Teripang, Sea Cucumber *(Stichopus* sp.*)* Fractions Induce Apoptosis in Liver Cancer Cell Line (HepG2)

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Abstract

Cancer remains a major global health problem, prompting continuous exploration of marine organisms as sources of novel bioactive compounds. This study evaluated the cytotoxic and apoptosis-inducing activities of F9 and F10 fractions obtained from methanolic extracts of sea cucumber (Stichopus sp.) against the human hepatocellular carcinoma cell line HepG2 as an in vitro cancer model. Sea cucumber samples were extracted using methanol maceration and subsequently fractionated through Medium Pressure Liquid Chromatography (MPLC) to obtain bioactive fractions. Cytotoxic activity was assessed using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay after 24, 48, and 72 h of incubation to determine cell viability. Apoptotic effects were examined using Annexin V and propidium iodide (PI) staining, followed by fluorescence microscopy to evaluate morphological changes associated with apoptosis. The results demonstrated that both fractions exhibited significant cytotoxic effects on HepG2 cells in a time-dependent manner, with IC_{50} values of 14 µg.ml^{-1} for the F9 fraction and 23 µg.ml^{-1} for the F10 fraction after 72 h of treatment. Among the tested fractions, F9 showed stronger cytotoxic activity at lower concentrations. Morphological observations revealed typical apoptotic characteristics such as cell shrinkage, membrane blebbing, nuclear condensation, and phosphatidylserine externalization, indicating apoptosis as the predominant mechanism of cell death. These findings demonstrate that Stichopus sp. contains bioactive compounds with anticancer potential. This study highlights the importance of marine invertebrates as natural resources for biomedical applications and supports the development of marine biotechnology strategies for cancer therapy. Further investigations are needed to identify the active compounds responsible and clarify their molecular mechanisms of action.

Keywords: *Sea Cucumber; Stichopus* sp.*; Teripang' apoptosis; HepG2*

Introduction

Sea cucumber, commonly known as Teripang by the local community, is a readily available fishery commodity that has been used as food and is believed to possess medicinal properties for treating various diseases through traditional medicine. In the world, sea cucumbers are utilized as medicinal substances, thought to address a variety of ailments (Mazlan *et al*., 2023; Ujianti *et al*., 2024). Sea cucumber (*Stichopus* sp.) is an invertebrate marine animal characterized by its high protein content and low fat. The unsaturated fatty acids found in sea cucumbers, including EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), are known to play significant roles in anti-thrombotic activity, slowing down cell degeneration, reducing the risk of heart disease and stroke, providing anti-aging benefits (Jesionowska *et al*., 2023; Yu *et al*., 2016, Yahyavi *et al*., 2012), and promoting wound healing (Fan *et al*., 2025). To date, there has been limited research on the mechanism by which sea cucumbers contribute to treatment. Sea cucumbers contain a wealth of bioactive compounds, including antioxidants, triterpene glycosides (saponins), glycosaminoglycans, chondroitin sulfate, sterols, sulfated polysaccharides,

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phenolics, peptides, collagen, cerebrosides, and lectins (Jahani *et al*., 2025). Triterpene glycosides are particularly noted for their ability to inhibit free radical formation, playing a critical role in preventing several degenerative diseases, including heart disease and cancer (Janakiram *et al.*, 2015).

Cancer remains the leading cause of death worldwide, arising from abnormal cells that grow and develop uncontrollably in bodily tissues. Each year, the incidence of cancer continues to rise, with the World Health Organization (WHO, 2022) reporting nearly 10 million cancer-related deaths in 2020, accounting for one in six deaths globally. Chemotherapy is a common treatment for cancer; however, the cost of chemotherapy and cancer treatment is substantial, and the success rate of these therapies remains suboptimal. Consequently, cancer treatment strategies that involve the consumption of natural bioactive compounds are considered safer alternatives.

