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Original article

Marine polychaete *Namalycastis* sp. extracts enhance proliferation and regeneration of mice 3T3 fibroblast and MCR-5 human fibroblast cells

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ABSTRACT

The Nereidid worm is a marine polychaete commonly found near the Nipa palm (*Nypa fructicans*) along the mangrove estuary. Recently, many usages have been documented for this polychaete family. Nevertheless, the true potentials of these marine worms, especially *Namalycastis* sp., from the medical perspective are still unknown. The current study investigated the cytotoxicity effect of *Namalycastis* sp. crude extracts on mice 3T3 fibroblast cells and human lung MRC-5 fibroblast cells. Thirteen concentrations (2, 4, 8, 16, 31, 63 μg/mL and 0.1, 0.3, 0.5, 1, 2, 4, 8 mg/mL) of the extracts were used as a treatment for 24 h, and cell viability was measured *via* the MTT assay. None of the 13 concentrations of *Namalycastis* sp. crude extracts showed cytotoxicity effects on the cell types investigated. However, based on the live images captured by the IncuCyte™ imaging system, the cells treated with *Namalycastis* sp. crude extracts showed an increased proliferation and growth rate in less than 10 h Furthermore, the extract concentration of 8 μg/mL induced the highest cell proliferation rate whereas 8 mg/mL led to the lowest cell proliferation rate following the treatment. Overall, *Namalycastis* sp. crude extracts were non-toxic on mice and human cells within the tested concentrations set. Still, it increased cell viability and proliferation compared with the control. This finding could pave the way for an alternative therapeutic strategy to treat debilitating disorders such as ageing, cardiovascular diseases, and neurodegenerative diseases.

1. Introduction

Polychaete is a marine worm from the family Nereidinae. Some of these species have a high tolerance and survival ability living in low salinity and polluted water or semi-terrestrial environments (Glasby, 1999). The Nereidinae have several modifications to their range of specialised morphology, physiology and reproductive adaptations compared to other families of polychaetes to survive in harsh environments. This marine worm was widely spread, inhabiting the Nipa palm and mangrove sediments along the coastal swamps in Malaysia (Junardi, 2018). Polychaetes have been documented for their various agricultural utilities, for example, as a medium for the degradation of vegetation, wood and roots, as a bio-indicator in a marine environment for pollution and also used as a bio-remediation of wastewater by fish and other aquafarming industries (Dean, 2008; Sibaja-Cordero and Echeverria-Saenz, 2015; Tomassetti and Porrello, 2005).

The *Namalycastis* sp. is also known as "umpun-umpun" by Malaysian locals for its popular usage as bait worms for fishing and contributing to the local capital income (Junardi et al., 2010). To date, polychaetes are rich in essential fatty acids, which are crucial for developing reproductive organs in fish and various crustaceans (Costa et al., 2003). Moreover, as they exert various tolerance and modification to adapt and survive in a harsh environment (Lucey et al., 2016), this makes them one of the possible applicants to be used as the future alternative treatment in treating various pathological diseases related to humans. Moreover, polychaetes have developed specialised characteristics to enhance self-repair, regenerate new body parts with high accuracy, and maintain their normal functions. However, little is known about research on *Namalycastis* sp. for medicinal benefits in humans.

The variation and diverse cell types in the human body have their own capacity in the growth and regeneration process depending on the nutrients, energy, location and surrounding environment (Zakrzewsko

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et al., 2019). The cell proliferation process is required to enable cells to initiate and maintain the proper function of all adult tissues. Depending on the proliferative stimuli, cells go through a cell cycle process to double up their daughter cells, thus increasing cell number. The accuracy and precision in producing new cells through the cell proliferation process yield normal, functional and healthy daughter cells (Matson and Cook, 2018). Dysfunction and lacking this process contribute to various disease development such as β-cells dysfunction and neurodegenerative diseases. It was known that *Namalycastis* sp. can regenerate lost or wounded body parts with high efficiency and accuracy. However, the underlying mechanism of *Namalycastis* sp. effects on human cells is unclear. In the present study, human and mice fibroblast cells were used as a model to investigate the effects of *Namalycastis* sp. extracts on cell viability and overall cell growth and to observe the ability of cells to proliferate and migrate in the presence of *Namalycastis* sp. as treatment.

This study aims to investigate the beneficial effects of *Namalycastis* sp. extracts on the viability, cytotoxicity and proliferation/migration rates of human and mice fibroblast cells. A total of 13 concentrations (2–8000 μg/mL) of *Namalycastis* sp. crude extracts were tested on mice 3T3 fibroblast cells and human lung MRC-5 fibroblast cells. A range of complementary methods, including MTT assay, cell growth and proliferation assay, and migration assay, were used in the present study.

