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THE EFFECT OF *Diopatra claparedii* Grube, 1878 AQUEOUS EXTRACT ON CHANG LIVER AND HUMAN PRIMARY GLIOBLASTOMA (U-87) CELL LINES

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Abstract: The distinctive regenerative ability of local marine worm (polychaete), *Diopatra claparedii* Grube, 1878, has the potential as a cellular growth agent. In this study, the growth effect was investigated in normal cells and cancer cells. Different concentrations (0-100 μ g/mL) of *D. claparedii* aqueous extract were tested on Chang Liver (normal cells), and Human Primary Glioblastoma (U-87) (cancer cells) cell lines for 24, 48 and 72 hours. Percentage of cell viability was evaluated by [2-(4, 5-dimethyl-2-thiazolyl)-3, 5-diphenyl-2H tetrazolium bromide] (MTT) assay. The findings suggested that the extract had a proliferative effect on normal cell growth when tested at lower doses (<60 μ g/mL) but inhibited normal cells at concentrations >80 μ g/mL in all incubation periods. Meanwhile, it showed the cytotoxic effect on cancer cells only after 48h when treated with all concentrations. As demonstrated, the extract could induce normal cell growth without causing abnormal or cancer cells progression at low concentrations after 48h and 72h.

Keywords: *Diopatra claparedii*, Ruat Sarung, marine polychaete, cell regeneration, cytotoxicity, Chang Liver cell, Human Primary Glioblastoma

Introduction

Degenerative disease is a disease resulted from the process of degenerative cell changes that will deteriorate the affected tissues or organ. A few examples are cirrhosis of the liver, Alzheimer's disease, amyotrophic lateral sclerosis, osteoporosis, and atherosclerosis. These degenerative diseases, especially neurodegenerative diseases, are becoming a major concern worldwide, as the deterioration of the nerves can greatly affect the patient's balance, coordination, heart function, and also cause respiratory and talking impairment. It is a serious and life-threatening health condition that requires further study on treatment to support healthy growth of normal cells. In fact, statistics have shown that chronic degenerative diseases are now the cause of three-quarters of worldwide mortality of middle and old age people (Doll, 1995). Alzheimer Disease (AD) was recently reported as the sixth-leading cause of death in the United States and is the only top ten mortality causes that cannot be prevented, nor curable, nor slowed.

Although normal and healthy cell growth is important in preventing degenerative diseases, uncontrolled proliferation overgrowth of unhealthy or abnormal cells, which deviate from the normal cells may turn out to be a risk for cancer development. Recent statistics from 2018 has proved that cancer is still one of the deadliest diseases of all times, only exceeded by cardiac diseases (Cancer Facts & Figures, 2018). While it is devastating, the search for new potent and fully effective cancer treatment is still actively ongoing and leaves room for

discoveries of a novel treatment in cancer research.

All these degenerative diseases with their increasing mortality rate have brought light to regenerative medicine. Regenerative medicine is a new field of medical science that is dedicated to reestablishing normal function of damaged cells through the approach of repair, replacement or regeneration of healthy cells (Sun and Kurtzberg, 2015). According to Vincent (2014), these cell-based therapies and products specializing in regenerative methods are now on the market demand and are believed to be increasingly developed in the near future. More investment and effort in this field of study could provide an alternative to the future approach in the treatment of degenerative diseases.

It is well known that marine natural products (MNPs) have been widely studied in scientific and medical research for a long time, and used in traditional medicine. The MNPs are increasingly on demand, especially in cancer therapy and prevention. According to Calcabrini *et al.* (2017), natural compounds have been proven to be effective alternatives to traditional antitumor therapy. Nowadays, almost all of the newly-discovered anticancer drugs are derived from natural compounds. Their properties like anti-mutagenic and anti-carcinogenic can potentially inhibit carcinogenesis, either by preventing or delaying cancer (Rajasekaran *et al.,* 2008). Wargasetia and Widodo (2017) highly supported the medicinal values of sea cucumbers, attributed by their marine-derived agents with the potential to inhibit human tumor cells through *in vitro* studies, *in vivo* models, and also human studies.

Today, polychaetes, or more commonly known as bristle worms from the phylum Annelida, are becoming one of the actively studied marine organisms. Most annelids, including polychaetes, have the ability for posterior regeneration, but anterior regeneration is less common (Bely, 2006). However, interestingly, polychaetes exhibit variable degrees of regeneration (Glasby *et al.*, 2000) and can regenerate both forward and backward (Goss, 2016).

Diopatra claparedii is a tropical polychaete species and can be found along the coastline of Malay Archipelago including the Philippines, its type locality (Paxton, 2002). In Malaysia, *D. claparedii* is recorded to be distributed along the mangrove facing tidal flats of the west coast of Peninsular Malaysia (Idris and Arshad, 2013). The species is commonly known as 'Ruat Sarung' or 'Pumpun Sarung' in the Peninsular Malaysia, and is known among recreational anglers as one of popular bait worm (Idris and Arshad, 2013).

Similar to other *Diopatra* species, for example *D. neopolitana* (Freitas *et al.*, 2015), *D. claparedii* found in the Peninsular Malaysia has anterior regenerative capability (Idris and Arshad, 2013). Thus, the study of *D. claparedii* regeneration could provide a new approach in dynamic process of replacing devitalized and missing cellular structure in human (Bely, 2014), as in degenerative diseases. Nevertheless, while it may promote normal cell growth, it may also have an adverse effect, such as promoting the proliferation of cancer cells. As such, instead of focusing solely on normal cells (Chang Liver), this study also focused on the effect of *D. claparedii* extract on the cellular growth of cancer cells (Human Primary Glioblastoma (U-87)) cell lines.