Currently, active compounds derived from marine invertebrates are being rigorously explored for their therapeutic properties. For instance, extracts from *Stichopus japonicus* have been shown to inhibit the growth of human colon adenocarcinoma (Caco-2) cells by inducing apoptosis (Dikmen *et al*., 2017). Additionally, sulfated saponins and triterpene glycosides from sea cucumbers have been reported as antitumor and antiangiogenic agents (Tian *et al*., 2005; Tong *et al*., 2005). Therefore, it is essential to investigate and identify potential new drugs for cancer treatment. Sea cucumbers (*Stichopus* sp.) are known to contain bioactive compounds that have shown cytotoxic activity in several studies, but the potential of selected fractions of *Stichopus* sp. against liver cancer cells has not been specifically studied. Based on this, this study aims to evaluate the cytotoxic effect of selected fractions of *Stichopus* sp. against human hepatocarcinoma HepG2 cells as an in vitro model.

Materials and Methods

Sea cucumber (*Stichopus* sp.) was collected from Bidong Island. The HepG2 cell line was obtained from the American Type Culture, Collection, USA. Dulbecco's minimal essential medium (DMEM) was used as media (Sigma-Aldrich, USA). The Cell Titer 96 Aqueous Non-Radioactive Cell, Proliferation Assay (Promega USA) was utilized for the growth inhibition assay, at the same time–ApoAlertTM Annexin V and Propidium Iodide (PI) (Clontech, USA) was employed for apoptosis analysis.

Preparation of fractions from sea cucumber (Stichopus sp.*)*

The sample used was the sea cucumber (*Stichopus* sp.). The samples were cleaned, cut, and freeze-dried. The dried samples were then ground into a powder for extraction. Ten grams of powdered samples were extracted overnight using three aliquots of 100 ml of methanol via the maceration method. The filtrate was separated using a vacuum filter (Buchner) equipped with Whatman filter paper. The filtrate was subsequently evaporated using a rotary evaporator at 45^{o}C to produce a methanol crude extract. Following this, the samples were fractionated using medium-pressure liquid chromatography (MPLC). Two fractions were obtained from the Sea cucumber (*Stichopus* sp.) sample. Five grams of the extract were reconstituted with 50 mL of methanol: water mixture containing 0.1% trifluoroacetic acid (TFA), and then centrifuged to produce a supernatant at 14,000 rpm for 15 min at 4°C. The supernatant was dried using a dryer. The sample mixture (dried sample dissolved in 3 ml of 90:10 methanol: water with 1% TFA) was loaded into MPLC flash chromatography (Buchi, Switzerland).

Cytotoxicity assay of fractions

HepG2 cells (American Type Cell Culture, USA) were seeded at a density of 8x10^{4} cells.well^{-1} in a 96-well plate and incubated at 37 °C in a 5% CO_2 atmosphere. Upon reaching 80% confluency, the medium was replaced with a fraction solution dissolved in the medium, and the cells were incubated for 24, 48, and 72 h. Following the treatment period, 20 µl of MTS reagent was added to each well, and the plates were incubated for an additional 3 h at 37°C in a 5% CO_2 atmosphere. Media (DMEM) was used as a negative control. The absorbance was measured at 490 nm using an ELISA reader (Multiskan, Thermo Fischer USA). The assay was conducted in triplicate, and the results were expressed as the percentage of growth inhibition compared to the control.

Early apoptosis in treated cells

The Apoptosis Detection Kit, including Annexin V and propidium iodide (PI) (APO Alert Annexin V, USA), was used to determine the exposure of phosphatidylserine (PS) induced by the fractions in HepG2 cells. HepG2 cells were seeded at a density of 8x10^{4} per well in a 96-wellt plate and incubated at 37 ^{o}C in a 5% (v/v) CO_2 atmosphere for 24 h. After 20 h, the medium was replaced with the fraction solution dissolved in the medium, corresponding to the IC_{50} value obtained from the 72-h cytotoxicity assay, and incubated for 4, 12, and 24 h (totaling three plates), with three replicates for each sample. Subsequently, the cells were rinsed once with a binding buffer. Five µL of Annexin V and 10 µL of PI were added to each well and incubated for 5–15 min in the dark. Observations were conducted under a fluorescence microscope.