2. Material and methods

2.1. Sample collection

Namalycastis sp. were collected from selected estuarine areas in Setiu Wetlands (Fig. 1) and mangrove areas of our university. Samples were taken from *Nypa fruticans* fronds, transferred into the plastic bottle, and returned to the laboratory. Living samples were kept alive in a tank containing brackish mangrove water at the Hatchery of the Faculty of Fisheries and Food Science until use. Samples were identified based on morphological characteristics, using identification keys in various publications, e.g. Glasby, 1999, Bakken et al., 2009 and Wilson et al., 2023.

2.2. Polychaete extraction

Namalycastis sp. were extracted using the NucleoSpin®RNA/Protein-Kit (Macherey-Nagel, Germany) protein purification protocol (Martin et al., 2010). According to the manufacturers' instructions, the *Namalycastis* sp. samples were washed thoroughly to remove foreign particles, and 30 mg of polychaetes were homogenised and lysed with 350 μL of ly sis buffer (RPI) and 3.5 μL of β-mercaptoethanol and vortexed vigorously. Next, the sample was centrifuged for 1 min at 11,000*g* to filter the lysate.

DNA and RNA binding conditions of the lysate were produced, and they were adjusted and mixed with 350 μL of 70 % ethanol. Finally, the lysate was loaded into the collection tube and centrifuged for 30 s at 11,000*g*. RNA and DNA were bounded to the column membrane, and proteins were eluded into the flow, which is used as the crude extract.

2.3. Cell culture

Both the mice 3T3 fibroblast and human lung MRC-5 fibroblast cells were cultured at 37 °C with 5 % CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % (v/v) heat-inactivated bovine serum (SIGMA), 2 mmol/mL L-glutamine, 100 U/mL of penicillin and 100 μg/mL streptomycin. Cells were seeded, used at ~90 % confluence, and treated with *Namalycastis* sp. crude extracts ranging from 2, 4, 8, 16, 31, 63 μg/mL and 0.1, 0.3, 0.5, 1, 2, 4, 8 mg/mL for 24 h. Cells were then washed and used for the following experiments.

