} of CM compared with control mutagen. Relating it to the previously described indicators of general toxicity, the observed reduction of root number supports the claim that at the highest concentration of the *P. graeffei* crude extracts, pronounced root malformations are exhibited causing a higher general toxicity level.

3.4 Cytological Observation and Parameters Observed in Allium Test

A genotoxicity test is an important procedure for the screening of potential drugs and their effects before application to humans. Oftentimes, it employs additional mutagenicity or genotoxicity tests to describe the consequences of the toxic component of a known genotoxic compound or a natural product under evaluation. To further investigate the genotoxic properties of the different concentrations of *P. graeffei* crude methanol, ethyl acetate and n-hexane extracts from the body wall and the cuvierian tubule, the mitotic indices and percent abnormalities were statistically analyzed. Table 2 suggests that all *P. graeffei* crude extracts across all concentrations were statistically comparable ($p < 0.05$; Tukey's test) with the negative control group except for 500 μg mL^{-1} of BM, which resulted in a lower MI. Remarkably, even the distilled water resulted in a comparable MI ($p < 0.05$) against the control mutagen and all other treatment groups except for the 500 μg mL^{-1} BM. This means that the 500 μg mL^{-1} of BM (having the lowest computed MI after 168 h) had higher inhibitory properties than the control mutagen or genotoxins.

Table 1. The mean number of roots and mean root lengths of *A. cepa* L. after 120, 144 and 168 h of exposure in the control and treatment concentrations

Treatment	Concentration ($\mu g\ mL^{-1}$)	Average root number ± SD			Average root length ± SD (mm)		
		120 h	144 h	168 h	120 h	144 h	168 h
Control (-)	dH_20	8.78±3.96^{a}	9.44±3.84^{a}	10.67±3.20^{a}	34.33±16.52^{a}	34.02±17.28^{a}	32.82±17.83^{a}
H_2O_2 (+)	300 mM	4.44±2.01^{b}	4.67±1.94^{b}	5.11±1.96^{b}	20.25±9.11^{ab}	19.74±9.61^{b}	19.34±9.35^{b}
BM	500	5.44±5.03^{ab}	5.78±5.59^{ab}	5.78±5.59^{ab}	17.43±8.93^{b}	17.47±8.91^{b}	17.50±8.91^{ab}
	1,000	4.00±2.65^{b}	4.56±3.32^{b}	4.56±3.32^{b}	11.35±3.50^{b}	10.62±3.14^{b}	10.56±3.30^{b}
	1,500	2.33±2.24^{b}	2.56±2.30^{b}	2.56±2.30^{b}	13.44±14.37^{b}	13.55±14.69^{b}	13.68±14.92^{b}
BEA	500	4.67±3.54^{ab}	5.00±3.74^{ab}	5.00±3.74^{ab}	10.68±6.01^{b}	10.43±6.16^{b}	10.44±6.16^{b}
	1,000	1.89±1.69^{b}	2.00±1.66^{b}	2.00±1.66^{b}	15.79±9.38^{b}	15.00±9.58^{b}	14.90±9.62^{b}
	1,500	2.67±1.87^{b}	2.78±1.72^{b}	2.78±1.72^{b}	10.71±7.57^{b}	10.72±7.57^{b}	10.75±7.58 ^{b}
BH	500	3.44±3.13^{ab}	3.44±3.13^{ab}	3.56±3.40^{ab}	19.05±12.47^{b}	19.10±12.48^{ab}	19.14±12.47^{ab}
	1,000	2.67±2.83^{b}	2.67±2.83^{b}	2.78±3.11^{b}	6.94±7.33^{b}	6.96±7.34^{b}	6.74±6.78^{b}
	1,500	2.44±1.67^{b}	2.44±1.67^{b}	2.44±1.67^{b}	11.81±8.33^{b}	11.84±8.34^{b}	11.85±8.33^{b}
CM	500	4.00±5.07^{ab}	4.00±5.07^{b}	4.00±5.07^{ab}	13.23±12.13^{b}	13.33±12.27^{b}	13.27±12.20^{b}
	1,000	2.33±2.87^{b}	2.33±2.87^{b}	2.33±2.87^{b}	5.66±7.89	5.66±7.89	5.67±7.89
	1,500	2.56±2.19^{b}	2.56±2.19^{b}	2.56±2.19^{b}	9.07±7.93^{b}	8.89±7.93^{b}	8.88±7.95^{b}
CEA	500	5.33±3.46^{ab}	5.67±3.64^{ab}	5.67±3.64^{ab}	13.95±11.72^{b}	15.40±8.23^{b}	14.69±8.00^{b}
	1,000	3.33±2.74^{b}	3.89±3.26^{b}	3.89±3.26^{b}	14.48±9.35^{b}	14.43±7.91^{b}	14.36±7.85^{b}
	1,500	2.78±2.59^{b}	3.11±2.57^{b}	3.11±2.57^{b}	14.09±8.86^{b}	13.89±8.40^{b}	14.06±8.52^{b}
CH	500	4.67±4.18^{ab}	4.56±4.07^{ab}	4.33±3.64^{ab}	21.24±7.05^{ab}	19.98±7.72^{ab}	20.62±8.30^{ab}
	1,000	3.11±2.57^{b}	3.22±2.73^{b}	3.11±2.57^{b}	15.05±7.00^{b}	15.34±7.19^{b}	15.43±7.11^{b}
	1,500	1.56±0.88^{b}	2.11±0.93^{b}	2.11±0.93^{b}	7.29±6.02 ^{b}	7.11±5.27^{b}	7.12±5.26^{b}

Values with similar superscript letter under same column are not significantly different ($p < 0.05$).

