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Anticancer activity of *Stichopus vastus* extract against MCF-7 and HeLa cells from Natuna waters

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ABSTRACT: Sea cucumbers (*Stichopus vastus*) contain bioactive compounds with potential anticancer properties, making them a promising alternative raw material for pharmaceutical development. This study aimed to isolate and fractionate bioactive compounds from *S. vastus* and evaluate their anticancer activity against MCF-7 and HeLa cell lines. Isolation was performed using Thin Layer Chromatography (TLC), and cytotoxic activity was assessed in vitro using the MTT assay. Phytochemical screening revealed the presence of steroids, terpenoids, and saponins. IC_{50} values were calculated through linear regression analysis. The methanol extract exhibited IC_{50} values of 13.50 µg/mL (HeLa) and 17.25 µg/mL (MCF-7), indicating active anticancer potential. The n-hexane fraction showed IC_{50} values of 10.09 µg/mL (HeLa) and 15.11 µg/mL (MCF-7), also categorized as active. Notably, the ethyl acetate fraction demonstrated superior activity, with an IC_{50} of 7.00 µg/mL against HeLa (very active) and 13.32 µg/mL against MCF-7 (active). These findings suggest that the ethyl acetate fraction of *S. vastus* holds significant promise as an anticancer agent, particularly against HeLa cells.

KEYWORDS: Breast cancer; bioactive compounds; cervical cancer; MTT assay; sea cucumber.

INTRODUCTION

Sea cucumbers are harvested globally for various uses, particularly in traditional medicine and culinary applications. However, due to high demand, they are often overexploited, leading to the depletion or significant reduction of natural populations in many regions. Despite this, not all species have been utilized to their full potential. One such underutilized species is *Stichopus vastus*, which currently holds low economic value and is not widely commercialized. Nevertheless, emerging research indicates that *S. vastus* contains bioactive compounds with promising anticancer properties. In the pharmaceutical field, the ongoing search for novel compounds with anticancer potential is a crucial step in the development of more effective and targeted therapies. Sea cucumbers, including *S. vastus*, present significant interest in this context due to their high nutritional content—particularly calories and proteins—and their diverse array of secondary metabolites. Specifically, *S. vastus* has been found to exhibit antioxidant activity and contains several biologically active compounds such as steroids, terpenoids, and saponins, which may contribute to its anticancer effects [1].

Cancer is one of the leading causes of morbidity and mortality worldwide, affecting millions of people each year. It poses a significant public health challenge due to its complex nature and the burden it places on healthcare systems globally. According to the World Health Organization (WHO) in 2018, it was estimated that there would be approximately 18.1 million new cancer cases and around 9.6 million cancer-related deaths globally. These alarming statistics highlight the urgent need for effective prevention, early detection, and treatment strategies. Several types of cancer contribute significantly to the global death toll, including lung, cervical, liver, esophageal, colorectal, and breast cancers. Among these, breast and cervical cancers are particularly prevalent in women. Breast cancer accounts for approximately 43.3% of all cancer cases in women, while cervical cancer represents about 14%. These figures underscore the importance of continued research into innovative therapies and preventive measures, especially those targeting cancers that predominantly affect women [2].

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Cancer treatment strategies currently include surgery, radiotherapy, chemotherapy, and immunotherapy. While these conventional approaches can be effective, they often come with significant limitations. Among them, chemotherapy remains one of the most commonly used methods; however, it is associated with high treatment costs, severe side effects, and often a relatively low success rate, particularly in advanced stages of cancer. These challenges highlight the urgent need for the development of alternative therapies that are not only more effective but also more selective and affordable. One promising source of such alternative treatments lies in bioactive compounds derived from marine organisms, particularly sea cucumbers. According to previous study, extracts from various species of sea cucumbers have shown potential as anticancer agents [3]. This is largely attributed to their rich content of secondary metabolites, including compounds such as saponins, terpenoids, and steroids, which have demonstrated biological activities relevant to cancer inhibition. These promising findings have drawn increasing interest from researchers to further investigate the anticancer potential of sea cucumber-derived compounds, as highlighted in previous study [4]. As such, sea cucumbers represent a valuable natural resource in the ongoing search for novel anticancer drugs.

Previous research on the anticancer activity of sea cucumbers has primarily focused on certain species, such as *Holothuria atra*, which has been studied extensively for its bioactive properties and cytotoxic effects against various cancer cell lines. However, despite the growing interest in marine organisms as sources of potential anticancer agents, there is a notable lack of scientific data regarding the cytotoxic activity of *Stichopus vastus*. To date, studies specifically investigating the anticancer potential of *S. vastus* remain limited or unreported in the scientific literature. Given this gap, the present research was undertaken to evaluate the anticancer activity of *S. vastus* by identifying its secondary metabolic compounds and assessing its cytotoxic effects on selected cancer cell lines. This study aims to contribute new insights into the pharmacological potential of *S. vastus*, particularly its possible application in the development of novel anticancer agents.

▪ MATERIALS AND METHODS

Materials

S.vastus weighing between 600 to 700 g were collected from the Natuna Waters, Riau Island. The sample was identified in Marine Biology Laboratory, Jakarta Oceanography Research Center. Breast cancer cells MCF-7 (ATCC HTB 22), cervical cancer cells HeLa (ATCC CCL-2), doxorubicin, MTT (Merck, Germany), RPMI media (Sigma, USA), methanol p.a (Merck, Germany), Phosphate buffer saline (Sigma, USA), trypsin-EDTA (Sigma, USA), n-hexane p.a (Merck, Germany), DMSO 100% (Merck, Germany), and Sodium Dodecyl Sulfate (SDS) 10% (Sigma, USA), ethyl acetate p.a (Merck, Germany), and butanol p.a (Merck, Germany).