Materials and Methods

Sample Collection

Specimens of *D. claparedii* used in this study were collected from Pantai Kelanang, Morib, Selangor during low tide in July, 2017. The tubes were carefully removed from the sediment and checked to make sure the worms were inside. All tubes were covered with damp clothes to keep them moist until arrival at the laboratory. The sediment was also collected to keep the specimens alive prior to experiment.

Preparation of Extract

Extracts of *D. claparedii* were prepared according to Mazliadiyana *et al.,* (2017) and Hussain *et al.,* (2018), with a few modifications. Six samples of *D. claparedii* freshly killed by freezing were weighed without the tubes and cut into smaller pieces in one beaker. Then, they were pulverised using pestle and mortar. The pulverised samples were later centrifuged at 4 °C, 4000 rpm for 20 minutes for three times until a clear, homogenized supernatant was collected. The whole process was carried out in cold condition all samples and apparatus were kept in ice-filled container to prevent protein degradation. 20 mL of the supernatant obtained from the centrifugation was collected and stored in the freezer overnight. The frozen supernatant was later freeze-dried using FreeZone Labconco 18 freeze-dryer to produce extract in powder form. The aqueous extract was then prepared by dissolving 0.012 g powder extract in 12 mL deionized water. It was first filtered through a 0.2 μm syringe. The final volume of the aqueous extract stock prepared was 1000 μg/mL.

Cell Culture

This study only focused on two cell lines. The Chang Liver cell growth represented the normal cell growth, while U-87 cell growth represented cancer cell growth. The stock for Chang Liver cell was obtained from UMT, while the brain cancer cell (U-87) was obtained from Universiti Sultan Zainal Abidin (UniSZA). Routine cell culture works including thawing, subculturing and freezing of cells were carried out according to the Gibco Cell Culture Basics Handbook (Thermo Fisher Scientific, 2015).

Both cell lines were cultured and grown in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), as both cell lines were observed to prefer the condition in this preliminary study. The laboratory conditions for both cells were monitored and the air was controlled regularly, containing 95% air and 5% CO_2 at temperature 37 °C.

Treatment of Extract on Cell

Five different concentrations of aqueous extract of *D. claparedii* 20, 40, 60, 80, and 100 μg/mL were used on each cell of both types, Chang liver and U-87 cells. These concentrations were chosen as treatments according to the methods used in a study by Patar *et al.,* (2012) but with a slight increase in all concentrations used. The wider range of concentrations was designed to determine the most optimum concentration that would show the best result on normal cell growth. Positive effect on cell growth shown at a small concentration would depict the high efficiency of the extract, compared to a higher concentration of extract. Cells in culture media without any treatment of extract were used as the negative control of this experiment.

Both types of cells were then seeded in 100 μL complete media in the 96 well plate for 24, 48 and 72 hours of treatment. They were incubated at temperature 37 °C with 5% CO_2 overnight. After 24 hours, the media in the wells were removed, leaving only the attached cells, which were then mixed with 200 μg/mL extract of the five chosen extract concentrations and a negative control. The plates were then incubated again at 37 °C with 5% CO_2 until [2-(4, 5-dimethyl-2-thiazolyl)-3, 5-diphenyl-2H tetrazolium bromide] (MTT) assay was performed after 24 hours, 48 hours, and 72 hours of incubation respectively. All tests were done in quadruplicates.

MTT Assay

Percentage of cell viability was evaluated by MTT assay. After the respective intervals for the test, the old medium in the 96 well plates was removed, and all wells were washed with phosphate buffered saline (PBS) before 100 μL fresh medium was added to each well. MTT assay treatment was performed in the dark, as it is light-sensitive. 20 μg of MTT solution was added into each well, and the plates were incubated for another three hours. After that, the supernatant was removed and 100 μL

DMSO was added to the wells as a solvent to dissolve the formazan crystals. To ensure that the crystals were completely dissolved, the plates were shaken for 5 minutes and the reading was recorded using a micro plate reader with the absorbance wavelength set at 570 nm.

Data Analysis

IBM SPSS Statistics 20 software was used for all statistical analyses. The differences between the values of relative proliferation were analysed by paired sample t-test. Data were expressed as mean ± standard deviation. Significance level was set at $p = 0.05$ for all tests with 95% confidence level.

Results and Discussion

Chang Liver Cell (Normal Cell)

Microscopic observation using light microscope was conducted to visually observe the effect of *D. claparedii* extract on the growth of cells. Observation of Chang Liver cells for all three incubation periods **(**Figures 1, 2, and 3) showed no obvious comparison in term of cell confluency. All wells showed high confluency with limited space and healthy cell lineage due to the high viability of cells demonstrated. This observation was in accordance with the MTT assay results, which proved that no significant difference ($p > 0.05$) was recorded in all tests, except for concentration 100 μg/mL Figure 2 (F) and 3 (F) after 48 hours and 72 hours. Even so, from the microscopic observation, well F for both 48 hours and 72 hours still demonstrated high cell confluency, despite the decrease in live cells.

The MTT assay results in **Figures 4, 5**, and **6** showed similar trends in cell viability of normal cell for all incubation periods. An increasing trend was observed when lower concentrations of *D. claparedii* extract (20 μg/mL 40 μg/mL, and 60 μg/mL) were used, compared to the negative control. However, when the extract concentration was increased to 80 μg/mL and 100 μg/mL, normal cell viability started to decrease significantly at 100 μg/mL after 48 and 72 hours. This result suggested that the extract could only support normal cell proliferation at lower concentrations for all three periods, but it inhibited cell growth when used at high doses. The highest promotion of cell viability was recorded when concentration of extract used was at the lowest (20 μg/mL) for all three incubation periods. But when used at concentration >60 μg/mL, the extract appeared to be cytotoxic to the normal cells.