Table 2. MI and PCA of *A. cepa* root tip cells exposed to the increasing concentrations of different crude extracts of *P. graeffei* within 72 h

Treatment groups	Concentrations (µg mL^{-1})	MI (Mean ± SD)	(%) Abnormalities (Mean ± SD)
Control (-)	Distilled water	59.600±2.778[a]	0.000±0.000[a]
H_2O_2	300 mM	65.033±5.281[a]	7.433±2.947[b]
BM	500	46.267±1.320	16.183±1.446[b]
	1,000	53.767±21.298[a]	18.383±6.676[b]
	1,500	48.967±2.444[a]	19.230±5.262[b]
BEA	500	58.300±2.955[a]	30.067±7.177
	1,000	62.200±1.136[a]	26.650±2.429
	1,500	52.333±3.564[a]	34.623±3.777
BH	500	54.400±7.817[a]	24.557±4.018
	1,000	49.967±4.285[a]	27.073±12.676
	1,500	53.933±4.900[a]	24.270±2.583
CM	500	53.467±3.707[a]	17.947±0.446
	1,000	47.033±2.050[a]	18.867±4.184[b]
	1,500	53.267±2.916[a]	22.977±1.969[b]
CEA	500	59.667±3.842[a]	8.167±2.392[ab]
	1,000	69.733±1.721[a]	15.727±2.810[b]
	1,500	57.833±1.893[a]	19.333±1.395[b]
CH	500	56.300±1.400[a]	22.623±1.591
	1,000	53.633±4.474[a]	20.920±1.788[b]
	1,500	55.767±0.777[a]	20.623±0.119[b]

Values with similar superscript letter under same column are not significantly different ($p < 0.05$).

3.5 Chromosomal Abnormalities and Determination of MI in Allium Test

Chromosomal abnormalities observed in this study were results of an error in the mitotic cell division of meristem cells of *A. cepa* roots. This phase of the investigation sought to describe the consequences of the toxicity of *P. graeffei* crude extracts in terms of MI (actively dividing cells) and genotoxicity level/percent chromosomal aberration (M-phase). The parameters to evaluate the genotoxicity of the *P. graeffei* crude extracts included chromosomal aberrations, malformations in the cell's nucleus or membrane and the computation of the degree of incidence of the abnormalities in the cell. The latter parameter was taken as a whole considering aberrations under the prophase, metaphase, anaphase and telophase (Figure 6). Although interphase is not considered part of the M-phase, it was described in this study as an important part of the cell cycle, which is highlighted in the succeeding discussion.

Determination of the MI is necessary for the evaluation of genotoxic properties of a compound in the *Allium* test. It is often described that MI is a biomarker for cell proliferation, which means that a decrease in the MI of the root meristem of onion can be associated with the incidence of cell death (Abdel-Migid and Abdelrahman, 2013). In addition, toxicity tests can provide a holistic interpretation of the mutagenic effect of the crude extracts. Genotoxicity testing does not only provide information on how safe the test substance is but can also give descriptions or characterizations of the consequences of its toxic properties. Table 2 presents the MI and percent PCA of the *A. cepa* root tip cells properties, which were used to evaluate the genotoxic effects of the crude extracts from *P. graeffei*.

Table 2 shows that all concentrations of the *P. graeffei* crude extracts were statistically similar ($p < 0.05$; Tukey's test) to the negative control group except for 500 μg mL^{-1} of BM, which resulted in a lower MI. Remarkably, even the distilled water treatment resulted in a statistically similar MI ($p < 0.05$) when compared against the control mutagen and all other treatment groups except for the 500 μg mL^{-1} BM with higher mitodepressive property. It should be noted that in the counted 3000 cells observed per treatment group, the remaining percent includes cells that fall under the interphase stage. Consequently, though positive control and treatments exhibited similar MI to the negative control group ($p < 0.05$); it is not assumed that it has the ability to be mitogenic or non-cytotoxic or the reverse in the case of distilled water, which is now comparatively cytotoxic. It can be considered that even a mutated cell has the ability to proliferate and sometimes division is even faster than normal cells (Cancer Research UK, 2014; Eldridge, 2016). In line with this, despite the comparable MI, the crude extracts showed a high incidence of chromosomal aberrations comparable with the 300-mM H_2O_2 and, to some, an even higher genotoxic effect.

The genotoxic effect is not always manifested on the cells under M-phase but on the interphase stage (Zhao *et al*., 2011; Zhao *et al*., 2012; Aminin *et al*., 2015). In Figure 6, some concentrations (μg mL^{-1}) of crude extracts also showed abnormalities not on the M-phase but on the interphase. Figures 6j (500 CEA), 6k (1,500 CEA), 6l (1,000 CH), 6e (500 BH), 6f (1,500 CEA), 6m (1,500 BM), and 6r (1,500 BH) also illustrate the presence of damaged nuclei of cells at interphase stage.