The equipment used for cytotoxic test were spectrophotometric microplate reader (Thermo Fisher Scientific, USA), flask culture (Sigma, USA), CO_2 incubator (Sigma, USA), tube sterile conical (Sigma, USA), Centrifuge (Ortoalresa, Digicen 21, Spain), 96-hole microplate (Sigma, USA), water bath (Memmert, Germany), biosafety cabinet (Sigma, USA), ELISA reader (Thermo Fisher Scientific, USA), hemocytometer (Sigma, USA), inverted contrast microscope (Olympus, Cx23, Japan), scraper, ampoule, Laminar Air Flow, liquid nitrogen tank and pH meter.

Identification of secondary metabolic compounds

A qualitative analysis of secondary metabolites was conducted to identify the bioactive compounds present in the methanol extract of *Stichopus vastus*. This test aimed to determine the presence of specific classes of compounds known for their pharmacological activities. Based on the method described by [5], the methanol extract was found to contain several important secondary metabolites, including saponins, phenolic compounds, steroids, and terpenoids. These compounds are widely recognized for their biological properties, such as antioxidant, anti-inflammatory, and anticancer activities, thereby supporting the potential therapeutic value of the extract.

Extraction and fractionation

Extraction of bioactive compounds from the sea cucumber (*Stichopus vastus*) was carried out using the maceration method, which is a common and effective technique for obtaining secondary metabolites. A total of 200 g of fresh sea cucumber tissue was placed into a clean, dark glass bottle to prevent light-induced degradation of sensitive compounds. Subsequently, 200 mL of methanol was added as the extraction solvent.

The mixture was left to stand at room temperature for a period of three days, during which it was shaken occasionally to enhance solvent penetration and improve extraction efficiency. After the initial three-day period, the mixture was filtered to separate the liquid extract (macerate) from the solid residue. This maceration process was repeated two additional times using fresh methanol to ensure maximal extraction of the bioactive compounds. All collected methanol macerates were combined and then concentrated under reduced pressure using a rotary evaporator at a controlled temperature. This process continued until a thick, semi-solid extract was obtained. Finally, the concentrated extract was weighed and stored appropriately for further analysis.

The methanol extract obtained from *Stichopus vastus* was subjected to liquid-liquid fractionation in order to separate the bioactive compounds based on their polarity. Fractionation was performed using a separatory funnel and a series of immiscible solvents. Initially, the methanol extract was suspended in water and then partitioned with 800 mL of *n*-hexane. This step allowed the separation of non-polar compounds into the *n*-hexane layer, while the remaining polar compounds remained in the aqueous phase. The aqueous phase was subsequently subjected to further partitioning using other solvents of increasing polarity, including ethyl acetate and n-butanol, to obtain corresponding ethyl acetate and butanol fractions. Each fraction was collected separately and concentrated under reduced pressure using a rotary evaporator until a semi-solid or dry extract was obtained. Both the total methanol extract and each of the resulting solvent fractions (*n*-hexane, ethyl acetate, and butanol) were then evaluated for their anticancer activity through cytotoxicity assays against selected cancer cell lines.

TLC profiling and fractionation

Thin layer chromatography method was done according to [6]. About 2 mg of methanol extract was dissolved in 0.5 mL methanol, and extract was spotted on a 10 cm and 1.5 cm silica plate. The solvent was combined were n-hexane: ethanol (1:5). Spotting was carried out using a capillary tube at ±1 cm from the bottom. A long plate (10 cm) was eluted vertically in a developer vessel. The process of elution stopped at ¾ of the TLC plate because it's a standard practice to get reliable and readable results. The stains resulting from this separation were observed by spraying the reagent on the drip plate with two holes, and concentrated sulfuric acid was added to other holes to indicate terpenoid/steroid compounds in extract. $FeCl_3$ was added to other holes to indicate phenolic compounds in extract. Sulfuric acid Anisaldhyde was added to other holes to indicate saponin compounds in extract.The shape of each spot was observed, and distance traveled was measured. The retention factor (Rf) value was calculated using the formula:

$$Rf\ \ value\ =\ \frac{The\ distance\ traveled\ by\ the\ analyte\ (cm)}{The\ distance\ traveled\ by\ the\ eluent\ (cm)}$$

In vitro anticancer activity test [7]

Making media culture

The preparation of the cell culture media was carried out by combining several essential components to support optimal cell growth and maintain sterility. Initially, 10 mL of Fetal Bovine Serum (FBS) at a concentration of 10% was measured and added to a sterile container. To this, 0.5 mL of a 0.5% fungal growth zone solution was introduced, which is commonly used to simulate or study fungal interactions in cell-based assays. Additionally, 2 mL of a 2% Penicillin-Streptomycin (Pen-Strep) solution was added to the mixture to prevent bacterial contamination during cell culture. Following the addition of these supplements, 100 mL of Dulbecco's Modified Eagle Medium (D-MEM) was poured into the container to serve as the basal medium, providing essential nutrients, vitamins, and amino acids required for cell proliferation. The prepared culture medium was then thoroughly mixed to ensure even distribution of all components. Finally, the complete medium was stored at 4°C until further use in cell culture experiments to maintain its stability and sterility.

Cell preparation

Cryopreserved cells stored in cryotubes were retrieved from a liquid nitrogen storage tank maintained at a temperature of approximately -85°C. To initiate the revival process, the cryotubes were quickly thawed in a water bath set to 37°C to minimize thermal shock and promote cell viability. Before opening the cryotube, the exterior was thoroughly disinfected using 70% ethanol to prevent microbial contamination during handling.