2.4. MTT assay

Cell viability test was performed using a 3-(4, 5-dimethylthiazol- 2-

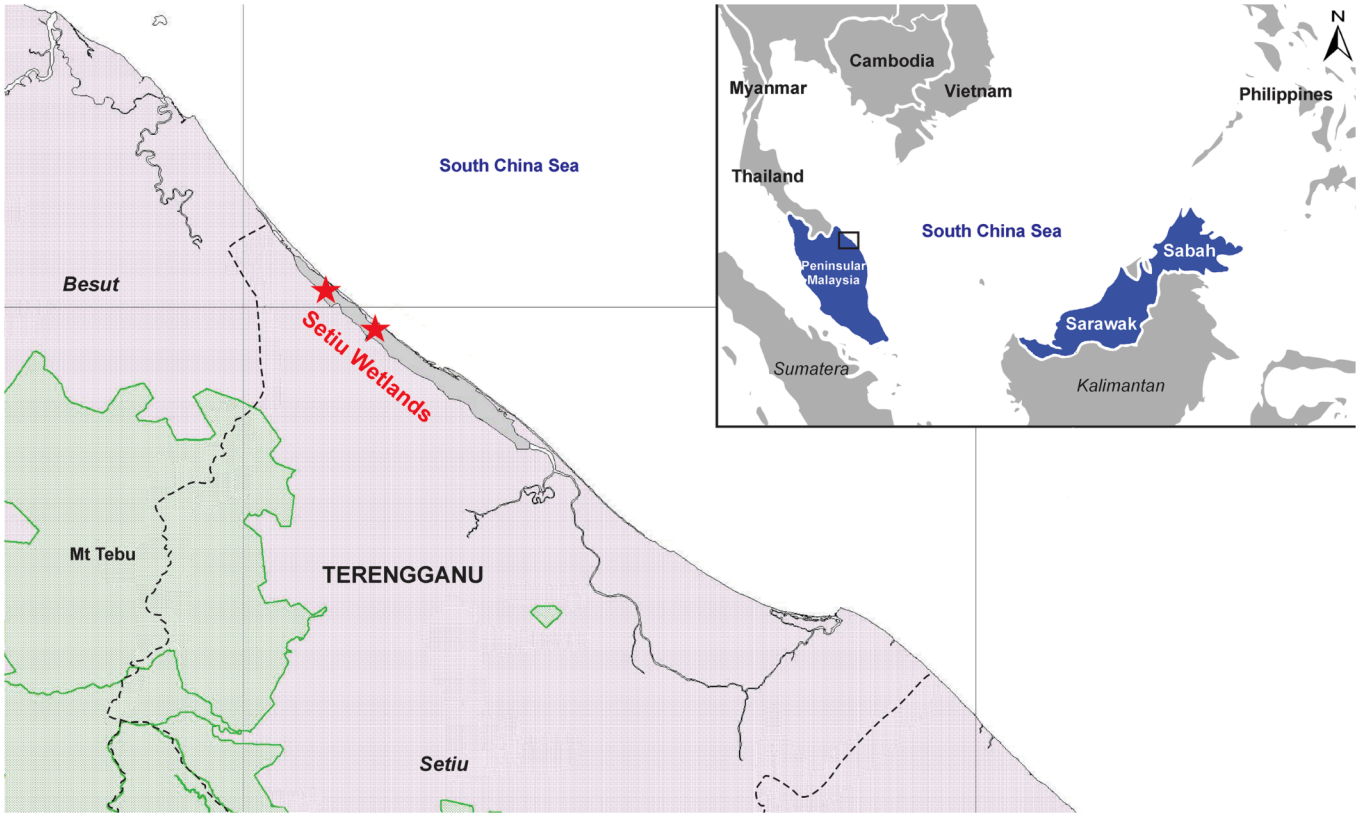

Fig. 1. Map showing the Setiu Wetlands in Terengganu, located on the East Coast of Peninsular Malaysia. Red stars indicate the location of the sampling sites. Map modified from the report on Hutan Simpan Kekal Terengganu 2021 on the Jabatan Perhutanan Negeri Terengganu website.

yl)-2, 5-Diphenyltetrazolium bromide (MTT) assay. Briefly, 1 x 10^4 cells were seeded into a 96-well plate supplemented with a complete medium of RPMI 1640. Then, cells were incubated for 24 h in a CO_2 incubator. The mice 3T3 fibroblast cells, and human MRC-5 fibroblast cells were treated with high and low concentrations of *Namalycastis* sp. crude extracts (0–8000 μg/mL) for 24 h. Then, 20 μL of MTT reagent was added to each well. The plate was then incubated for 4 h before the supernatant was discarded, and formazan crystals were dissolved in 100 μL of isopropanol. The absorbance was measured at 570 nm by a Multiskan™ Go microplate reader (Thermo Fisher Scientific).

2.5. Proliferation and migration assay

Mice 3T3 fibroblast cells were cultured into a 96-well plate. Cells at ~90 % confluence were used, and a straight-line scratch was made by the 96-pin WoundMaker™. Then, cells were washed with PBS to remove any remaining debris. Cells were treated with 13 concentrations of *Namalycastis* sp. crude extracts for 24 h. Images were automatically acquired and recorded by the Incucyte™ live imaging analysis monitoring system at 2 h intervals.