From the results, the optimum concentration of extract for positive growth of cells, despite the insignificant increase, was at the lowest concentration (20 μg/mL) after 24 hours. The extract was more cytotoxic to normal cells after longer incubation time and treated with higher extract concentrations as the percentage of cell viability significantly dropped. This result contradicted our initial hypothesis that suggested that the higher concentration of extracts will be an excellent cell growth promoter with no toxicity effect.

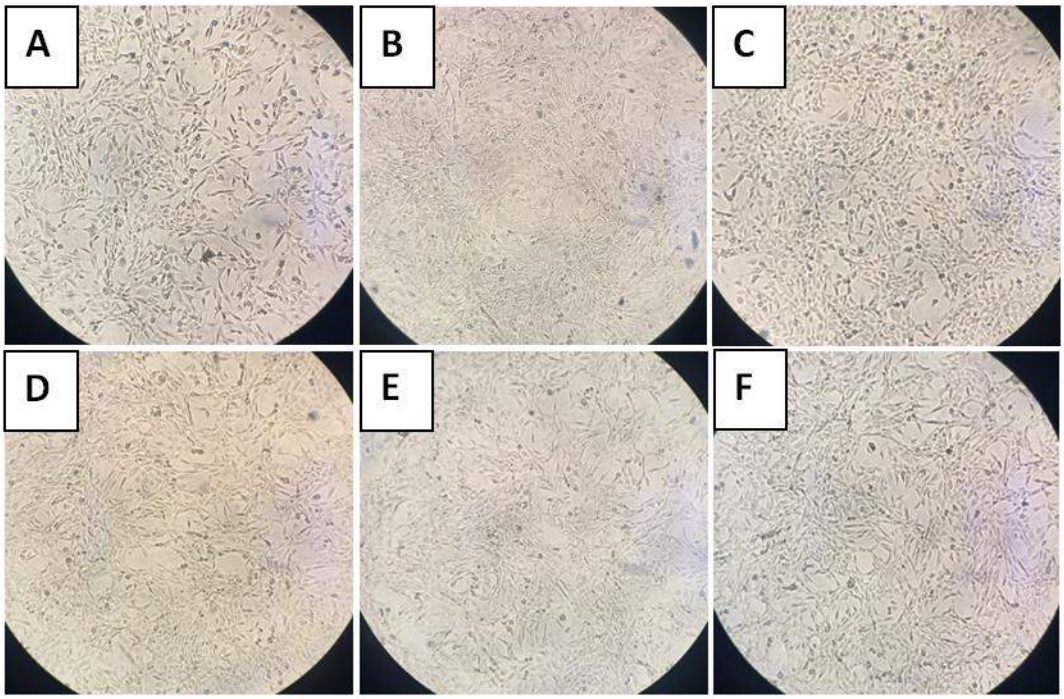

Figure 1: Microscopic appearance of Chang Liver cells, viewed under 20x magnification after 24-hour incubation period. (A: negative control; B: 20 μ g/mL; C: 40 μ g/mL; D: 60 μ g/mL; E: 80 μ g/mL; F: 100 μ g/mL concentrations of *D. claparedii* crude extract).

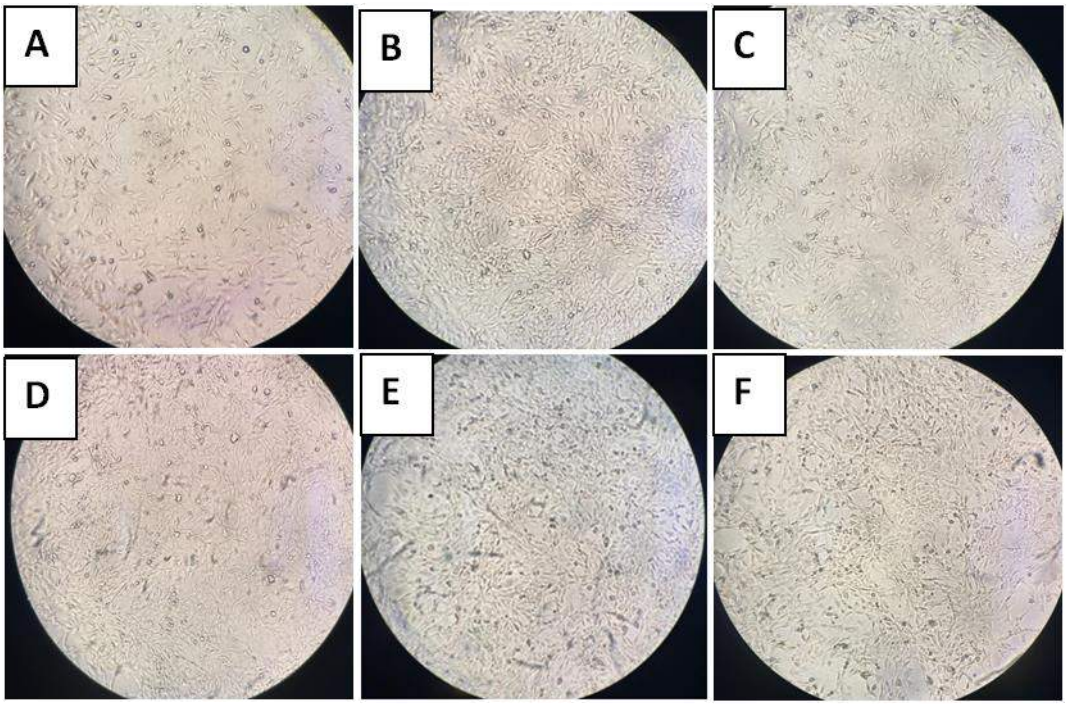

Figure 2: Microscopic appearance of Chang Liver cells, viewed under 20x magnification after 48-hour incubation period. (A: negative control; B: 20 μg/mL; C: 40 μg/mL; D: 60 μg/mL; E: 80 μg/mL; F: 100 μg/mL concentrations of *D. claparedii* crude extract).