Once disinfected, the cryotube was carefully opened under sterile conditions, and the thawed cell suspension was transferred into a sterile 15 mL conical centrifuge tube containing 10 mL of Dulbecco's Modified Eagle Medium (D-MEM) to dilute the cryoprotectant.

The cell suspension was then centrifuged at 7,500 rpm for 5 minutes to separate the cells from the cryoprotective agents and any debris. After centrifugation, the supernatant was discarded, and the resulting cell pellet was resuspended in 5 mL of fresh D-MEM growth medium. The mixture was gently pipetted up and down to achieve a homogeneous suspension, ensuring even distribution of cells for optimal growth.

The resuspended cells were then incubated at room temperature in a humidified incubator with a 5% CO_2 atmosphere for 24 hours to allow cell recovery and attachment. After the initial incubation period, the culture medium was carefully replaced to remove any residual cryoprotectants and dead cells. The cells were then allowed to continue growing under the same incubation conditions until they reached confluence and an adequate number of viable cells was obtained for use in subsequent experiments.

For routine cultivation, cells were maintained in D-MEM supplemented with 5% Fetal Bovine Serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin. All culture conditions were kept at room temperature in a humidified incubator containing 5% CO_2 to support optimal cell growth and viability.

Cells harvest

After obtaining sufficient number of cells, they were washed by adding Phosphate Buffered Saline solution. The medium and solutions were discarded and cells were used for further analysis. 700µL of 0.05% trypsin solution was then added to the cells and kept in a CO_2 incubator for 5-10 minutes at a temperature of 37^{o}C. An inverted microscope was used to observe cells. After cells were separated from the small tissue culture flask, to stop the trypsin reaction, 5 mL of culture medium was added. Cells were transferred into a sterile conical tube, and 10 mL PBS was added. After which, 10 µL was taken and a hemocytometer was used to count number of cells. The cell suspension was added with a certain amount of culture medium to obtained the required cell concentration (1.5 x 10^4 cells per 100 µL) and ready to be used for MTT assay.

Preparation of test solutions

Approximately 30 mg of the test sample was first dissolved in 150 µL of Dimethyl Sulfoxide (DMSO), a common solvent used to prepare stock solutions of bioactive compounds. This stock solution was then diluted with 900 µL of complete culture medium, resulting in a final stock concentration of 20,000 µg/mL. From this stock, a working concentration of 500 µg/mL was prepared for cytotoxicity testing.

To establish a range of concentrations for the assay, serial dilutions were prepared in sterile microcentrifuge tubes. Each dilution was thoroughly mixed to ensure homogeneity and accuracy in the test concentrations. Once the dilution series was ready, aliquots were carefully transferred into the wells of a sterile 96-well microplate. All sample preparation and transfer steps were carried out under aseptic conditions inside a laminar airflow cabinet to maintain sterility and prevent contamination. These prepared plates were then ready for use in the MTT assay to evaluate the cytotoxic effects of the test samples on the cultured cells.

MTT assay

A total of 5,000 cells were seeded and grown in complete culture medium to ensure healthy attachment and proliferation. For the cytotoxicity assay, 100 µL of the prepared cell suspension—containing a cell density of 1.5 × 10^4 cells per well—was dispensed into each well of a sterile 96-well microplate. The plate was then incubated in a humidified incubator set at 37°C with 5% CO_2 for 24 hours to allow the cells to adhere to the well surface and reach optimal conditions for treatment.

Following the initial incubation, 100 µL of the test samples at varying concentrations were added to the respective wells. The final concentrations tested were 1,000; 500; 250; 125; 62.50; and 31.25 µg/mL. Each concentration was prepared in culture medium, and wells were treated in triplicate to ensure statistical accuracy. After the addition of the test samples, the microplate was returned to the incubator and allowed to incubate for an additional 24 hours under the same conditions (5% CO_2, 37°C) to assess the cytotoxic effect of the compounds on the cultured cells.

Following the treatment period, 110 µL of a solution containing a mixture of culture medium and MTT reagent (at a concentration of 5 mg/mL) was added to each well. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide) is a yellow tetrazole that is metabolically reduced by viable cells to form purple formazan crystals, which indicate cell viability. The plate was then returned to the incubator for further incubation, allowing the MTT reagent to react with the viable cells.

Following the addition of the MTT reagent, the cells were incubated in a CO_2 incubator at 37°C to allow viable cells to metabolize the MTT into insoluble purple formazan crystals. After a 4-hour incubation period, 100 µL of 10% Sodium Dodecyl Sulfate (SDS) solution was added to each well. SDS acts as a solubilizing agent that breaks down cell membranes and dissolves the formazan crystals, making them suitable for spectrophotometric measurement. The plate was then placed on a microplate shaker and gently agitated for 5 minutes to ensure thorough mixing of the SDS and complete solubilization of the formed formazan.

Subsequently, the microplate was incubated at 37°C for 24 hours to allow complete dissolution of the formazan, ensuring accurate and consistent absorbance readings. After the incubation, the optical density (OD) of each well was measured at a wavelength of 595 nm using a microplate spectrophotometer. The intensity of the purple color is directly proportional to the number of viable (metabolically active) cells in each well.