Statistical analysis

The experimental data were subjected to one-way ANOVA and expressed as mean ± SD *p<0.05, with triplicate. Data was analyzed using the Shapiro-Wilk test to assess normality. Levene's test was conducted to evaluate the homogeneity of variances. To compare the effects of the F9 and F10 fractions on HepG2 cells at various incubation times, one-way ANOVA was employed, followed by Tukey's HSD post-hoc test to identify significant differences between groups. A *P*-value of < 0.05 was considered statistically significant. Linear regression analysis was also performed to evaluate the relationship between fraction concentration and percentage inhibition.

Result and Discussion

Fractionation of *Stichopus* sp. yielded 10 fractions (F1 to F10). At the 1st stage we have tested all the fraction with 1 designated concentration only (1 mg.ml^{-1}). Fraction F1-F8 were found not active against HepG2 (IC_{50} value higher than 30 µg.ml^{-1}, while 2 others were active. Hence, F9 and F10 were proceed for stage 2 with two-fold dilution concentration (start from 100 µg.ml^{-1}). Two fractions coded ECH10170039 (F9) and ECH101700310 (F10), which were used for the cytotoxicity assay against HepG2 cells. Based on the IC_{50} value, both F9 and F10 fractions demonstrated toxicity to liver cancer cells (HepG2), with IC_{50} values below 30 µg.ml^{-1} after 72-hour incubation (Table 1). The fraction has a fairly strong cytotoxic potential against HepG2 liver cancer cells in vitro. The F9 fraction of *Stichopus* sp. showed cytotoxicity against HepG2 cell lines with an IC_{50} value of 14 µg.ml^{-1} after 72 h. In contrast, the F10 fraction showed cytotoxicity against HepG2 cell lines with IC_{50} values of 28 µg.ml^{-1} after 48 h and 23 µg.ml^{-1} after 72 h.

The F9 fraction inhibited cell growth at a concentration of 12.5 µg.ml^{-1} after 24 h. Interestingly, this fraction exhibited potent cytotoxic effects on the HepG2 cell line at lower concentrations, with an IC_{50} value of 1.5625 µg.ml^{-1} after 72 h. At a concentration of 12.5 µg.ml^{-1}, inhibition caused the F9 fraction to increase by 37.2 % from 86% (24 h) to 54 % (72 h). At an effective concentration of 50 µg.ml^{-1}, the inhibition of HepG2 cell growth by the F9 fraction increased by 56.5 % from 65 % (24 h) to 28 % (72 h).

The F10 fraction inhibited cell growth at a concentration of 50 µg.ml^{-1} after 24 h. Additionally, this fraction demonstrated cytotoxic effect on the HepG2 cell line at lower concentrations, with an IC_{50} value of 1.5625 µg.ml^{-1} after 72 h. At a concentration of 50 µg.ml^{-1}, the inhibition of the F9 fraction of *Stichopus* sp. on HepG2 cell growth by the F10 fraction increased by 43 % from 56.6 % (24 h) to 31 % (72 h).

Some studies have reported that bioactive compounds from sea cucumbers possess potential anticancer properties against certain types of cancer. Tian *et al.* (2005) investigated the anti-angiogenic properties of the sea cucumber compound philinopside E (PE). The findings indicated that these compounds inhibited the migration, proliferation, tube formation, and adhesion of human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMECs) in vitro. PE has the potential to be explored as an anti-angiogenic agent due to its ability to prevent the proliferation of HMECs and HUVECs.

Furthermore, *Stichopus chloronotus* demonstrated antiproliferative activity against the tested cancer cells. The antiproliferative and anticancer properties of sea cucumber extracts can be attributed to a variety of phenolic and flavonoid compounds that act as antioxidants, protecting against degenerative diseases and oxidative stress, including certain cancers (Hossain *et al*., 2022). Peptides derived from sea cucumbers have also been reported to provide anticancer and antioxidant potential, which may be utilized in cancer therapy (Huang *et al*., 2023; Liu *et al*., 2025). Antioxidants are widely recognized for their health benefits, as they help shield the body from reactive oxygen species (ROS).