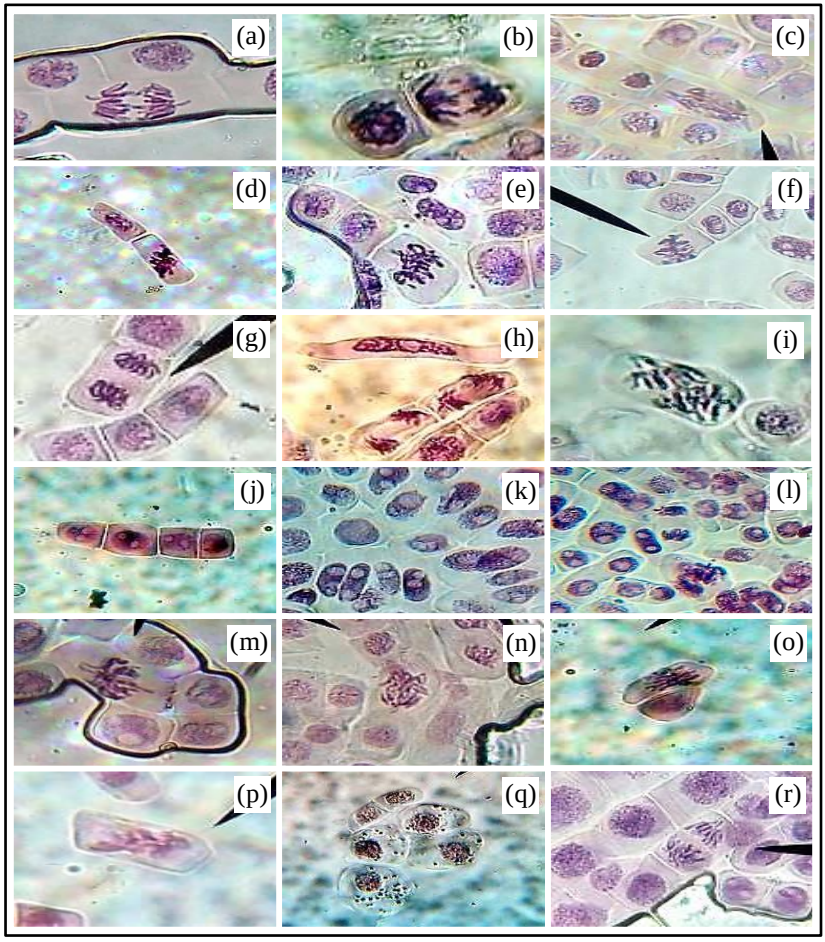

Figure 6. Photomicrographs of the different chromosomal and nuclear aberrations induced by 500, 1,000 and 1,500 μg mL^{-1} *P. graeffei* body wall and cuvierian tubule crude methanol, ethyl acetate and n-hexane extracts. (500 CM) bridged and fragmented anaphase (a); (500 BM) bridge and chromosome fragments at anaphase (b); (1,500 BM) bridged and pulverized chromosome at anaphase (c); (1,500 CM) sticky and diagonal metaphase (d); (500 BH) stickiness and chromosome fragments (e); (1,500 CEA) diagonal metaphase/polar deviation (f); (500 CM) sticky anaphase (g); (1,000 BEA) polar deviation and sticky telophase (h); stekinesis (i); (500 CEA) (j), (1,500 CEA) (k) and (1,000 CH) (l) cells with damaged nucleus and nuclear membrane; (1,500 BM) vagrant metaphase and cells with damaged nucleus (m); vagrant and multipolar anaphase (n); (500 CM) sticky chromosomes at metaphase (o); (1,000 BM) pulverized chromosome (p); (1,500 CM) nuclear fragments and apoptotic bodies (q); (1,500 BH) early cell plate formation in anaphase (r)

Although this is not the case at the M-phase stage, extracts definitely caused abnormalities in the cell cycle stages, which may lead to high MI and PCA when allowed to develop. This mutagenic effect also alters the computed MI and PCA most likely when observation time is extended and even if the damaged cell would not proceed to the M-phase of the cell cycle. In fact, there is a high incidence of observed cells under the interphase in all concentrations of crude extracts that exhibit damaged nuclei, which may sequentially spawn apoptosis or the production of abnormal chromosomes in the succeeding stages in the cell cycle. Consequently, this could be manifested in general toxicity as either root discoloration or root growth inhibition. The same damaging effect in the interphase cells was also noticed in the control mutagen H_2O_2, yet interphase abnormality was more prevalent in the onions treated with *P. graeffei* crude extracts.

Various species of sea cucumbers were found to be a rich source of saponins, which are usually in the form of triterpenes, glycosides, or triterpene glycosides commonly termed as holothurin (Silchenko *et al*., 2007; Caulier *et al*., 2011; Mohklesi *et al*., 2012). Holothurin in the form of triterpene glycoside was reported to exhibit genotoxic and/or cytotoxic effects and antitumor properties by cell cycle arrest at S or G_2/M phase, activation of cell death pathways and the up-down regulation of proteins (receptors and enzymes) primarily involved in metastasis (Aminin *et al*., 2015).

Among the many different cytotoxic properties of extracts from sea cucumbers, the effect of saponin (triterpene glycosides/holothurin), the echinoside A and ds-echinoside A from *P. graeffei* are the most critical in this discussion. Echinoside A and ds-echinoside A were both found to stop the cell cycle process at G_0/G_1 phase. In a concentration-dependent manner, ds-echinoside A was found to suppress adhesion, migration and invasion of human hepatocellular liver carcinoma cells. The said non-sulfated triterpene glycoside was able to down-regulate or suppress the matrix metalloproteinase-9 (MMP-9) essential in destroying the basement membranes involved in metastasis and angiogenesis. In this case, the ds-echinoside A can enhance the release of TIMP-1 (tissue inhibitors of metalloproteinase-1), thereby regulating activation of MMP-9, reduction in the VEGF (vascular endothelial factor) and the NF-κB (Zhao *et al*., 2011; Zhao *et al*., 2012).

Holothurin was also proven to have antimitotic properties against onion root cells in the *Allium* test by generally causing arrest in mitosis specifically arrest at any stage in the cell cycle. It was also confirmed to be genotoxic (through

H-thymidine autoradiography) due to its ability to inhibit DNA and ribonucleic acid (RNA) synthesis although, at some point, may show replication of nucleolar DNA in some cells treated with low dosage. Holothurin (a water-soluble saponin), which was isolated from the body wall of sea cucumber *Holothuria vagabunda*, caused cytological abnormalities too like star metaphase, formation of pycnotic nuclei, splitting chromosomes at late prophase, double-stranded chromosomes at anaphase and further subdivision of chromatid pairs. In a previous study, a 1,000 ppm of holothurin resulted in lethal necrosis of onion root tip cells; even crude holothurin from a sea cucumber *Actinopyga agassizi* caused antitumor effect (Santhakumari and Stephen, 1988).

Previous studies (Santhakumari and Stephen, 1988; Zhao *et al.*, 2012; Aminin *et al.*, 2015) found the antimitotic property of holothurin which is also present in *P. graeffei*. The said studies also described that the cytotoxicity may be manifested in inhibitory action or death of cells both in *A. cepa* or animal cells. It was further presented that cytological observation may result in abnormalities that include types that were recognized in the present study. Furthermore, to fully evaluate the genotoxic effect of the crude extracts, statistical analysis was performed to interpret the result for the mean PCA.