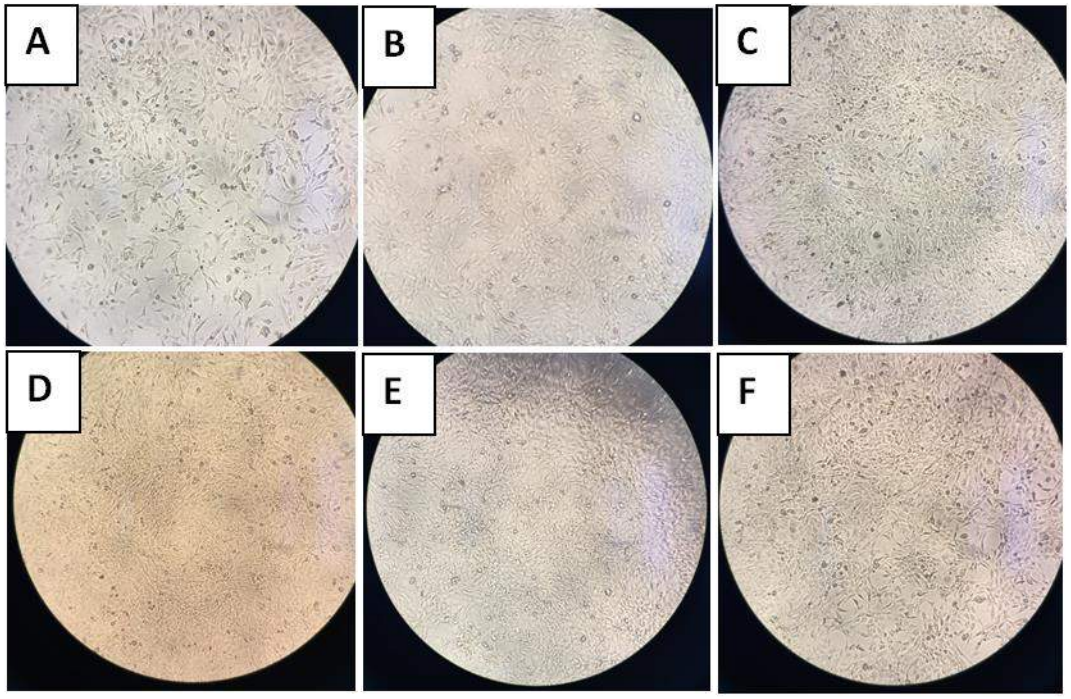

Figure 3: Microscopic appearance of Chang Liver cells, viewed under 20x magnification after 72-hour incubation period. (A: negative control; B: 20 μg/mL; C: 40 μg/mL; D: 60 μg/mL; E: 80 μg/mL; F: 100 μg/mL concentrations of *D. claparedii* crude extract).

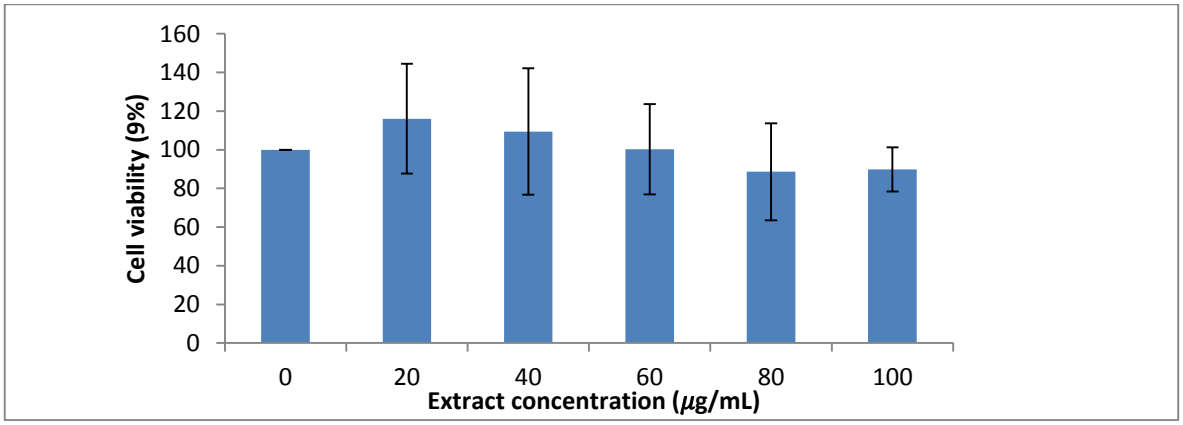

Figure 4: Percentage of Chang Liver cell viability (%) against extract concentration (μ g/mL) for incubation period of 24 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when co mpared to negative control, $p < 0.05$, n = 3.