The cytotoxic effect of the test samples was then quantified by calculating the percentage of cell death relative to the untreated control. The results were expressed as a percentage of dead cells, and the concentration of the sample required to inhibit cell growth by 50% (IC_{50}) was determined. This was done using linear regression analysis, applying the formula Y = a + bx, where Y represents the absorbance, and x is the concentration of the test sample. The IC_{50} was calculated as:

$$IC_{50}\,\frac{50\ -\ a}{b}$$

Where
a : intercept
b: slope
IC_{50} > 100 is weak, 10-100 µg/mL is active, and IC_{50} <10 µg/mL is very active [8]

▪ RESULTS

The secondary metabolite analysis was conducted to identify the active compounds present in the crude extracts of *Stichopus vastus*. This qualitative test serves as an essential preliminary step in the discovery of naturally occurring bioactive substances, particularly those with potential pharmaceutical applications. Secondary metabolites, unlike primary metabolites, are not directly involved in the organism's growth or reproduction, but they often exhibit significant biological activities, including antimicrobial, antioxidant, and anticancer properties.

By conducting this analysis, researchers aim to detect specific classes of compounds—such as saponins, steroids, terpenoids, and phenolics—that are commonly associated with therapeutic potential. This approach not only aids in characterizing the chemical profile of *S vastus* but also helps to guide further fractionation and purification steps in the search for novel medicinal agents derived from marine organisms.

According to the results of the phytochemical screening, *S. vastus* was found to contain several bioactive components, as outlined in Table 1. These findings support the hypothesis that sea cucumbers, particularly *S. vastus*, are a valuable source of natural compounds that may contribute to the development of alternative treatments for diseases such as cancer.

Table 1. Secondary metabolite content and TLC profiles of *S. vastus* extracts.

Fraction	Compound	Rf	Spot
N-hexane	steroid, saponin	0.9;0.9	Weak yellow
Ethyl acetate	phenolic, steroid, saponin	0.9 ; 0.88; 0.91; 0.85; 0.93	Weak yellow, and Strong yellow
Butanol	phenolic	0.98	very weak yellow

Thin Layer Chromatography (TLC) analysis was performed to investigate the presence and nature of bioactive compounds in the different solvent fractions obtained from *Stichopus vastus*. The TLC results revealed that distinct spots were produced in the eluate of both non-polar (n-hexane) and semi-polar (ethyl acetate) solvent fractions. These spots appeared in vials coded 1, 2, 3, 4, and 6, indicating the presence of compounds that were successfully eluted from the stationary phase. In contrast, vial code 5 did not exhibit any staining, suggesting that no compounds were eluted from the corresponding fraction.

The retention factor (Rf) values, which provide insight into the polarity and mobility of the compounds, were calculated based on the distances traveled by the spots relative to the solvent front. A prominent Rf value of 0.9 was observed in both the n-hexane fraction (vials coded H-1 and H-6) and the ethyl acetate fraction (vials coded E-3 and E-6). Additionally, a slightly lower Rf value of 0.8 was observed in the ethyl acetate fraction coded E-4, suggesting that the compounds in this fraction were somewhat more polar than those with an Rf of 0.9.

The UV254 nm results further corroborated these findings, with the strongest spot being observed at an Rf value of 0.9, indicating the presence of highly absorbent compounds at this location on the TLC plate. The Rf value provides a direct correlation to the polarity of the compounds; compounds with higher Rf values typically have lower polarity, as they move further along the TLC plate under the influence of the solvent. These results suggest that both non-polar and semi-polar compounds are present in the *Stichopus vastus* extracts, which may possess potential bioactivity. The full TLC profile can be visualized in Figure 1.

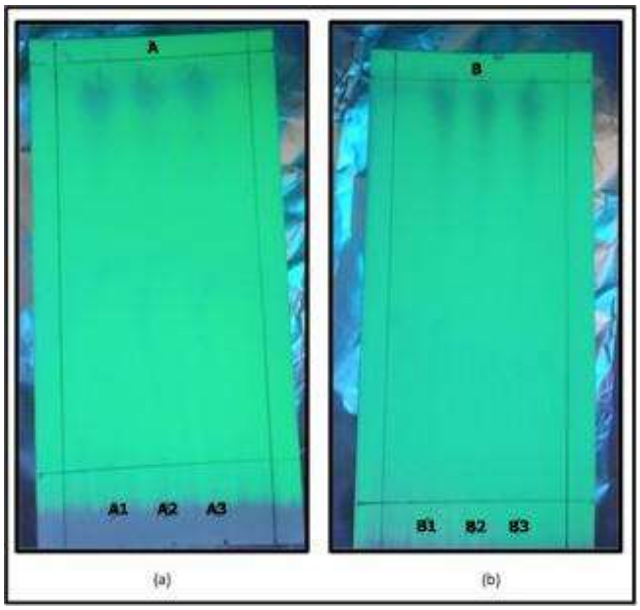

Figure 1. TLC plate under UV light 254-366nm: (a). Spot of hexane fraction. (b). Spot of ethyl acetate fraction.

Cytotoxic activity

Cytotoxic activity of S.vastus against MCF-7 (ATCC HTB 22) cells

The cytotoxicity of the hexane, ethyl acetate, and methanol fractions derived from *Stichopus vastus* was evaluated on two different cancer cell lines, HeLa cells and MCF-7 cells, to assess their potential anticancer properties. These cell lines were selected due to their relevance in cancer research: HeLa cells are a well-established cervical cancer cell line, while MCF-7 cells are commonly used to study breast cancer. The fractions were introduced to the cells at varying concentrations, and their effects on cell viability were monitored through a series of assays.

The anticancer potential of *Stichopus vastus* on the MCF-7 cell line (ATCC HTB 22) was specifically tested, and the results are illustrated in Figure 2. This figure provides a detailed visual representation of the cytotoxicity assay, showcasing the dose-dependent response of MCF-7 cells to the different solvent fractions. The results of these tests are crucial for determining the effectiveness of these fractions

in inhibiting cancer cell proliferation and assessing the therapeutic potential of compounds derived from *Stichopus vastus*. These findings contribute valuable insights into the bioactivity of sea cucumber extracts and highlight their potential for use in the development of new anticancer treatments.