The analysis ($p < 0.05$; Tukey's test) of the mean PCA of the crude extracts showed that all crude extracts except 500 µg mL^{-1} of CEA resulted in a higher incidence of chromosomal aberrations compared with the treatment under distilled water. In addition, all concentrations of BEA, BH and the 500 µg mL^{-1} of CM had a higher genotoxic effect than H_2O_2. The *A. cepa* treated with 1,500 BEA had the highest genotoxicity level in terms of PCA while the lowest genotoxicity was manifested in onion treated with control groups and 500 µg mL^{-1} CEA. This only proves that crude extracts derived from *P. graeffei* possess genotoxic components.

3.6 Other Sea Cucumber Species' Bioactive Compounds and their Genotoxic, Mitogenic and Antimitotic Properties

Several studies revealed that the active derivatives of species of sea cucumbers can alter the proliferation of cells both in the in vivo and in vitro assays. Some of the reported cytotoxic properties of active derivatives of different species of sea cucumbers were antimetastatic, antimitotic, antiangiogenesis and even against human immunodeficiency virus 1 (HIV-1) (Zhao *et al*., 2009; Hamid *et al*., 2013). In a study by Ridzwan *et al*. (2014), water extracts of sea cucumber (*Holothuria atra*) Jaeger exhibited dose-dependent (10, 20, 30, 50,

100, 150 and 200 mg/kg) toxicity and apoptotic properties in mouse hepatocytes. Increased doses of water extracts resulted in an increased degree of formation of abnormal hepatocytes characterized by distorted shape, undefined cell lining, and enlarged nuclei and even necrosis. Similarly, hot water extract of sea cucumber (*Stichopus japonicas*) was also found to induce apoptosis via Caspase-3 activation (Ogushi *et al*., 2005). Khodayar *et al*. (2015) showed that methanol extracts of *Holothuria leucospilota* time-dependently promoted apoptosis and successfully enhanced the apoptotic effect of lovastatin against colon tumor cells. In another study by Seydi *et al*. (2015), methanol extracts from another sea cucumber also exhibited a considerable cytotoxic effect. The methanol extracts of sea cucumber (*Holothuria parva*) increased ROS formation and mitochondrial membrane potentials (MMP), which were contributory to cell apoptotic events. The methanol extract was further described to activate the Caspase-3 mechanism (essential for apoptosis) in male Sprague-Dawley rats' liver cells. In the present study, crude cuvierian tubule extracts were found to exhibit mitodepressive and genotoxic effects on onion root meristem cells. Crude toxins from sea cucumber *Holothuria atra*'s cuvierian organ were previously described to induce cytotoxic effects specifically causing leukocytosis, thrombocytosis and mild polycythemia (Zaki, 2005).

Lectins were previously found to be present in some species of sea cucumbers (Mojica and Merca, 2005; Gowda *et al*., 2009). These bioactive compounds, which are usually isolated from plants, were also evaluated for their antitumor properties. Isolated lectins from both terrestrial and marine organisms possessed the properties of an anticancer agent. Hamid *et al*. (2013) reported that isolated lectins' property as antimetastatic is linked to their ability to affect surface markers in tumor cell recognition, involvement in cell-to-cell communication and adhesion, mitogenic property, immune system booster, cytotoxicity and apoptotic effects. The apoptotic property of lectins is attributed to their ability to activate death receptors commonly in the form of FAS receptors (Liu *et al*., 2009; Lam and Ng, 2010a) or the down-regulation of anti-apoptotic factors of the βcl-family (Lam and Ng, 2010a, 2010b). In addition, these reported lectin-induced apoptosis was described to involve antimitotic activity by cell cycle arrest at G_0/G_1 (Park *et al*., 2001; Liu, *et al*., 2009; Lam and Ng, 2010a) and at G_2/M phases of mitosis (Lam and Ng, 2010a).

Classes of peptides are also one of the components of some reported sea cucumber extracts (Rafiuddin *et al*., 2004; Zhao *et al*., 2011). According to

Cheung *et al*. (2015), marine-derived peptides can be grouped into linear depsipeptides, cyclic depsipeptides, linear peptides and marine hydrolysates. Cytotoxic depsipeptides and cyclic peptides from marine sources have structurally differentiated amino acid structures such as carbamylation or *N*- and *O*-methylation, which is not present in common proteins. This implies that, oftentimes, they are taxa or species-specific. Linear depsipeptides like dolastatins 10 and 15, hemiasterlin A and B, monomethyl auristatin E (a derivative) and HT1-286 are among the reported marine-derived peptides with the cytotoxic property through either inhibition of tubulin polymerization, promotion of mitotic arrest and abnormal spindle formation, or the initiation of apoptosis. Furthermore, cyclic depsipeptides like pardaxin can induce apoptosis by targeting the ER and inducing c-FOS.

The crude extracts of *P. graeffei* may contain similar forms of cytotoxic peptides which caused the inhibition of the growth of cells. Microscopically, the cytotoxic effect may be linked to the mutagenic action or genotoxic effect of the present active components of crude extracts that may have interfered with the normally nuclear process of the onion root tip cells. Holothurians are also a rich source of essential fatty acids, which include classes of long-chain polyunsaturated fatty acids (PUFA) like arachidonic, eicosapentaenoic and docosahexaenoic acids (Anisuzzaman *et al*., 2019) and the monounsaturated fatty (MUFA), namely gadoleic and cis-oleic acids (Yahyavi *et al*., 2012). Like docosahexaenoic acid, which was found to inhibit the growth of cancer cells, a long-chain saturated fatty acid called stearic acid from sea cucumber (*Holothuria* sp.) exhibits high cytotoxic property comparable with an anti-cancer drug (doxorubicin) (CAS: 23214-92-8) (Januar *et al*., 2014). A study also proved that saturated long-chain fatty acid like stearic acid is considered a cytotoxic compound (Brinkmann *et al*., 2013). Similar research also supports the claim that saturated fatty acids have chemotherapeutic properties – significant cytotoxic effect, anti-proliferative and oxidative property and ability to interfere in the signaling pathway of the cell (Fauser *et al*., 2011). Meanwhile, the cytotoxic effect is also dependent on the length of the carbon chain wherein the shorter the chain, the more powerful it is as a cytotoxic agent. In the study by Siegel *et al.* (1987), palmitic acid (16 C) was found to be more toxic than arachidonic acid (20 C).