Usually, observation of cell morphology using Annexin V+/PI was used to show apoptotic cells occurred at early stages. At an advanced stage of apoptosis, Annexin V+/PI- cells were used to show the apoptotic cells. While using Annexin V−/PI+ cells was used to display necrotic cells. Observation on the cell

Table 1. IC_{50} value of the F9 and F10 fractions of *Stichopus* sp. (µg.ml^{-1})

Fractions	IC_{50} (µg.ml^{-1})		
	24 h	48 h	72 h
F9 Fraction of *Stichopus* sp.	>60	50	14
F10 Fraction of *Stichopus* sp.	>60	28	23

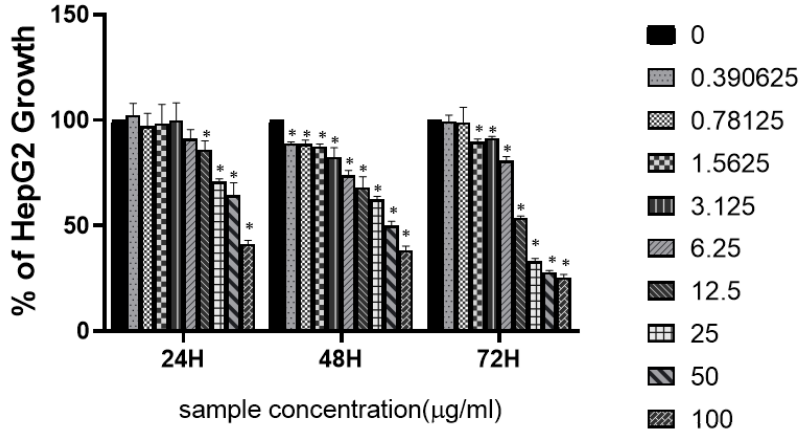

Figure 1. Percentage of growth inhibition ± SD for F9 (10170039) fraction of *Stichopus* sp. against HepG2 Cells at 24 h (24H), 48 h (48H), and 72 h (72H). *The mean of treatment is significantly different with the mean of the control at the 0.05 level.

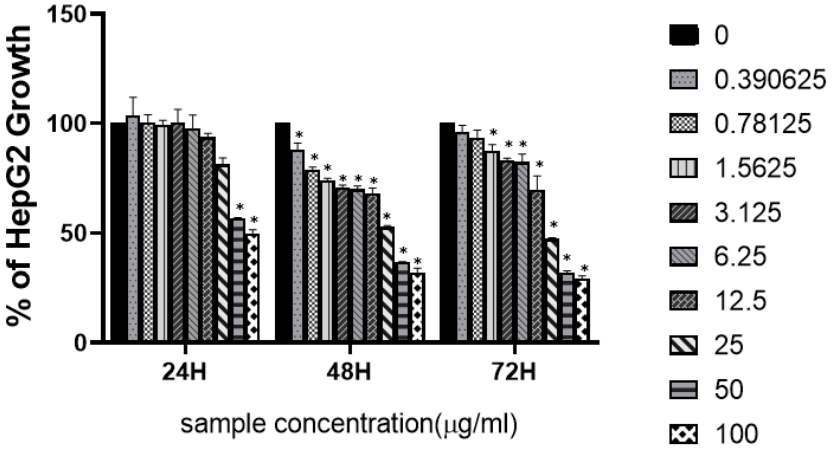

Figure 2. Percentage of growth inhibition ± SD for F10 fraction (101700310) of *Stichopus* sp. against HepG2 Cells at 24 h (24H), 48 h (24H) and 72 h (72H). *The mean of treatment is significantly different from the mean of the control at the 0.05 level.