2.6. Statistical analysis

All data are presented as mean ± S.D. of at least three different

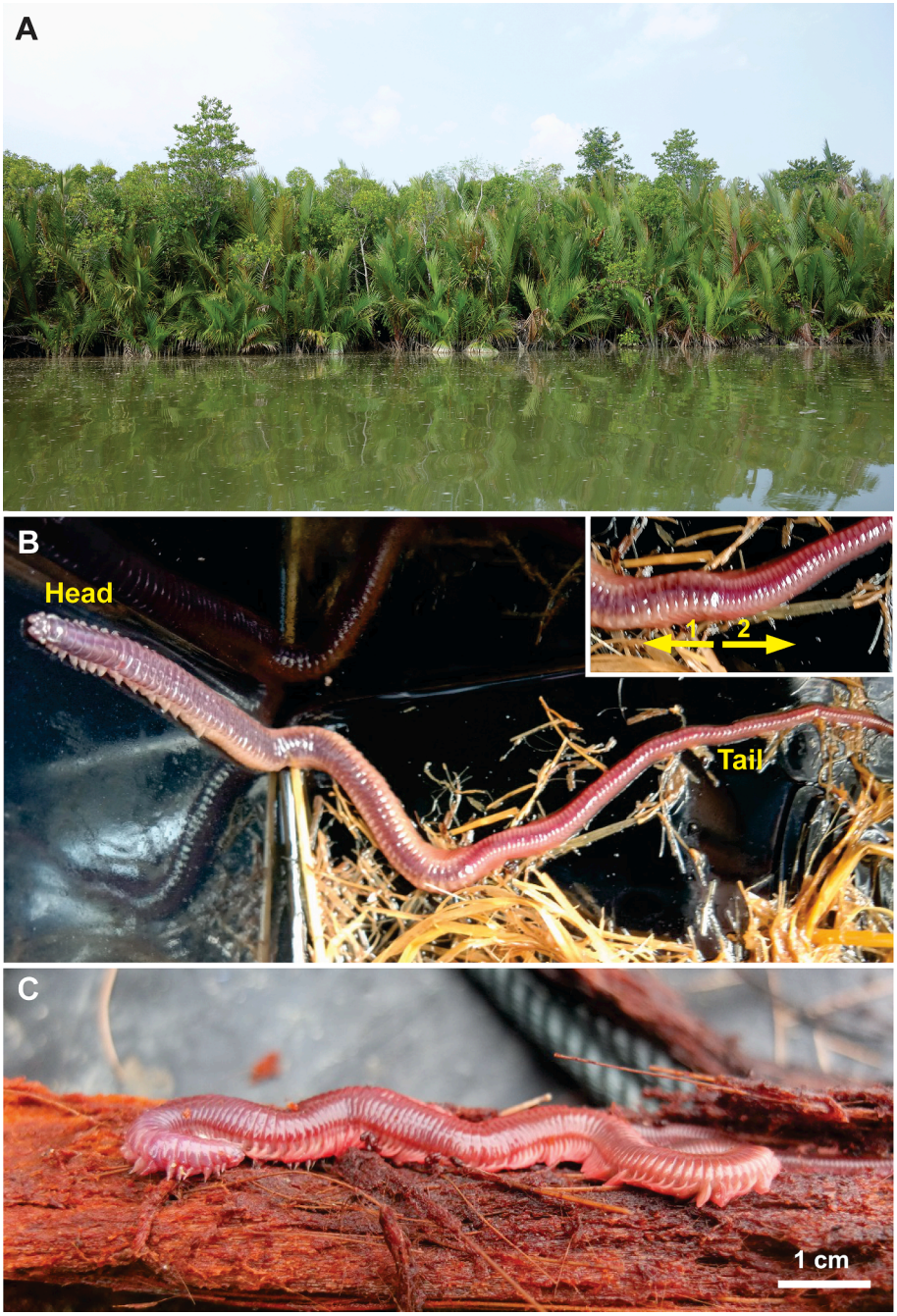

Fig. 2. A. Habitat of *Namalycastis* sp. in the Nipa palm (*Nypa fruticans*) population from the Setiu Wetlands. B. Image of an adult *Namalycastis* sp. with its initial and regenerated body part (inset: regenerated segments of *Namalycastis* sp. at the rear end, towards the tail, yellow arrow 1: initial body part, yellow arrow 2: regenerated body part). C. The average adult size of the wild *Namalycastis* sp., collected from rotten Nipa palm frond.

culture experiments for each cell type from three experimental results taken from three independent cell cultures for each condition. Comparisons were made by an unpaired *t*-test and a one-way ANOVA test. $p < 0.05$ was considered statistically significant.

3. Results

Following the treatment of *Namalycastis* sp. crude extracts on both mice, and human fibroblast cells, various effects on the viability and growth of these cells were discovered. Naturally, *Namalycastis* sp. can be found in the mangrove area by the shorelines between Terengganu and the South China Sea area (Fig. 2A). This marine polychaete can undergo consistent self-repair into full adult size ranging between 5 and 15 cm in length (Fig. 2C). From the laboratory observation, it was found that *Namalycastis* sp. can grow and regenerate its rear segments, from the midway body part down towards the tail end, accurately (Fig. 2B).

3.1. Effects of Namalycastis sp. crude extracts on mice 3T3 fibroblast cell viability

Overall, treatment of mice 3T3 fibroblast cells with *Namalycastis* sp. crude extracts significantly increased cell viability with all concentrations over 24 h of incubation. Incubation of cells from 0 to 63 μg/mL indicated that 8 μg/mL caused the highest growth in cells, and with a concentration above 8 μg/mL, cell growth gradually reduced with a concentration in a dose-dependent manner (Fig. 3A). Moreover, we also examined the effects of *Namalycastis* sp. on cells with higher concentrations ranging from 0 to 8 mg/mL with the same condition (Fig. 3B). A similar effect from cells treated with the lower concentrations where all *Namalycastis* sp. extract concentrations significantly increased cell viability at higher concentrations. The highest cell viability was seen at 0.1 mg/mL and gradually reduced to 8 mg/mL (Fig. 3B).