Classes of saponins from sea cucumbers are identified as cytotoxic compounds (Mohammadizadeh *et al*., 2013). Previous studies also demonstrated that both steroidal and triterpenoid saponins showed significant cytotoxic properties against different cancer cell lines. Importantly, at times,

a certain class of saponins like steroid or triterpenoid types has high or sometimes higher cytotoxic properties than the positive control (Podolak *et al*., 2010). In the study of Zhang and Li (2007), a certain kind of saponin known as holostane saponin, isolated from sea cucumber *Pseudocolochirus violaceus*, showed considerably higher cytotoxic effects compared with the positive control (10-hydroxy-camptothecin) against the colon cancer (HCT-116). Similarly, holostane-type triterpene glycoside from sea cucumber (*Pentacta quadrangularis*) exhibited cytotoxic properties (Han *et al*., 2010). Moreover, a novel sulfated saponin philinopside A also from *P. quadrangularis* exhibited dual antiangiogenic and antitumor effects (Mayer and Gustafson, 2009).

4. Conclusion and Recommendation

The genotoxic property of the crude extracts of *P. graeffei* can be linked to the presence of bioactive compounds in the body wall and cuvierian tubules. The bioactive components may include the same compounds previously isolated from the same species, namely holothurin or triterpene glycosides, echinoside A, holothurin-A, 24-dehydroechinoside and ds-echinoside. Root growth inhibition in terms of root number and root length comparable with the cytotoxicant (H_2O_2) was a result of the present genotoxic component in the evaluated crude *P. graeffei* extracts. The observed genotoxicity of the crude extracts was attributed to the mitodepressive, mutagenic, necrotic, or apoptotic effects, which were previously described in several studies. It is interpreted that the genotoxic property of *P. graeffei* body wall and cuvierian tubule crude extracts on root tip cells may not be highly manifested in the M-phase of the cell cycle. The high incidence of abnormalities in the interphase stage of observed cells, induced by the crude extracts, suggests that genotoxic property was also exhibited in the non-actively dividing cells of the observed onion root tips which consequently contributed to its genotoxic property. Purification of the crude extracts (BM, BEA, BH, CM, CEA and CH) is necessary to obtain the bioactive components and fully evaluate their genotoxic and mitodepressive properties.

The remarkable genotoxic properties of all the evaluated *P. graeffei* Semper's crude body wall and the cuvierian tubule extracts will be helpful in the future use of the extracts in other biological studies. The result will be highly valuable in studies leading to the discovery of drugs for cancer. The key concept in discovering the genotoxic property of the crude extracts from the

sea cucumber was not to emphasize its danger for human consumption. In fact, commercial drugs of any kind for any disease can be toxic or poisonous if taken in large amounts. The science of toxicology will also agree that even freshwater or drinking water can be toxic to human cells if not used appropriately and is taken in excessive amounts. Therefore, the unknown genotoxic compounds or active components from the evaluated crude extracts from *P. graeffei*, once elucidated, may serve as a model or blueprint for developing drugs that can limit the mitotic growth of and promote apoptotic effect on unwanted cells that cause diseases.

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6. References

Abdel-Migid, H.M., & Abdelrahman, H.M.H. (2013). Phyto-genotoxicity assessment of sewage water for agriculture applications. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4(2), 511-529.

Akamine, J. (2010). Problem on sea cucumber conservation. In globalization, food, and social identities in the Asia Pacific region. Tokyo: Sophia University Institute of Comparative Culture.

Aminin, D.L., Menchinskaya, E.A., Pisliagin, A.S., Silchenko, A.S, Avilov, S.A., & Kalinin, V.I. (2015). Anticancer activity of sea cucumber triterpene glycosides. Marine Drugs, 13(3), 1202-1223. https://doi.org/10.3390/md13031202

Anisuzzaman, Md., Jin, F., Kabery, K., Jeong, U.-C., Jung, H.-C., Lee, S.R., & Kang, S.-J. (2019). Lipid class and fatty acid compositions of dried sea cucumber *Apostichopus japonicus*. The Open Food Science Journal, 11, 79-86. https://doi.org/10.2174/1874256401911010079

Azwinida, N.N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. Medicinal & Aromatic Plants, 4(3), 1000196. https://doi.org/10.4172/2167-0412.1000196

Bácskay, I., Nemes, D., Fenyvesi, F., Váradi, J., Vasvári, G., Fehér, P., Vecsernyés, M., & Ujhelyi, Z. (2018). Role of cytotoxicity experiments in pharmaceutical development. In T. A. Celik (Ed.), Cytotoxicity (pp. 132-146). United Kingdom: IntechOpen Limited.

Benhusein, G.M., Mutch, E., Aburawi, S., & Williams, F.M. (2010). Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay. Libyan Journal of Medicine, 13(5), 1-6. https://doi.org/10.3402/ljm.v5i0.4637

Brambilla, G., & Martelli, A. (2009). Genotoxicity and carcinogenicity studies of analgesics, anti-inflammatory drugs and antipyretics. Pharmacological Research, 60(1), 1-17. https://doi.org/10.1016/j.phrs.2009.03.007

Brambilla, G., Mattioli, F., Robbiano, L., & Martelli, A. (2012). Studies on genotoxicity and carcinogenicity of antibacterial, antiviral, antimalarial and antifungal drugs. Mutagenesis, 27(4), 387-413. https://doi.org/10.1093/mutage/ger094

Brinkmann, C.R., Brodkorb, A., Thiel, S., & Kehoe, J.J. (2013). The cytotoxicity of fatty acid/á lactalbumin complexes depend on the amount and type of fatty acid. European Journal of Lipid Science and Technology, 115(6), 591-600. https://doi.org/10.1002/ejlt.201200165

Brown, E.O., Perez, M.L., Garces, L.R., Ragaza, R.J., Bassig, R.A., & Zaragoza, E.C. (2010). Value chain analysis for sea cucumber in the Philippines. Retrieved from http://hdl.handle.net/1834/22288

Cancer Research UK. (2014). How cancers grow. Retrieved from http://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancers-grow

Cantoni, O., Brandi, G., Salvaggio, L., & Cattabeni, F. (1989). Molecular mechanisms of hydrogen peroxide cytotoxicity. Annali dell'Istituto Superiore di Sanità, 25(1), 69-74.