morphology using Annexin V+/PI on Figures 3 and 4 showed that there were apoptotic cells occurring at early stages, after 4 h of incubation cells with fractions 9 and 10 (the induction of apoptosis by both fractions started at 4 h). Moreover, observation using Annexin V+/PI- on cell's morphology found the apoptotic cells were continued at 12 h until 24 h observation (advanced stage of apoptosis). Compared to the observation using Annexin V-/PI+, we can see that only a small amount of necrotic cells occurred at the same time. All of the cells displayed red stain after 12 h or longer, indicating that late apoptosis had taken place. The process of apoptosis, sometimes referred to as programmed cell death, involves the activation of a few intercellular enzyme activities that ultimately result in DNA damage and protein degradation. The apoptosis process is indicated by nuclear condensation, cell shrinkage, cell separation, blebbing of the plasma membrane, and DNA damage (Elmore, 2007). Early apoptosis prevents direct tissue damage by keeping the cell membrane intact, which stops the internal components from leaking out. Furthermore, during early apoptosis, phosphatidyl serine (PS) is transferred from the inner to the outer layer of the cell membrane (Nagata *et al.*, 2016). The results show both fractions could have a potency as an anticancer agent against the HepG2 cell line. Some investigations have been reported by other researchers on the anticancer potency of Sea cucumbers from different species.

Additionally, three novel triterpene oligoglycosides, okhtosides B1, B2, and B3, were isolated from the sea cucumber *Cucumaria okhotensis* together with compounds frondoside A and cucumarioside A2-5. These compounds showed anticancer properties (Silchenko *et al*., 2008). The