3.2. Effects of Namalycastis sp. crude extracts on mice 3T3 fibroblast cell growth

To measure the effect *Namalycastis* sp. crude extracts has on mice 3T3 fibroblast cell growth, we randomly selected two concentrations; 8 μg/mL from the lower concentration group and 8 mg/mL from the higher concentration group and treated cells with a kinetic overview of mice 3T3 fibroblast cell growth and proliferation live measurements were acquired and measured by the Incucyte® S3 live-Cell Analysis Imaging System. The total overview of cells treated with both concentrations indicated an increase in cell growth versus cells in the vehicle control (Fig. 4). Cells treated with 8 μg/mL of *Namalycastis* sp. crude extracts had a significant cell growth versus cells in the control group for 24 h. However, cells treated with 8 mg/mL of *Namalycastis* sp. crude extracts had a similar growth trend as cells in the vehicle control group when observed in real-time.

3.3. Effects of Namalycastis sp. crude extracts on mice 3T3 fibroblast cell migration

We assessed this with a migration assay to investigate the effects of *Namalycastis* sp. crude extracts on cells. Mice 3T3 fibroblast cells were treated with *Namalycastis* sp. crude extracts, and images were taken live every 2 h. The images in Fig. 5 are represented four major time points of the observation; 0, 8, 16, and 24 h. There was a significant acceleration in cell migration when treated with 8 μg/mL of *Namalycastis* sp. crude extracts compared to the vehicle control group at 8 h post-treatment. Moreover, there was also a rapid migration on cells treated with 8 mg/mL compared to the control at 16 h post-treatment. We observed that the closure area was filled with migrated cells at 24 h for cells treated with 8 μg/mL compared to cells in the control group and cells treated with 8 mg/mL (Fig. 5).

3.4. Effects of Namalycastis sp. crude extracts on human MRC-5 fibroblast cell viability

We investigated the effects of *Namalycastis* sp. crude extracts treatment on human MRC-5 fibroblast cells for 24, 48 and 72 h. Overall, there was no significant reduction in the cell viability following treatment at all time points. However, cell viability was significantly increased when cells were treated with 1 μg/mL and 4 mg/mL for 24 h (Fig. 3C,D). In addition, there was a significant increase in cell viability observed when cells were treated with 1 μg/mL and 2 mg/mL incubated at 48 h (Fig. 3E, F). Nonetheless, when cells were treated with all the concentrations for 72 h (Fig. 3G,H), there was no significant difference in cell viability. Still, they exhibited a reduction in the trend of distribution.

4. Discussion

The significant findings of the present study are (1) all the *Namalycastis* sp. crude extracts concentrations are none toxic when challenged on both mice 3T3 fibroblast cells, and human MRC-5 lung fibroblast cells; (2) all the *Namalycastis* sp. crude extracts concentrations significantly increased mice 3T3 fibroblast cell viability when treated for 24 h; and (3) there were only selected concentrations that exhibited a significant increase on human MRC-5 lung fibroblast cell viability at different time points. Our results suggest that *Namalycastis* sp. crude extracts may play a crucial pathophysiological role in the progression of cell growth in both species.

Interestingly, the same set of *Namalycastis* sp. crude extract concentrations have variable effects on both types of fibroblast cells from different species. Both mice and human fibroblast cells are frequently used for cytotoxicity tests, and cultures of these cells have been commonly used in many previous studies (He et al., 2020; Kim et al., 2020; Xu et al., 2019). Moreover, the MTT assay is a standard for measuring the cytotoxicity of any new therapeutic treatments (Terpilowska et al., 2018). Through MTT assay, we observed that *Namalycastis* sp. crude extracts are not cytotoxic to both mice 3T3 fibroblast cells and human MCR-5 lung fibroblast cells following treatment. Compared to earthworm and leech extract with IC_{50} of 689.68 μg/mL and 119.844 μg/mL, respectively (Ling and Gurupackiam, 2017; Merzouk et al., 2012), *Namalycastis* sp. extracts may have higher IC_{50}, following treatment with the highest concentration of 8 mg/mL, inhibition of cell growth was not observed. This phenomenon occurred partly because polychaete is rich in proteins and lipids. Together with its essential nutritional compositions, the constituent of marine polychaete may be highly valuable in assisting, directing and enhancing cell growth and proliferation (Wang et al., 2019). This data was complemented by a live overview of the hourly kinetic result of proliferation and the migration assay. There were apparent differences in the responsiveness of mice 3T3 fibroblast cell proliferation and migrational activity when treated with 8 μg/mL and 8 mg/mL compared to the vehicle control group. A lower concentration of *Namalycastis* sp. crude extracts, 8 μg/mL, had significantly enhanced cell proliferation and growth compared to cells treated with the highest concentration, 8 mg/mL. When challenged between these two concentrations, this disparity in mice 3T3 fibroblast cell proliferation and growth might imply that this cell line demonstrates significantly higher relative sensitivity against this treatment (Boncler et al., 2019).