Caulier, G., Van Dyck, S., Gerbaux, P., Eeckhaut, I., & Flammang, P. (2011). Review of saponin diversity in sea cucumbers belonging to the family Holothuridae. SPC Beche-de-mer Information Bulletin, 31(1), 48-54.

Cheung, R.C.F., Ng, T.B., & Wong, J.H. (2015). Marine peptides: Bioactivities and applications. Marine Drugs, 13(7), 4006-4043. https://doi.org/10.3390/md13074 006

Collin, P.D. (1999). U.S. Patent No. US 5985330 A. Retrieved from ttps://patents.google.com/patent/US5985330A/en

Conand, C., Gamboa, R., & Purcell, S. (2013). *Pearsonothuria graeffei*. Retrieved from https://www.iucnredlist.org/species/180248/1605468

Egito, L.C.M., Medeiros, M.D.G., Batistuzzo, D.M., & Agenez-Lima, L.F. (2007). Cytotoxic and genotoxic potential of surface water from Pitumbu river, northeaster/RN Brazil. Genetics and Molecular Biology, 30(2), 435-441. https://doi.org/10.1590/S1415-47572007000300023

Eldridge, L. (2016). Cancer cells vs. normal cells – A dozen differences. Retrieved from https://www.verywell.com/cancer-cells-vs-normal-cells-2248794

Fauser, J.K., Prisciandaro, L.D., Cummins, A.G., & Howarth, G.S. (2011). Fatty acids as potential adjunctive colorectal chemotherapeutic agents. Cancer Biology Therapy, 11(8), 724-731. https://doi.org/10.4161/cbt.11.8.15281

Firbas, P. (2011). *Allium* metaphase genotoxicity assay for the testing of drinking water, chemicals, and environmental samples. Private Laboratory of Plant Cytogenetics, 1(1), 1-6.

Firbas, P., & Amon, T. (2013). *Allium* chromosome aberration test for evaluation effect of cleaning municipal water with constructed wetland (CW) in Sveti Tomaz, Slovenia. Journal Bioremediation and Biodegradation, 4(4), 1-6. https://doi.org/10.4172/2155-6199.1000189

Firbas, P., & Amon, T. (2014). Chromosome damage studies in the onion plant *Allium cepa* L. Caryologia: International Journal of Cytology, Cytosystematics, and Cytogenetics, 67(1), 25-35. https://doi.org/10.1080/00087114.2014.891696

Fiskesjö, G. (1985). The *Allium* test as a standard in environmental monitoring. Hereditas, 102(1), 99-112.

Gajalakshmi, K., & Ruban, P. (2014). Evaluation of physico-chemical parameters and cytotoxic effect of Orathupalayam dam in Tirupur District. International Journal of Agricultural Policy Research, 2(5), 191-197.

Galdiero, E., Siciliano, A., Maselli, V., Gesuele, R., Guida, M., Fulgione, D., Galdiero, S., Lombardi, L., & Falanga, A. (2016). An integrated study on antimicrobial activity and ecotoxicity of quantum dots and quantum dots coated with the antimicrobial peptide indolicidin. International Journal of Nanomedicine, 11, 4199-4211. https://doi.org/10.2147/IJN.S107752

Gamboa, R., Gomez, A.L., & Nievales, M.F. (2007). The status of sea cucumber fishery and mariculture in the Philippines. FAO Fisheries Technical Paper, 463(1), 69-78.

Gowda, N.M., Goswami, U., & Islam-Khan, M. (2009). Purification and characterization of a T-antigen specific lectin from the coelomic fluid of a marine invertebrate, sea cucumber (*Holothuria scabra*). Fish and Shellfish Immunology, 24(1), 450-458. https://doi.org/10.1016/j.fsi.2008.01.002

Gwozdzinski, K., & Lichota, A. (2018). Anticancer activity of natural compounds from plant and marine environment. International Journal of Molecular Sciences, 19(11), 3533, 1-38. https://doi.org/10.3390/ijms19113533-

Hamid, R., Masood, A., Wani, I.H., & Rafiq, S. (2013). Lectins: Proteins with diverse applications. Journal of Applied Pharmaceutical Science, 3(4), 2231-3354. https://doi.org/10.7324/JAPS.2013.34.S18

Han, H., Xu, Q., Tang, H., Yi, Y., & Gong, W. (2010). Cytotoxic holostane-type triterpene glycosides from the sea cucumber *Pentacta quadrangularis*. Planta Medica, 76(16), 1900-1904. https://doi.org/10.1055/s-0030-1249854

International Union for Conservation of Nature and Natural Resources. (2013). IUCN red list of threatened species. Retrieved from http://www.iucnredlist.org

Januar, H.I., Nursid, M., & Ekowati, C. (2014). Cytotoxic saturated fatty acids from the Indonesian sea cucumber *Holothuria* sp. Squalen. Bulletin of Marine and Fisheries Postharvest and Biotechnology, 9(1), 11-15. http://dx.doi.org/10.15578/squalen.v9i1.69

Khodayar, M.J., Keshtar, E., Javadipour, M., Hosseini, M., Hashemitubar, M., Ahmadi, S., & Rezaei, M. (2015). Sea cucumber enhances apoptotic activity of lovastatin. International Journal of Experimental Pharmacology, 5(1), 37-41.