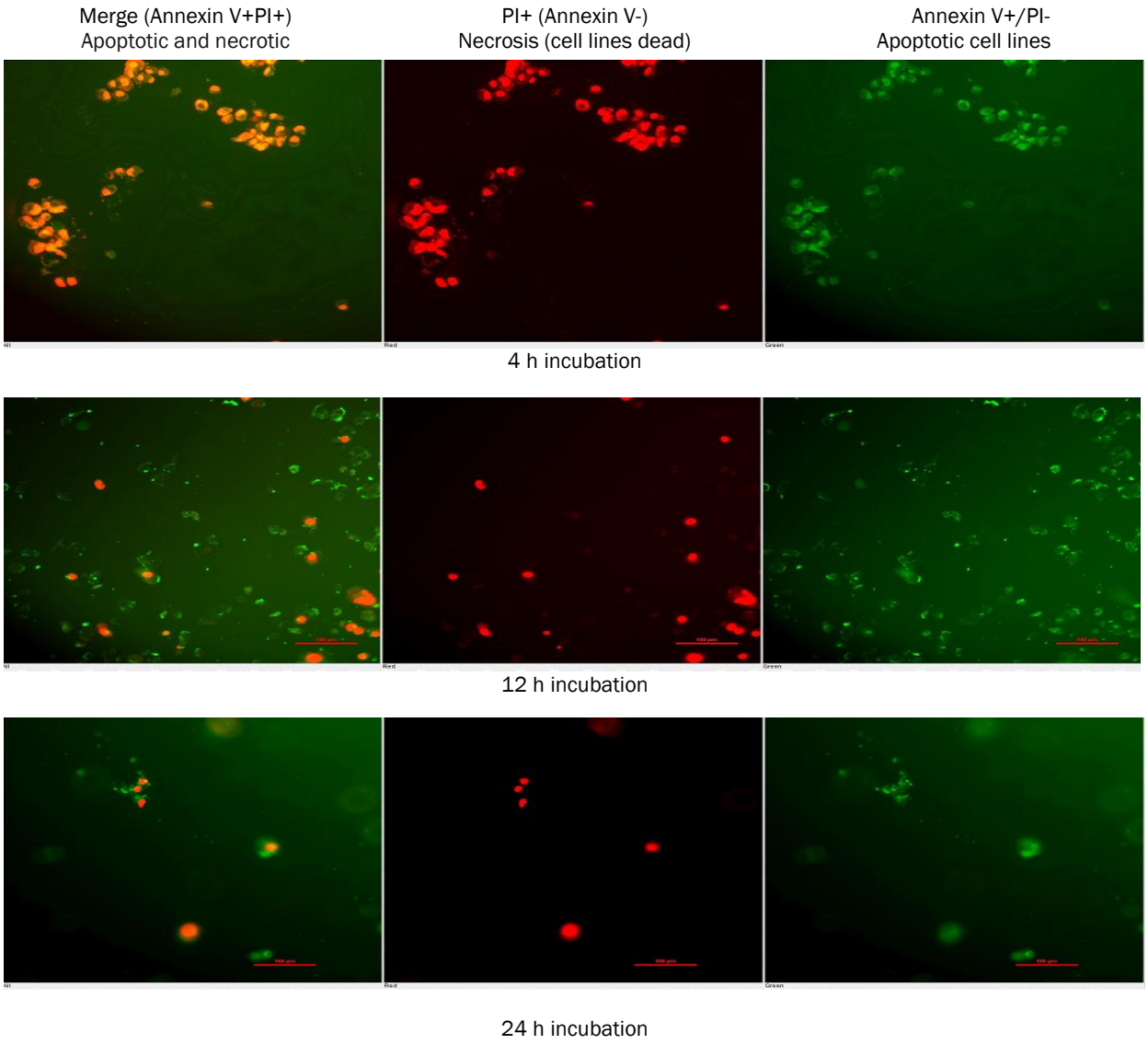

Figure 3. Morphology of HepG2 cell lines after treatment with F9 fraction (at 4, 12, and 24 h of incubation), stained with Annexin V (green) and PI (red), and then analyzed by fluorescence microscopy.

results of their research indicated that triterpene oligoglycosides were quite toxic to HeLa cancer cells, while Frondoside A was more cytotoxic to HeLa cancer and THP-1 cells. Triterpenoid compounds and frondoside A derived from *Cucumaria frondosa* species effectively inhibit the growth of human pancreatic cancer cells, and frondoside A inhibits proliferation followed by apoptosis. Frondoside A is capable of inducing apoptosis through the mitochondrial pathway and cascade activation. Pranweerapaiboon *et al*. (2021) reported the impact of extracts from three species of sea cucumbers (*Holothuria scabra, Holothuria leucospilota, Stichopus chloronotus*) on the development of two human cancer cells, specifically C33A (cervical cancer cells) and A549 (human non-small lung carcinoma cancer cells). Only extracts from *Stichopus chloronotus* exhibited antiproliferative activity against the examined cancer cells, according to the data. The presence of several phenolic and flavonoid compounds, which function as antioxidants to prevent oxidative stress and degenerative diseases, including some types of cancer, is another factor contributing to the antiproliferation and anticancer properties of sea cucumber extracts.

Cancer is a disease caused by DNA damage, leading to mutations in vital genes that regulate cell division. Peptides and collagen derived from sea cucumbers can serve as cancer preventive agents (Suarez-Jimenez *et al*., 2012). The results of enzymatic hydrolysis are likely to enhance antioxidant activity. The bioactive peptides found in marine protein hydrolysates exhibit antioxidant potential, demonstrating anticancer, immunostimulating, and antiproliferative effects (Picot *et al*., 2006).