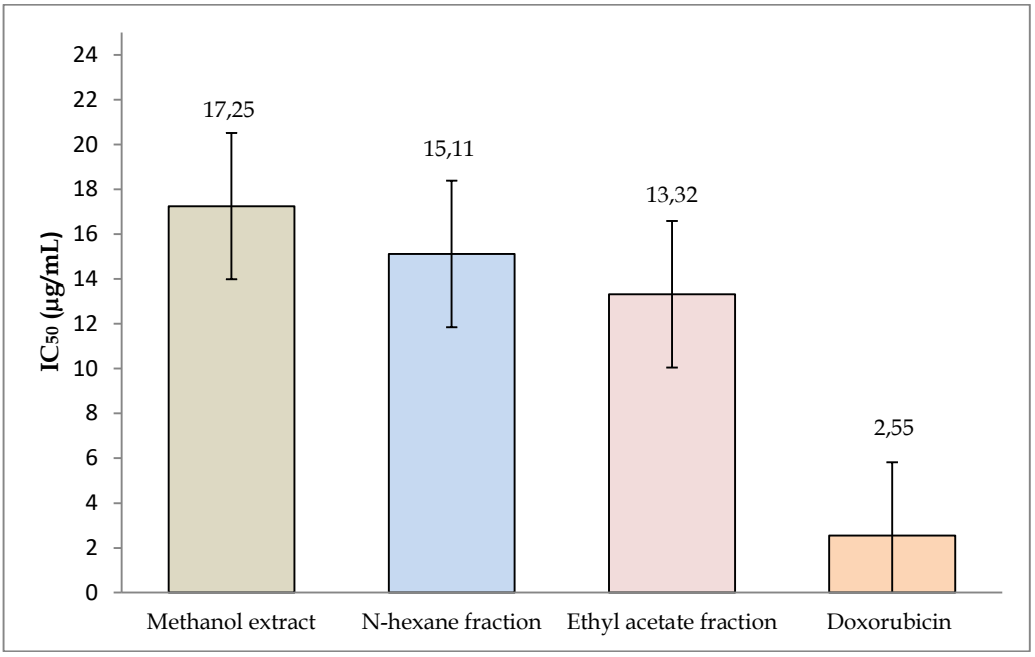

Figure 2. The IC_{50} values of *Stichopus vastus* fractions and control on MCF-7 cells.

Anticancer activity of S. vastus on HeLa cells (ATCC CCL-2)

The hexane, ethyl acetate, and methanol-water fractions of *Stichopus vastus* were subjected to cytotoxicity testing to evaluate their potential anticancer effects on two distinct human cancer cell lines: HeLa cells and MCF-7 cells. HeLa cells, originating from cervical cancer (ATCC CCL-2), and MCF-7 cells, representing breast cancer, were chosen for this study due to their wide use in cancer research and their relevance in testing the effectiveness of anticancer agents. The fractions were applied to both cell lines at various concentrations, and their impact on cell viability was assessed using standard cytotoxicity assays.

The anticancer potential of *Stichopus vastus* extracts on HeLa cells (ATCC CCL-2) was specifically evaluated, and the corresponding results can be observed in Figure 3. This figure visually represents the data from the cytotoxicity tests, providing insight into the dose-dependent effects of the different solvent fractions on HeLa cell proliferation and viability. The effectiveness of each fraction in inhibiting cancer cell growth was analyzed, with the aim of identifying promising fractions that may harbor bioactive compounds with potential therapeutic applications.

These results are essential for understanding the anticancer properties of marine-derived compounds, specifically from *Stichopus vastus*, and could serve as a foundation for further research into the development of novel treatments for cervical and other cancers.

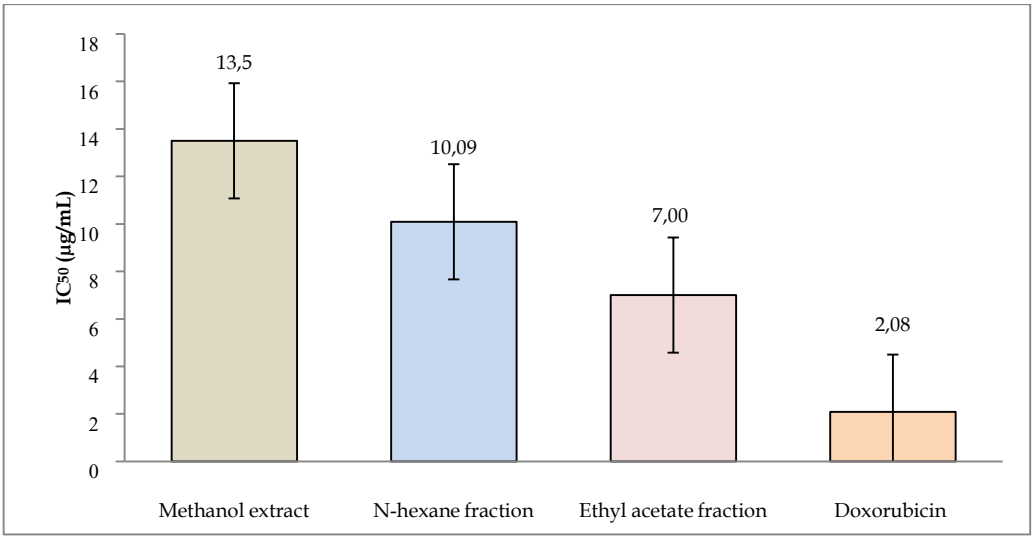

Figure 3. The IC_{50} values of *Stichopus vastus* fractions and control on HeLa cells.