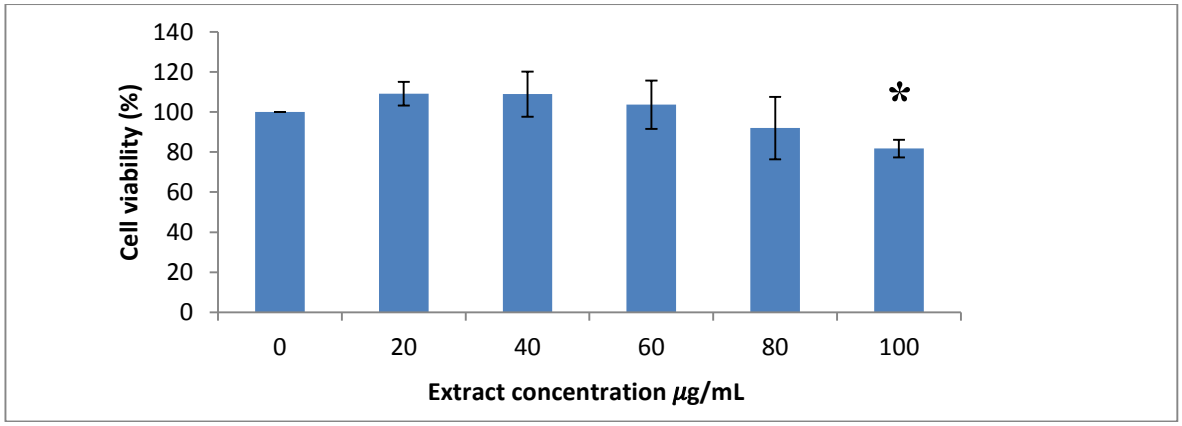

Figure 5: Percentage of Chang Liver cell viability (%) against extract concentration (μg/mL) for incubation period of 48 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when compared to negative control, $p < 0.05$, n = 3.

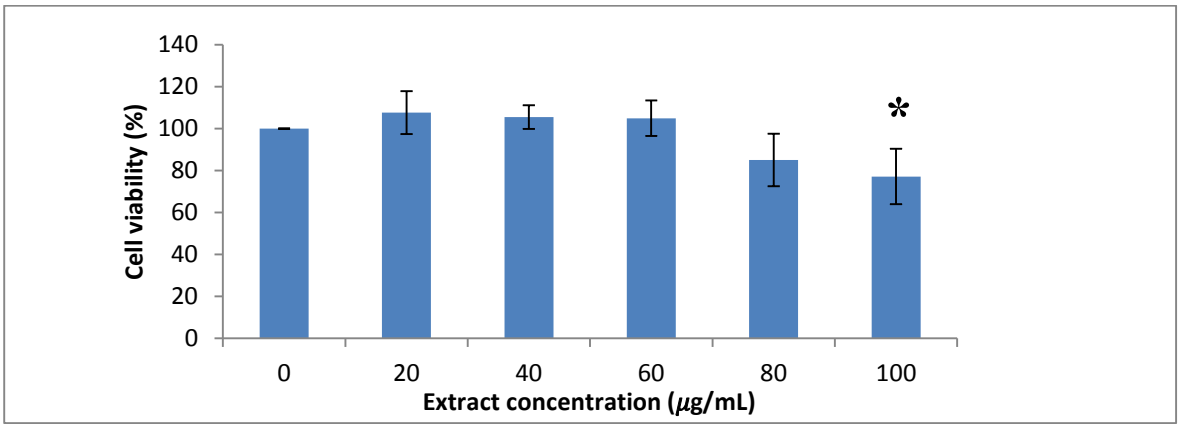

Figure 6: Percentage of Chang Liver cell viability (%) against extract concentration (μg/mL) for incubation period of 72 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when compared to negative control, $p < 0.05$, n = 3.

Human Primary Glioblastoma U-87 (Cancer Cell)

Microscopic observation of U-87 cancer cells after 24 hours (Figure 7) showed observable comparison in cell confluency when compared to the negative control (A). Wells D, E, and F demonstrated higher cell confluency due to the promoted cell viability. However, after 48 (Figure 8) and 72 hours (Figure 9), significant decrease in cancer cell confluency could be observed in wells E and F. The spaces between cells were also larger, which was most probably caused by the significant cell death. This observation was also in accordance with the results obtained from the MTT assay.

MTT assay readings showed that after 24 hours (Figure 10), a direct increasing trend of cancer cell viability and proliferation was recorded for all tested concentrations. The highest dose of extract (100 μ g/mL) appeared to promote the highest cell viability. This result suggested that at incubation period of 24 hours, the extract supported the growth of cancer cells, which was in contrast with our initial hypothesis. However, interestingly, at longer incubation period of 48 hours (Figure 11), significant decrease ($p < 0.05$) of cancer cell viability and proliferation could be observed at concentrations 60, 80, and 100 μ g/mL, compared to the control. After 72 hours (Figure 12), significant decrease in cancer cell viability was observed at concentrations 20 μ g/mL and 80 μ g/mL. This finding showed the extract was indeed cytotoxic and able to inhibit the growth of cancer cells as per our proposed hypothesis, only more effectively at longer incubation periods.

At incubation period of 48 hours, the lowest cancer cell viability was observed when

the extract concentration used was 80 μg/mL (77.45% inhibition). The IC_{50} was estimated at the concentration of 40 μg/mL. Meanwhile, at incubation period of 72 hours, the lowest cell viability was when 60 μg/mL of extract was used (65.85% inhibition), despite having no significant differences from the control ($p > 0.05$). The IC_{50} was estimated at 80 μg/mL. Thus, from the findings, we suggest that the optimum extract concentration for effective and cytotoxic on cancer cells is at 80 μg/mL, for with incubation period of 48 hours.