Kong, D.X., Jiang, Y.Y., & Zhang, H.Y. (2010). Marine natural products as sources of novel scaffolds: Achievement and concern. Drug Discovery Today, 15 (21-22), 884-886. https://doi.org/10.1016/j.drudis.2010.09.002

Lam, S.K., & Ng, T.B. (2010a). First report of a haemagglutinin-induced apoptotic pathway in breast cancer cells. Bioscience Report, 30(5), 307-317. https://doi.org/10.1042/BSR20090059

Lam, S.K., & Ng, T.B. (2010b). Isolation and characterization of a French bean hemagglutinin with antitumor, antifungal, and anti-HIV-1 reverse transcriptase activities and an exceptionally high yield. Phytomedicine, 17(6), 457-462. https://doi.org/10.1016/j.phymed.2009.07.017

Lawrence, A.J., Afifi, R., Ahmed, M., Khalifa, S., & Paget, T. (2009). Bioactivity as an options value of sea cucumbers in the Egyptian Red Sea. Society for Conservation Biology, Conservation Biology, 24(1), 217-225. https://doi.org/10.1111/j.1523-1739.2009.01294

Li, B., Lu, F., Wei, X., & Zhao, R. (2008a). Fucoidan: Structure and bioactivity. Molecules, 13(8), 1671-1695. https://doi.org/10.3390/molecules13081671

Li, C., Haug, T., Styrvold, O.B., Jørgensen, T., & Stensvåg, K. (2008b). Strongylocins, novel antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*. Developmental & Comparative Immunology, 32(12), 1430-1440. https://doi.org/10.1016/j.dci.2008.06.013

Mattana, C.M., Cangiano, M.A., Alcaraz, L.E., Sosa, A., Escobar, F., Sabini, C., Sabini, L., & Laciar, A.L. (2014). Evaluation of cytoxocicity and genotoxicity of

Acacia aroma leaf extracts. The Scientific World Journal, 380850. https://doi.org/10.1155/2014/380850

Mayer, A.M.S., & Gustafson, K.R. (2009). Marine pharmacology in 2005-6: Antitumor and cytotoxic compounds. European Journal of Cancer, 44(16), 2357-2387. https://doi.org/10/1016/j.ejaca.2008.07.001

Mohammadizadeh, F., Ehsanpor, M., Afkhami, M., Mokhlesi, A., Khazaali, A., & Montazeri, S. (2013). Antibacterial, antifungal, and cytotoxic effects of a sea cucumber *Holothuria leucospilota*, from the north coast of the Persian Gulf. Journal of the Marine Biological Association of the United Kingdom, 93(5), 1401-1405. https://doi.org/10.1017/S0025315412001889

Mohklesi, A., Saeidma, S., Gohari, A.R., Shaverdi, A.R., Nasrolahi, A., Farahani, F., Khoshnood, R., & Es'haghi, N. (2012). Biological activities of the sea cucumber *Holothuria leucospilota*. Asian Journal of Animal and Veterinary Advances, 7(3), 243-249. https://doi.org/10.3923/ajava.2012.243.249

Mojica, E-R., & Merca, F.E. (2005). Biological properties of lectin from sea cucumber (*Holothuria scabra* Jaeger). Journal of Biological Sciences, 5(4), 472-477. https://doi.org/10.3923/jbs.2005.472.477

Montaser, R., & Leusch, H. (2011). Marine natural products: A new wave of drugs? Future Medicinal Chemistry, 3(12), 1475-1489. https://doi.org/10.4155/fmc.11.118

Ogushi, M., Yoshie-stark, M., & Suzuki, T. (2005). Cytotoxic activity of hot water extracts from sea cucumber in Caco-2. Food Science and Technology Research, 11(1), 202-206. https://doi.org/10.3136/fstr.11.202

Podolak, I., Galanty, A., & Sobolewska, D. (2010). Saponins as cytotoxic agents: A review. Phytochemistry Review, 9(3), 425-474. https://doi.org/10.1007/s11101-010-9183-z

Purcell, S.W. (2010). Managing sea cucumber fisheries with an ecosystem approach. FAO Fisheries and Aquaculture Technical Paper, 520, 1-158.

Purcell, S.W., Samyn, Y., & Conand, C. (2012). Commercially important sea cucumbers of the world. Food and Agriculture Organization (FAO) Species Catalogue for Fishery Purposes, 6(1), 150.

Rafiuddin, A.M., Venkateshwarlu, U., & Jayakumar, R. (2004). Multi-layered peptide incorporated collagen tubules for peripheral nerve repair. Biomaterials, 25(13), 285-294. https://doi.org/10.1016/j.biomaterials.2003.09.075

Ranganatha, R., Chakravarthy, S., & Sukumaran, S.K. (2016). High-throughput approaches for genotoxicity testing in drug development: Recent advances. International Journal of High Throughput Screening, 2016(6), 1-12. https://doi.org/10.2147/IJHTS.S70362

Ridzwan, B.H., Azizan, N.A., Zamli, Z., Zulkipli, F.H., Mazlan, F.H., & Althunibat, O.Y. (2014). Toxicity effects of water extracts of *Holothuria atra* Jaeger in mice. Asian

Pacific Journal of Tropical Biomedicine, 4(8), 614-617. https://doi.org/10.12980/APJTB.4.201414B154

Santhakumari, G., & Stephen, J. (1988). Antimitotic effects of holothurin. Cytologia, 53(1), 163-168. https://doi.org/10.1508/cytologia.53.163

Sarker, U., & Oba, S. (2018a). Drought stress effects on growth, ROS markers, compatible solutes, phenolics, flavonoids, and antioxidant activity in *Amaranthus tricolor*. Applied Biochemistry and Biotechnology, 186, 999-1016. https://doi.org/10.1007/s12010-018-2784-5

Sarker, U., & Oba, S. (2018b). Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. Scientific Reports, 8, 16496. https://doi.org/10.1038/s41598-018-34944-0