Surprisingly, when human MCR-5 lung fibroblast cells were treated with *Namalycastis* sp. crude extracts for 24, 48 and 72 h, not all of the concentrations significantly enhanced cell proliferation and growth. This is likely due to it being less sensitive toward certain agents as a treatment (Havelek et al., 2017). In this case, the *Namalycastis* sp. extracts at all three-time points. These effects may also be potentially explained as there is an inter-species difference in both types of these cells (Johnston et al., 2010). The observed enhancement in cell proliferation and growth on human MRC-5 cells following treatment appears to be distinct to only specific concentrations at different time points.

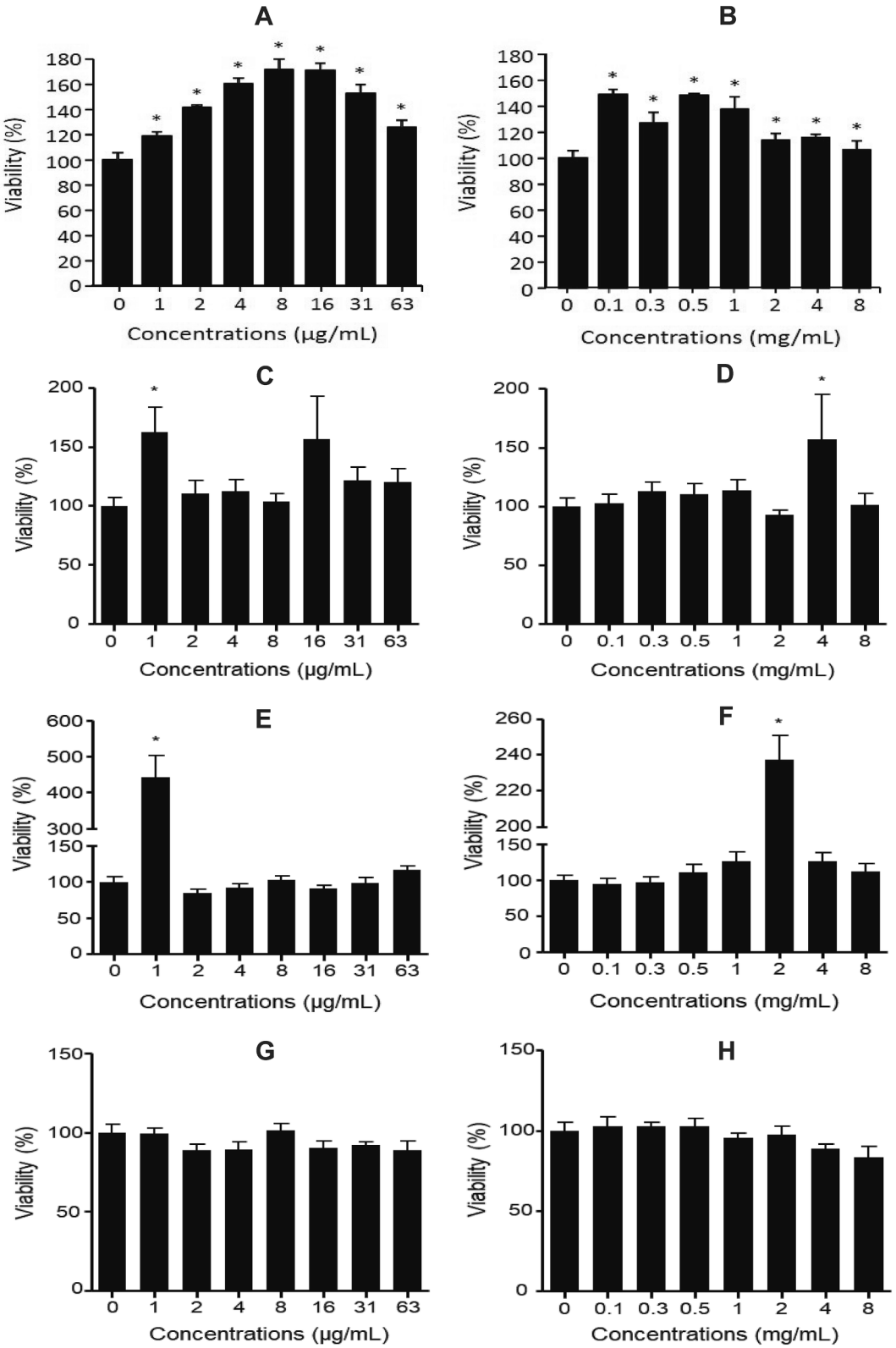

Fig. 3. Mice 3T3 fibroblast cell viability treated for 24 h (A,B) with lower concentrations of *Namalycastis* sp. crude extracts ranging from 1 to 63 µg/mL (A) and 0.1–8 mg/mL (B). Human MRC-5 lung cell viability challenged for 24 h (C,D), 48 h (E,F) and 72 h (G,H) with lower concentrations of *Namalycastis* sp. crude extracts ranging from 1 to 63 µg/mL (C,E,G) and 0.1–8 mg/mL (D,F,H). *$p < 0.05$ for indicated values versus the value of vehicle control in the same group. Error bars, mean ± S.D. n = 3.

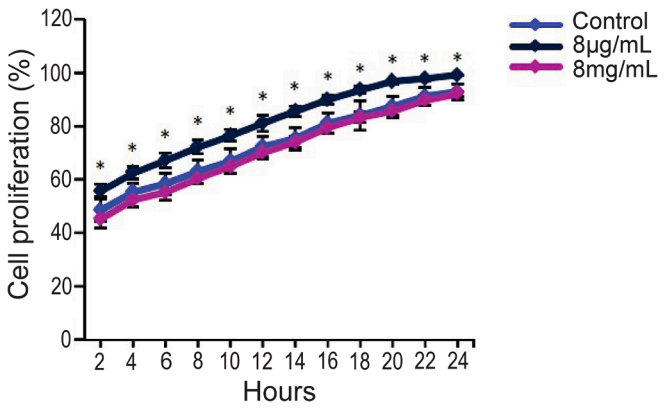

Fig. 4. A kinetic overview of mice 3T3 fibroblast cell growth and proliferation treated with two different concentrations of *Namalycastis* sp. crude extracts (navy; 8 µg/mL, pink; 8 mg/mL) over 24 h. The kinetic measurement of growth and proliferation was added after every 2 h during the experiment. $^{*}p < 0.05$ for indicated values versus values of vehicle control in the same group. Error bars, mean ± S.D. n = 3.