DISCUSSION

Among the different solvent fractions obtained from *Stichopus vastus*, the butanol fraction was found to be the most abundant, yielding 14.32 g of extract. Despite the high yield, this fraction did not contain saponins, as these compounds were not detected during the secondary metabolite screening. This outcome was surprising, as saponins are often associated with significant biological activity, including anticancer effects.

The Thin Layer Chromatography (TLC) test, which is used to separate and identify different compounds based on their mobility on the chromatographic plate, showed no distinct spots or stain separation in the butanol fraction. This lack of separation suggests that the compounds in the butanol fraction may not have the necessary properties to be detected by this method, possibly indicating a lack of suitable bioactive components or compounds with low polarity. The standard Rf values for typical bioactive compounds are usually between 0.2 and 0.8, according to previous studies [9]. However, in the case of the butanol fraction, the absence of any visible separation further indicated that it was not suitable for anticancer testing, leading to its exclusion from further biological assays.

Interestingly, the butanol fractionation process resulted in the recovery of more n-hexane and ethyl acetate fractions, which are known to dissolve non-polar and semi-polar compounds, respectively. This suggests that the crude extract of Holothuridae (sea cucumber) contains more polar compounds, which are typically more soluble in polar solvents like butanol. However, the polar nature of these compounds did not appear to contribute to the presence of saponins in the butanol fraction, as evidenced by the absence of saponin detection during the secondary metabolite analysis. As noted in previous research [10], sea cucumbers such as *Stichopus vastus* often contain a complex mixture of bioactive compounds, and the fractionation process can help isolate specific compounds based on their solubility and polarity.

The bioactive compounds screening revealed the presence of steroids in both the ethyl acetate and n-hexane fractions of *Stichopus vastus*. The detection of steroids was confirmed by a positive result in the color change test, where the appearance of a red color indicated the presence of steroid compounds. This color change is a well-established method for identifying steroids, as it occurs due to a specific reaction between the steroid molecules and the test reagent.

Steroids are known to possess a wide range of biological activities, and their presence in the extracts of *Stichopus vastus* suggests that these fractions could have significant therapeutic potential. In addition to steroids, saponins and terpenoids are also secondary metabolites with recognized anticancer potential, making them key compounds of interest in marine pharmacology. These compounds are frequently found in sea cucumbers, where they contribute to the organism's defense mechanisms and have shown various bioactive

effects. As noted in previous studies, saponins, steroids, and terpenoids are all known to exhibit anticancer properties, among other biological activities [11].

Saponins, in particular, were also detected in both the ethyl acetate and n-hexane fractions, highlighting their presence in *Stichopus vastus* extracts. Saponins are a diverse group of triterpene glycosides, consisting of a terpene backbone attached to one or more carbohydrate chains. These compounds are widely distributed in the plant kingdom, as well as in various marine organisms, including sea cucumbers. Saponins have been extensively studied due to their remarkable biological activities, such as anticancer, antibacterial, and antifungal effects [12]. Their ability to disrupt cell membranes and inhibit tumor cell proliferation has garnered attention in cancer research, making them a valuable target for further exploration in drug development.

In summary, the identification of steroids and saponins in the extracts of *Stichopus vastus* emphasizes the anticancer potential of this marine organism and provides a promising foundation for the development of novel bioactive compounds from marine sources.

Phenolic compounds were also detected in *S.vastus* by the formation of a red color after adding sulfuric acid. Aqueous and methanolic extracts were analyzed by using HPLC for quantification. Both extracts can cause low cell viability at a certain extract concentration. The lowest IC_{50} value from sea cucumber extract was found in HeLa cancer cells at 21.01 µg/mL [13].

When comparing two different samples under the same chromatographic conditions, the Rf values of the compounds can offer insight into their polarity and interactions with the stationary and mobile phases of the chromatographic system. Compounds that exhibit high Rf values typically originate from low-polarity solvents, meaning that these compounds are less attracted to the polar stationary phase and move further along the plate. Conversely, compounds that show low Rf values are generally derived from high-polarity solvents and tend to interact more strongly with the stationary phase, leading them to travel shorter distances on the plate [14]. The Rf value serves as a characteristic identifier for specific compounds, allowing researchers to deduce their general chemical properties, including polarity, when the chromatographic conditions are consistent.

In Thin Layer Chromatography (TLC), compounds with higher Rf values are often considered less polar, as they exhibit greater mobility on a polar stationary phase, such as silica gel. This movement indicates that the compound's interaction with the stationary phase is weak, allowing it to travel faster and farther. On the other hand, lower Rf values correspond to more polar compounds, which are more strongly retained by the stationary phase, hence moving more slowly.

In the process of visualizing and identifying the compounds, TLC plates are often sprayed with reagents like anisaldehyde and H_2SO_4 (sulfuric acid). These reagents result in the development of different colors on the chromatogram, each corresponding to specific classes of compounds. For instance, compounds such as phenols typically produce blue spots, while steroids and triterpenoids often yield purple or yellow colors upon reaction. These color changes aid in identifying the presence of certain metabolites, facilitating the identification of bioactive compounds in the sample.

Moreover, observation of the TLC plate before and after the addition of LB (Liebermann-Burchard reagent) to the fractions revealed a significant color change, with a yellow/orange hue developing. This is indicative of the presence of saponin compounds, particularly steroidal saponins, which were found in both the hexane and ethyl acetate fractions of *Stichopus vastus*. Saponins are known to have a diverse range of bioactivities, and their detection on the TLC plate further supports the anticancer potential of these fractions.