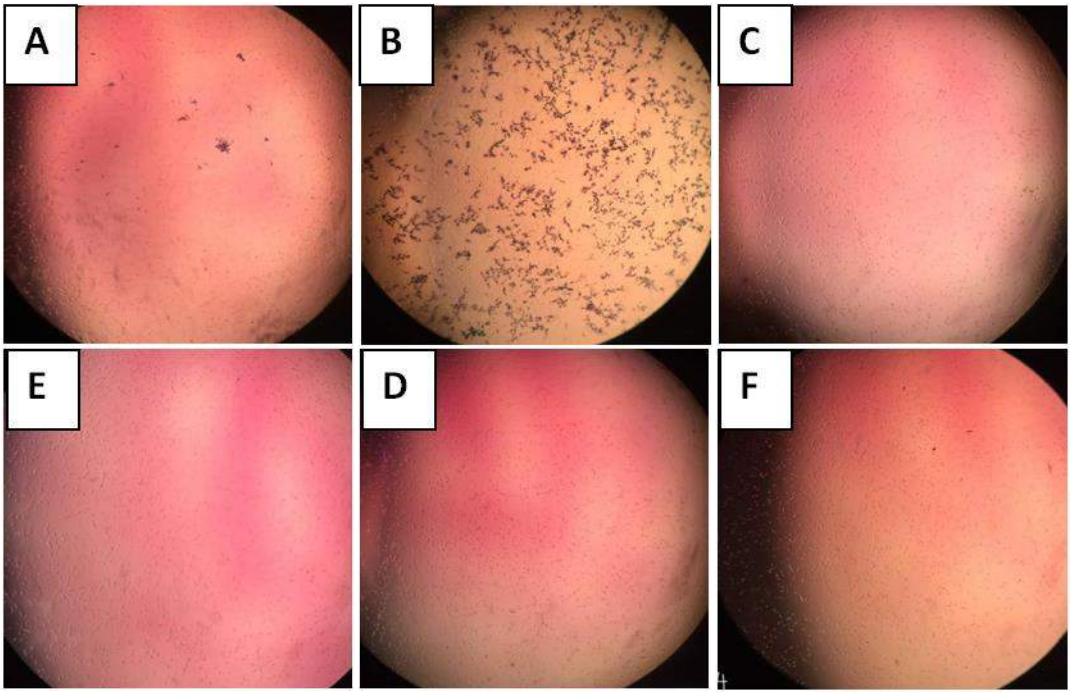

Figure 7**:** Microscopic appearance of U-87 cells, viewed under 4x magnification after 24-hour incubation period. (A: negative control; B: 20 μg/mL; C: 40 μg/mL; D: 60 μg/mL; E: 80 μg/mL; F: 100 μg/mL concentrations of *D. claparedii* crude extract).

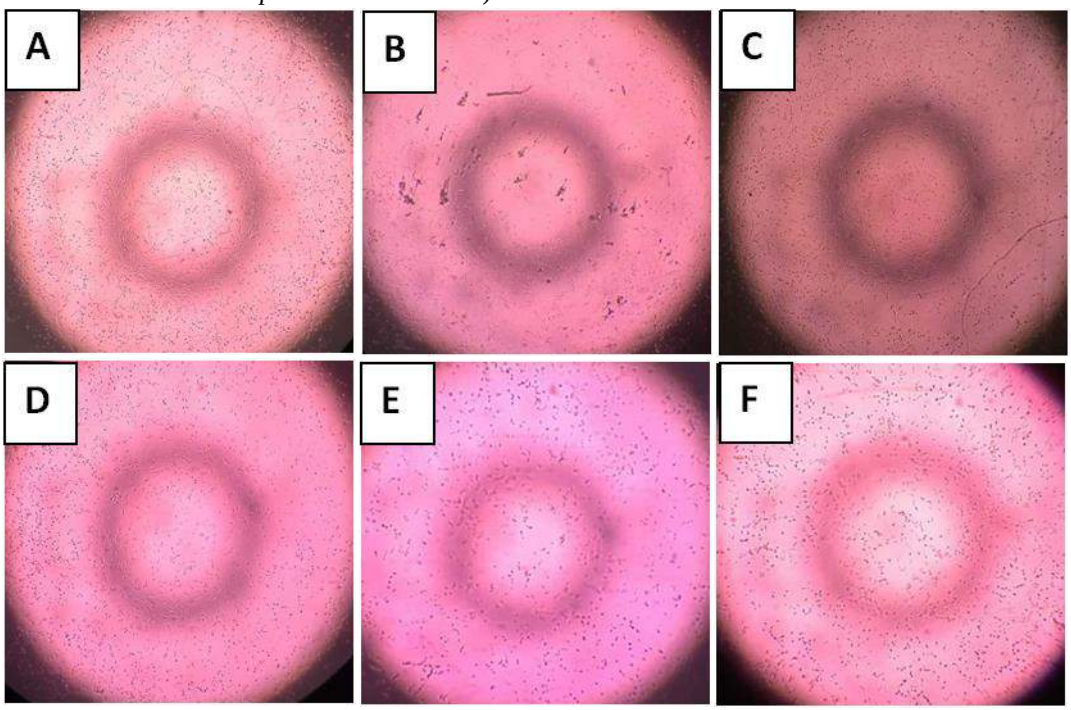

Figure 8: Microscopic appearance of U-87 cells, viewed under 4x magnification after 48-hour incubation period. (A: negative control; B: 20 μg/mL; C: 40 μg/mL; D: 60 μg/mL; E: 80 μg/mL; F: 100 μg/mL concentrations of *D. claparedii* crude extract).