Treatment of human MCR-5 lung fibroblast cells at 1 µg/mL and 4 mg/mL as well as 1 µg/mL and 2 mg/mL, at 24 and 48 h, respectively, significantly enhanced cell viability. This notable effect could be attributed to the differential activation of pathways within the cells by the stimulating agent at these concentrations. At lower concentrations, specific growth-related pathways may be triggered, promoting cellular proliferation. In contrast, higher concentrations may engage alternate pathways that still support cell growth, suggesting there are distinct effects on cell proliferation based on concentration. For example in normal cells, low concentrations of insulin stimulate growth-related pathways. At lower doses, insulin activates the PI3K/AKT pathway, promoting cell growth and metabolism (Boucher et al., 2014). Also, at low concentrations, fibroblast growth factor (FGF) engages MAPK pathways, supporting cell proliferation and differentiation in non-cancerous cells (Beenken and Mohammadi, 2009). Conversely, higher concentrations of the same substances can potentially trigger pathways related to compensatory mechanisms. Elevated doses of insulin may activate pathways involving negative feedback loops to regulate excessive growth or induce autophagy (Saltiel and Kahn, 2001). Likewise, this outcomes may also be related to stress response activation, for instance, increased concentrations of FGF may induce stress-related pathways in normal cells to regulate cell proliferation (Jain, 2003).