Secondary metabolites from sea cucumbers, such as *Stichopus vastus*, include a wide array of bioactive compounds, including steroids, phenolic compounds, collagen, polysaccharides, chondroitin sulfate, glycosaminoglycans, glycosides, and N-acetylglucosamine, as well as lectins. These metabolites are not only important for the organism's own biological functions but also offer promising therapeutic applications. Sea cucumbers are renowned for their diverse biological activities, including anticancer, anti-inflammatory, antimicrobial, and antioxidant effects [15]. These findings underline the importance of sea cucumbers as a source of valuable natural products with significant medicinal potential.

Cytotoxic activity of *S.vastus* on MCF-7 (ATCC HTB 22) cells

The cytotoxic analysis of various *Stichopus vastus* fractions against MCF-7 cells (a human breast cancer cell line) revealed that the ethyl acetate fraction exhibited stronger cytotoxic activity compared to the n-hexane fraction. Specifically, the IC_{50} value (the concentration of the compound required to inhibit 50% of cell viability) for the ethyl acetate (EtOAc) fraction was 13.32 µg/mL, indicating that this fraction had a relatively potent effect on cell viability. In comparison, the n-hexane fraction showed a higher IC_{50} value of 15.11 µg/mL, suggesting that it was slightly less cytotoxic than the ethyl acetate fraction. For reference, the positive control, doxorubicin, which is a well-known chemotherapeutic agent, demonstrated an IC_{50} of 2.55 µg/mL, indicating its potent cytotoxicity against the MCF-7 cells.

These findings suggest that the ethyl acetate fraction of *S. vastus* contains bioactive compounds that have the potential to be developed as anticancer agents. The fact that the ethyl acetate fraction exhibited a lower IC_{50} value compared to the n-hexane fraction suggests that the compounds in the ethyl acetate fraction may be more effective at targeting cancer cells or may act more selectively, making them more promising for future therapeutic applications.

Previous studies have also highlighted the cytotoxic potential of other sea cucumber species, including *Holothuria atra* and *Bohadschia marmorata*. In research conducted by [16], these species demonstrated high cytotoxicity against T47D cells, another breast cancer cell line, with IC_{50} values of 23.0 µg/mL for *H. atra* and 28.1 µg/mL for *B. marmorata*. While these values are slightly higher than the IC_{50} observed for the *S. vastus* fractions, they still indicate a significant anticancer potential for these marine organisms. These studies reinforce the idea that sea cucumbers, including *S. vastus*, may be rich sources of bioactive compounds with promising anticancer properties, contributing to the growing interest in marine-derived natural products for cancer treatment.

The ethyl acetate fraction of *Stichopus vastus* exhibited anticancer activity that can be categorized as active (IC_{50} 10-100 µg/mL) against MCF-7 cells, a commonly used human breast cancer cell line. When compared to the methanol extract (IC_{50} of 17.25 µg/mL) and the n-hexane fractions, the ethyl acetate fraction demonstrated relatively stronger cytotoxic effects, indicating its potential as a source of anticancer compounds. These results suggest that the compounds present in the ethyl acetate fraction may have a more potent effect on cancer cells, making it a promising candidate for further development in cancer therapy [8].

Previous studies have also explored the anticancer properties of sea cucumbers, including the polar fractions from *Holothuria atra*. A study conducted by [12] investigated the anticancer activity of different solvent fractions derived from *H. atra* against MCF-7 and HeLa cancer cell lines using the MTT assay. The results showed that the polar fractions of *H. atra* exhibited higher cytotoxicity towards both MCF-7 and HeLa cells compared to other fractions. This suggests that the more polar compounds in *H. atra* might be more effective in targeting cancer cells, similar to the findings observed in *S. vastus*'s ethyl acetate fraction. These findings further emphasize the importance of solvent polarity in isolating effective anticancer compounds from sea cucumbers.

Furthermore, a study by [17] reported the anticancer potential of compound 1 isolated from *Holothuria spinifera*, which demonstrated antiproliferative activity against the MCF-7 cell line with an IC_{50} value of 20.6 µM. This was compared to the chemotherapeutic drug cisplatin, which exhibited an IC_{50} of 15.3 µM. The study showed that compound 1 from *H. spinifera* had a comparable antiproliferative effect to cisplatin, highlighting its potential as an anticancer agent. In the same study, other isolated compounds from the extract of *H. spinifera* were also tested for their cytotoxicities against the MCF-7 cell line, further supporting the idea that sea cucumbers are a valuable source of bioactive compounds with significant anticancer properties.

These studies collectively underscore the importance of marine organisms like sea cucumbers, which possess a rich array of bioactive compounds, including steroids, terpenoids, saponins, and phenolic compounds, all of which contribute to their anticancer potential. The results highlight the need for further research to isolate and characterize these compounds, as they may offer novel treatments for breast cancer and other malignancies.

In recent studies, four compounds isolated from sea cucumbers have shown significant anticancer activities against MCF-7 cells, a human breast cancer cell line. These compounds demonstrated IC_{50} values of 13.83 µM, 8.13 µM, 8.27 µM, and 35.56 µM, respectively, which indicates their potential as effective anticancer

agents. When compared to doxorubicin, a well-established chemotherapeutic drug, which has an IC_{50} value of 8.64 µM, these sea cucumber compounds show varying levels of potency. The compounds with IC_{50} values closer to doxorubicin's suggest that they could be developed into promising alternatives or adjunct therapies for breast cancer treatment [18].