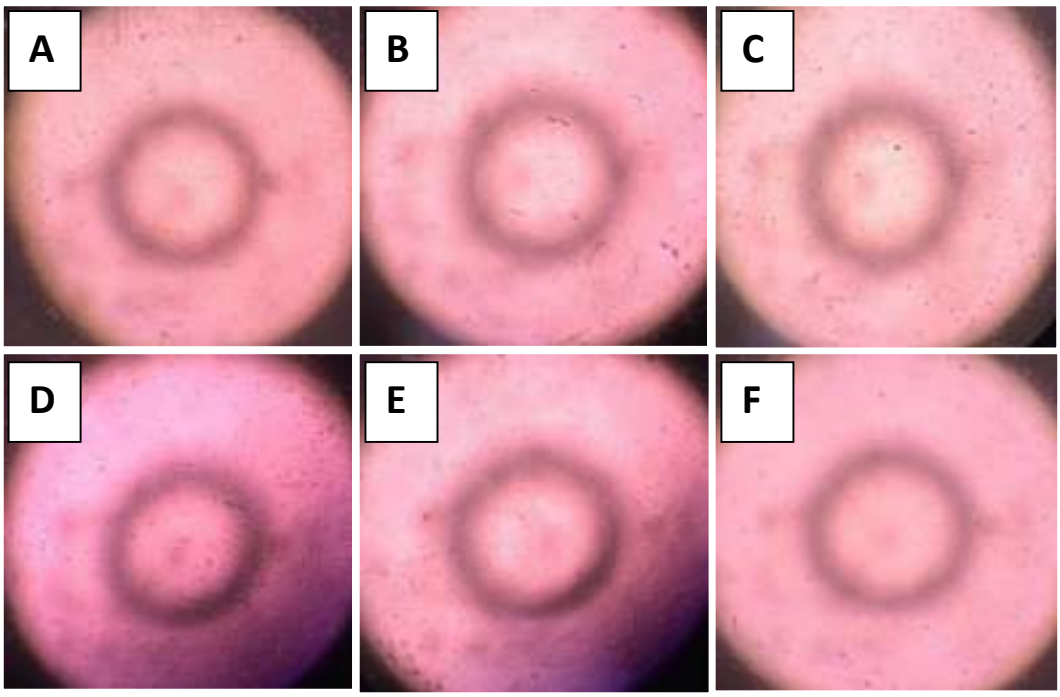

Figure 9: Microscopic appearance of U-87 cells, viewed under 4x magnification after 72-hour incubation period. (A: negative control; B: 20 μg/mL; C: 40 μg/mL; D: 60 μg/mL; E: 80 μg/mL; F: 100 μg/mL concentrations of *D. claparedii* crude extract).

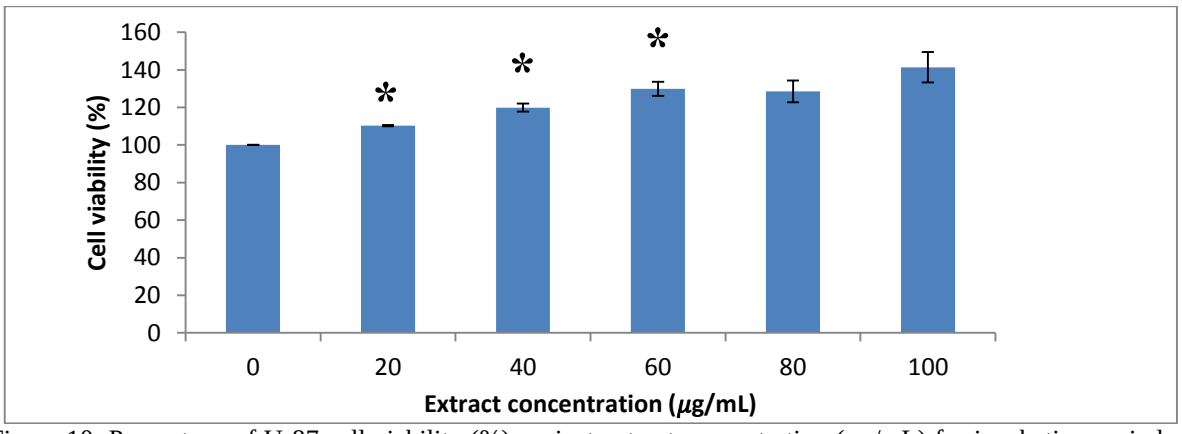

Figure 10: Percentage of U-87 cell viability (%) against extract concentration (μg/mL) for incubation period of 24 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when compared to negative control, $p < 0.05$, n = 2.

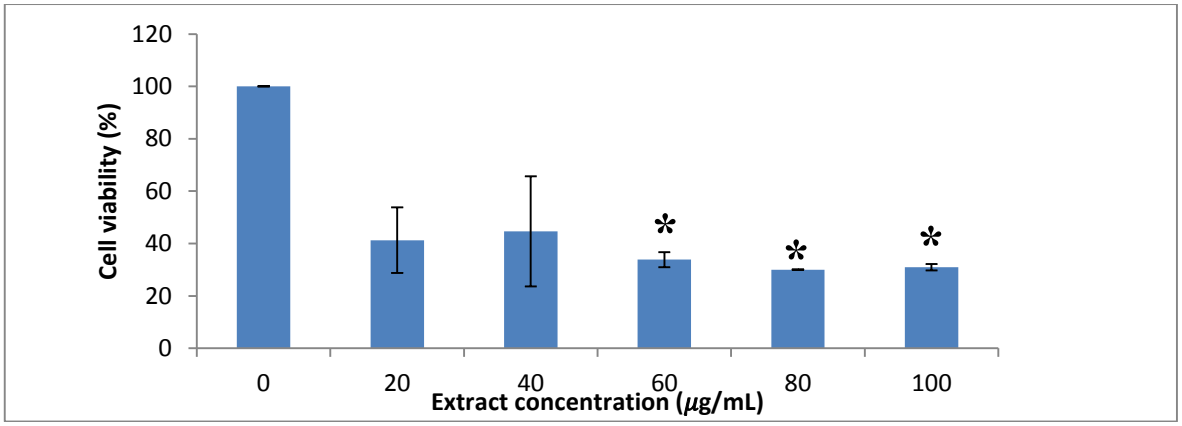

Figure 11: Percentage of U-87 cell viability (%) against extract concentration (μg/mL) for incubation period of 48 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when compared to negative control, $p < 0.05$, n = 2.