Sarker, U., & Oba, S. (2020). The response of salinity stress-induced *A. tricolor* to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. Frontiers in Plant Science, 11, 1354. https://doi.org/10.3389/fpls.2020.559876

Seydi, E., Motallebi, A., Dastbaz, M., Dehghan, S., Salimi, A., Nazemi, M., & Pouhrahmad, J. (2015). Selective toxicity of Persian Gulf sea cucumber (*Holothuria parva*) and sponge (*Haliclona oculata*) methanol extracts on liver mitochondria isolated from an animal model of hepatocellular carcinoma. Hepatitis Monthly, 15 (12), e33073. https://doi.org/10.5812/hepatmon.33073

Siegel, I., Yaghoubzadeh, E., Keskey, T.S., & Gleicher, N. (1987). Cytotoxic effects of free fatty acids on ascites tumor cells. Journal of the National Cancer Institute, 78(2), 271-277. https://doi.org/10.1093/jnci/78.2.271

Silchenko, A.S., Avilov, S.A., Kalinin, V.I., Kalinovsky, A.I., Dmitrenok, P.S., Fedorov, S.N., Stepanov, V.G., Dong, Z., & Stonik, V.A. (2007). Constituents of the sea cucumber *Cucumaria okhotensis*. Structures of okhotosides B1–B3 and cytotoxic activities of some glycosides from this species. Journal of Natural Products, 71(3), 351-356. https://doi.org/10.1021/np0705413

Southeast Asian Fisheries Development Center. (2012). Status and issues relevant to sea cucumber fisheries in Southeast Asia. 44th Meeting of the Council, Southeast Asian Fisheries Development Center, SEAFDEC Secretariat, REF07c, 1-14.

Swift, L.H., & Golsteyn, R.M. (2014). Genotoxic anti-cancer agents and their relationship to DNA damage, mitosis, and checkpoint adaptation in proliferating cancer cells. International Journal of Molecular Sciences, 15(3), 3403-3431. https://doi.org/10.3390/ijms15033403

Tehranifard, A., Oryan, S., & Vosoghi, G. (2011). Introducing a Holothurian sea cucumber species *Stichopus hermanni* from Kish Island in the Persian Gulf in Iran. Proceedings of the International Conference on Environment and Industrial Innovation IPCBEE, Kuala Lumpur, Malaysia, 138-143.

Tedesco, S.B., & Laughinghouse, H.D., IV. (2012). Bioindicator of genotoxicity: The *Allium cepa* test. In J. Srivastava (Ed.), Environmental contamination (pp. 137-156). United Kingdom: IntechOpen Limited.

Toral-Granda, M.V. (2006). Fact sheets and identification guide for commercial sea cucumber species. SPC Beche-de-mer, 24(1), 49-52.

Toral-Granda, V., Lovatelli, A., & Vasconcellos, M. (2008). Sea cucumbers. A global review of fisheries and trade. FAO Fisheries and Aquaculture Technical Paper. No. 516, 317.

Torres, J.R.D. (2017). Antibacterial and antifungal property of extracts derived from the body wall and cuvierian tubules of *Pearsonothuria graeffei* Semper (black-spotted sea cucumber). Frontiers in Medicinal Chemistry and Drug Discovery, 2(1), 010-018.

Tripathy, S.K., Bijayinee, S., Samad, I., & Das, R.K. (2013). Endosulfan: A potential genotoxicant on *Allium cepa* root tip cells. Journal of Agricultural Biotechnology and Sustainable Development, 5(2), 29-35. https://doi.org/10.5897/JABSD12.0001

Uthicke, S., & Conand, C. (2005). Local examples of beche-de-mer overfishing: An initial summary and request for information. SPC Beche-de-mer Information Bulletin, 21, 7-14.

Yahyavi, M., Afkhami, M., Javadi, A., Ehsanpour, M., Khazaali, A., Khoshnood, R., & Mohklesi, A. (2012). Fatty acid composition in two sea cucumber species, *Holothuria scabra* and *Holothuria leucospilata* from Qeshm Island (Persian Gulf). African Journal of Biotechnology, 11(12), 2862-2868. https://doi.org/10.5897/AJB11.3529

Zaki, M.A. (2005). Effects of crude toxin of sea cucumbers *Holothuria atra* on some hematological and biochemical parameters in rats. Egyptian Journal of Natural Toxins, 2(1), 71-86.

Zhang, Q.W., Lin, L.G., & Ye, W.C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. Chinese Medicine, 13, 20. https://doi.org/10.1186/s13020-018-0177-x

Zhang, Z., & Li, S. (2007). Cytotoxic triterpenoid saponins from the fruits of *Aesculus pavia* L. Phytochemistry, 68(15), 2075-2086. https://doi.org/10.1016/j.phytochem.2007.05.020

Zhao, J.K., Wang, H.X., & Ng, T.B. (2009). Purification and characterization of a novel lectin from the toxic wild mushroom *Inocybe umbrinella*. Toxicon, 53(3), 360-366. https://doi.org/10.1016/j.toxicon.2008.12.009

Zhao, Q., Xue, Y., Liu, Z., Li, H., Wang, J., Li, Z., Wang, Y., Dong, P., & Xue, C. (2011). Ds-echinoside A, a new triterpene glycoside derived from sea cucumber, exhibits antimetastatic activity via the inhibition of NF-κB-dependent MMP-9 and VEGF expressions. Journal of Zhejiang University-SCIENCE B (Biomedicine and Biotechnology), 12(7), 534-544. https://doi.org/10.1631/jzus.B1000217

Zhao, Q., Xue, Y., Wang, J.F., Li, H., Long, T.T., Li, Z., Wang, Y.M., Dong, P., & Xue, C. (2012). In vitro and in vivo anti-tumor activities of echinoside A and ds-echinoside A from *Pearsonothuria graeffei*. Journal of the Science of Food and Agriculture, 9(1), 965-974. https://doi.org/10.1002/jsfa.4678