Moreover, this outcome may also be related to hormesis, a phenomenon where low doses of a compound stimulate growth while higher doses induce stress responses, prompting survival-based growth. Cells can positively respond to low stress levels, promoting growth, while higher levels may trigger stress responses for survival. Besides, cells may adapt differently to the presence of a stimulating agent at varying concentrations. For example, at lower concentrations, initial growth stimulation may occur, while at higher concentrations, cells may adapt to continue growing despite potential inhibitory effects. This was discussed by researchers investigating the effects of insulin on cell growth (Iwakami et al., 2011; Calabrese and Mattson, 2017). Additionally, the concentration-dependent activation of cell surface receptors or intracellular components by the stimulating agent may result in the activation of different pathways or functions depending on the varying concentrations of the stimulating agent. Concentration-dependent receptor activation refers to the impact of a stimulating agent on cells which can differ according to the concentration level, leading to diverse cellular responses thus may be explaining the cellular responses on MRC-5 following treatment at 24 and 48 h. This variability may be affected by several factors such as cell surface receptors, type of receptors, and extracellular matrix (ECM) (Futosi et al., 2013; Philip, 2016; Hastings et al., 2019).

Nevertheless, the mechanism of this relationship still needs to be clarified. Thus, further mechanistic studies are required to understand the underlying mechanisms that caused these differences. This is the first study showing the effects of *Namalycastis* sp. crude extracts impacting

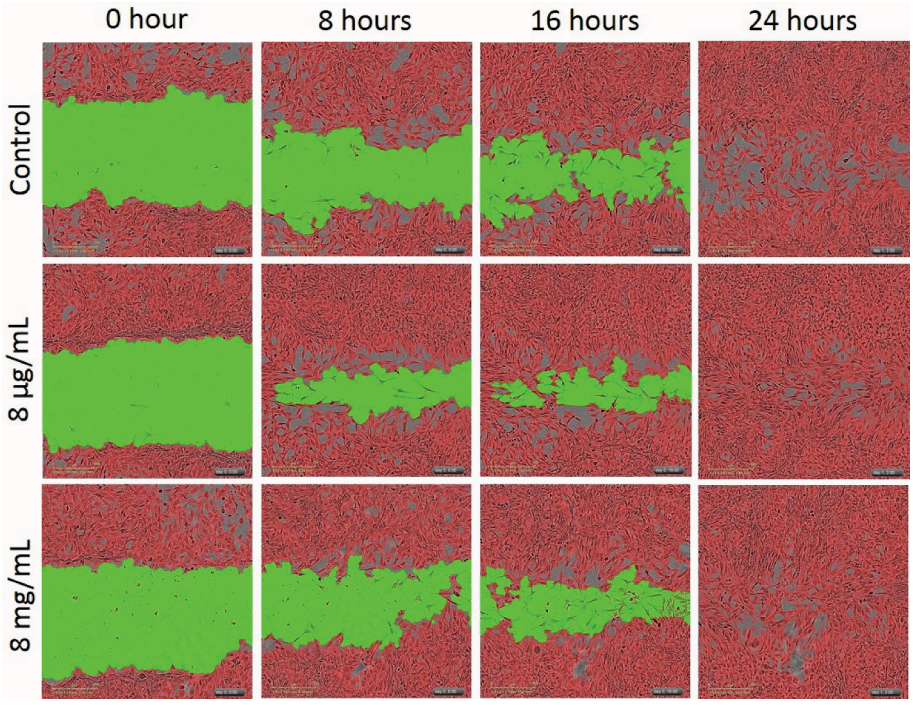

Fig. 5. Representative images of mice 3T3 fibroblast cell migration assay. A straight line scratch wound was made by a 96-pin WoundMaker™ and the process of migration and proliferation of the mice 3T3 fibroblast cells were observed and captured through an Incucyte® S3 live-Cell Analysis Imaging System with a 10x objective lens. Top row: cells in the control group; middle row: cells treated with 8 µg/mL *Namalycastis* sp. crude extract; bottom row: cells treated with 8 mg/mL of crude extract. Cells were treated for 24 h, and data were captured and acquired every 2 h.

both mice and human fibroblast cells. These data indicated that *Namalycastis* sp. crude extracts could enhance cell growth in both mice and human cells by about one-third compared to the control without exerting cytotoxic effects on cells at the investigated concentrations. However, future studies should include analysing the cell cycle, and a cellular redox profile would be required before a conclusive usage of this marine polychaete could be used.

Author contributions

Conceptualization and conceived the project, M.M. Ghazaly; Fieldwork and Identification, M.M. Ghazaly, Y.S. Ibrahim; Laboratory work and analyses, M.M. Ghazaly, M.F.N. Tajudin; Writing – original draft preparation, M.M. Ghazaly; Writing-review and editing, M.M. Ghazaly, G.E. Lee, N.F.M. Shaipulah, A. Mohammed; Funding acquisition: N.F.M. Shaipulah. All authors have read and agreed to the version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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