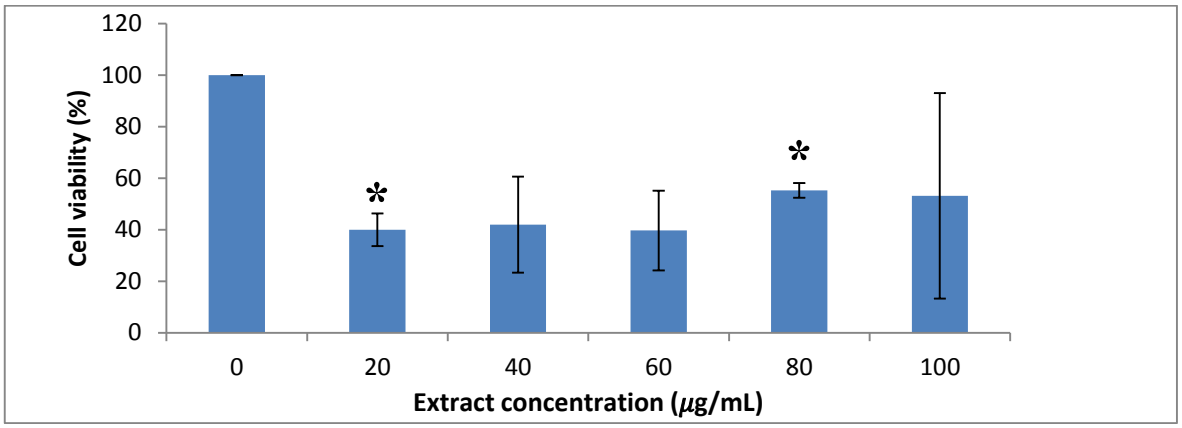

Figure 12: Percentage of U-87 cell viability (%) against extract concentration (μg/mL) for incubation period of 72 hours. Data are expressed as mean ± standard deviation. (*) shows significant difference when compared to negative control, $p < 0.05$, n = 2.

From the findings, although at lower concentrations (<80 μ g/mL) the extract showed a positive promoting effect in normal cells for all incubation periods, there was an inverse relationship between concentration and cell viability when higher extract doses (80 and 100 μ g/mL) were used. Significant decrease in normal cell viability was observed at the maximum concentration (100 μ g/mL) for incubation periods of 48 and 72 hours. Instead of promoting regeneration of cells, high doses of extract appeared to be cytotoxic to the normal cells and not able to promote normal cell growth. Whereas, the highest proliferative effect was demonstrated particularly when the extract used was at the lowest concentration (20 μ g/mL) for the shortest incubation period (24 hours), which was at 119.34% viability. However, this slight increment in cell viability was not significant ($p > 0.05$) when compared to the negative control. The result obtained was in line with a study by Patar *et al*., (2012) using aqueous extract of sea cucumber *Stichopus variegates*; which is one of the widely-used MNPs in regeneration studies. The extract showed an increased proliferation of rat spinal astrocytes cell lines at concentrations as low as 5µg/mL and 10µg/mL, after 72h. This finding further supports our result that the bioactive compounds present in marine products are able to promote healthy cell growth at low concentrations.

While low extract concentrations (<80 μ g/mL) and short incubation period showed a positive effect on normal cells, the same result was also observed in the cancer cell viability as well for incubation period of 24 hours. After 24 hours, the cancer cell viability was unexpectedly promoted when tested with all

extract concentrations. Significant increase in cancer cell viability was observed at lower concentrations of 20 μg/mL (p = 0.010), 40 μg/mL (p = 0.037), and 60 μg/mL (p = 0.045). This finding contradicted our initial hypothesis and main purpose of finding a mutual optimum concentration that would promote normal cell and inhibit cancer cell at the same time. It also suggested that this extract could be a risk for cancer cell development when used for short incubation period.

On another note, very different observation was seen for cancer cell viability after 48 and 72 hours, compared to 24 hours. Even though we did not manage to find a consistency in the trend of cancer cell inhibition, the results showed a general decreasing trend in cancer cell viability for prolonged incubation periods. The results suggested that the optimum concentration that could effectively inhibit cancer cell viability was at 80 μg/mL for 48 hours incubation period. It showed 77.45% of cell inhibition when compared to the negative control, which means that the extract had very high cytotoxic property on cancer cells. The IC_{50} was estimated at 40 μg/mL.

Conclusion

From this study, the regenerative ability of *D. claparedii* was evidently not applicable in *in-vitro* cellular growth. The normal cell viability was only slightly promoted when the lower concentrations of extract were used, compared to the negative control. Whereas, at higher concentrations, the extract appeared to be cytotoxic and had the ability to inhibit cell growth for both normal and cancer cells. However, there was an inconsistency throughout the results. Therefore, further and improved research is required for more reliable results, using cell lines from similar origins. Further studies to isolate the specific bioactive compounds in *D. claparedii* that is responsible for cellular regeneration may also be helpful for future research.

Acknowledgements

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