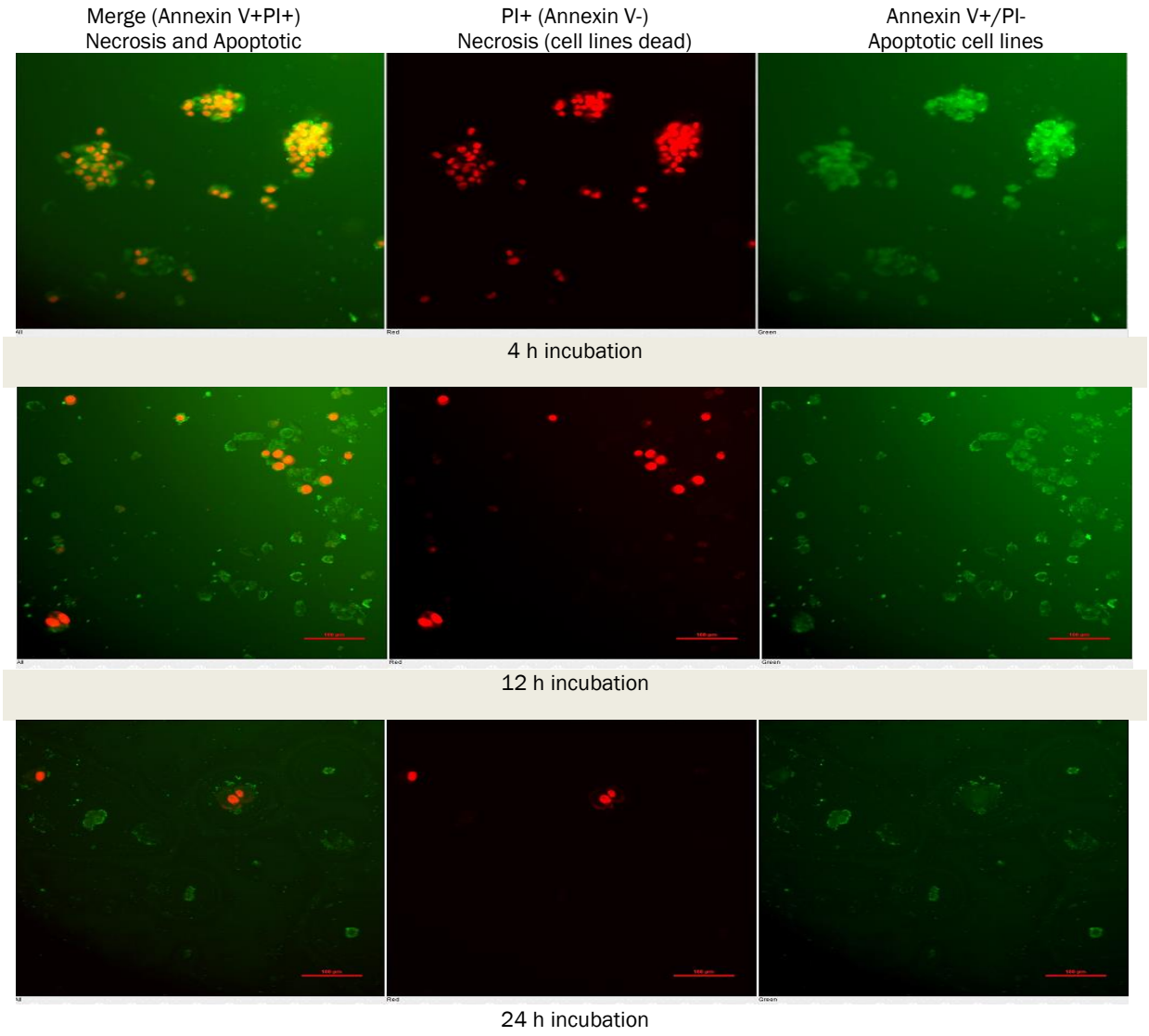

Figure 4. Morphology of HepG2 cell lines after treatment with F10 fraction (at 4, 12, and 24 h of incubation), stained with Annexin V (green) and PI (red), and then analyzed by fluorescence microscopy.

Conclusion

This study demonstrates that the F9 and F10 fractions of *Stichopus* sp. exhibit significant cytotoxic effects against the HepG2 cell line, with the IC_{50} values below 30 $\mu g.ml^{-1}$. The morphological analysis indicated that these fractions induce apoptosis in cancer cells, suggesting their potential as anticancer agents. These findings indicate that sea cucumber fractions have cytotoxic activity against cancer cells, thus having the potential to be an early candidate in the development of natural-based anticancer agents. This research contributes to the growing field of marine biotechnology by providing evidence of the anticancer properties of marine-derived compounds, thereby emphasizing the importance of exploring marine resources for innovative therapeutic strategies. Furthermore, the study paves the way for future investigations to identify specific bioactive compounds responsible for these effects and to elucidate their mechanisms of action, which could lead to the development of novel and effective cancer therapies.

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