Further supporting the anticancer potential of sea cucumbers, a study conducted by [19] examined the hexane extract of several sea cucumber species, including *Stichopus vastus*, *Actinopyga lecanora*, and *Holothuria atra*. This research revealed that the IC_{50} values of the hexane extracts against the T47D cell line (another breast cancer cell line) were 3.25 ± 0.53 µg/mL, 6.25 ± 0.50 µg/mL, and 7.5 ± 1.39 µg/mL, respectively. Notably, the IC_{50} value of S. vastus was slightly higher than that of the other species, which could be attributed to the presence of specific triterpene compounds that were isolated from *Stichopus*. These findings suggest that the chemical composition of *S. vastus* may contribute to its anticancer properties, though the exact compounds responsible for its effects remain to be fully identified.

Beyond sea cucumbers, other marine organisms such as spirulina have also been explored for their bioactive compounds, which include saponins, steroids, alkaloids, and flavonoids. A study by [20] demonstrated that these extracts exhibited cytotoxic activity in MCF-7 cells, with an IC_{50} value of 36.23 ppm. This further reinforces the idea that marine biota—including sea cucumbers and other marine flora—contain a rich array of secondary metabolites with the potential to act as anticancer agents.

The accumulating evidence highlights the importance of marine organisms as a valuable resource for discovering bioactive compounds with anticancer properties. With further research, these compounds could play a significant role in the development of novel treatments for breast cancer, offering more selective and targeted therapeutic options for patients.

Anticancer activity of S. vastus on HeLa cells (ATCC CCL-2)

The anticancer activity of *Stichopus vastus* was assessed against HeLa cancer cells, and the methanol extract of sea cucumbers demonstrated promising results, with an IC_{50} value of 13.50 µg/mL. This IC_{50} value falls within the range that is considered active (IC_{50} 10-100 µg/mL), indicating that the methanol extract has notable anticancer potential against HeLa cells [8].

In addition to the methanol extract, the ethyl acetate fraction exhibited even stronger anticancer activity, with an IC_{50} value of 7.00 µg/mL. This value categorizes the ethyl acetate fraction as very active (IC_{50} < 10 µg/mL), suggesting that this fraction contains bioactive compounds that could be highly effective in the treatment of HeLa cancer cells [8]. These results further highlight the potential of polar solvents, such as ethyl acetate, in isolating compounds with strong cytotoxicity against cancer cells.

Previous studies have also examined the anticancer activity of extracts from other sources. For instance, a study by [21] reported that the IC_{50} of the *Phaseolus vulgaris* extract against HeLa cells was 182.4 mg/mL, indicating that while the extract has some activity, it is much less potent compared to the sea cucumber extracts tested in the current study. This comparison emphasizes the stronger anticancer potential of sea cucumber compounds when compared to certain plant extracts.

In another study, [22] found that *Holothuria nobilis Selenka* exhibited anticancer activity with an IC_{50} value of 2.90 µg/mL against HeLa cells, which is lower than the IC_{50} of doxorubicin (1.74 µg/mL), a commonly used chemotherapeutic agent. This indicates that the compounds in *H. nobilis* may have similar or even superior potency to doxorubicin against HeLa cells, making it a potentially valuable source of bioactive compounds for cancer therapy.

Furthermore, previous studies have demonstrated that the brown sea cucumber exhibited significant anticancer activity against multiple cancer cell lines, including HeLa, MCF-7 (human breast cancer cells), and MDA-MB-231 (a triple-negative breast cancer cell line). Notably, the nonpolar extracts of the brown sea cucumber were considered significantly active against these cell lines, whereas the polar extracts (such as the butanol fraction) showed no anticancer activity. This suggests that nonpolar compounds may be more effective in targeting cancer cells, further supporting the notion that nonpolar fractions of sea cucumbers contain potent anticancer compounds.

These studies collectively reinforce the growing body of evidence that sea cucumbers are a promising source of bioactive compounds with anticancer properties, particularly against aggressive cancer cell lines

such as HeLa. The comparison with other extracts, such as those from Phaseolus vulgaris and *Holothuria nobilis*, underscores the remarkable potential of sea cucumbers as a source of novel anticancer agents, warranting further research into their chemical composition and therapeutic applications.

This study presents the first report of the anticancer activity of *Stichopus vastus* species, highlighting its rich saponin fraction as a key contributor to its cytotoxic effects. The cytotoxicity test on different fractions of *S. vastus* against HeLa cells revealed that the ethyl acetate (EtOAc) fraction exhibited stronger cytotoxic activity compared to the n-hexane fraction. Specifically, the IC_{50} value of the ethyl acetate fraction was found to be 7.00 µg/mL, indicating that this fraction has significant anticancer potential against HeLa cells. In contrast, the n-hexane fraction demonstrated a slightly higher IC_{50} value of 10.09 µg/mL, suggesting it has a lower level of cytotoxicity when compared to the ethyl acetate fraction. For comparison, the chemotherapeutic agent doxorubicin, which was used as a positive control, exhibited an IC_{50} value of 2.08 µg/mL, confirming its potency as a well-established anticancer drug.

The IC_{50} values were calculated through interpolation using a specific equation, and the results showed that the IC_{50} values for ethyl acetate fraction, n-hexane fraction, and doxorubicin were 7.00 µg/mL, 10.09 µg/mL, and 2.08 µg/mL, respectively. These results indicate that the ethyl acetate fraction of *S. vastus* is more effective in inhibiting the growth of HeLa cells than the n-hexane fraction, which may be due to the different chemical compositions and polarity of the fractions. Moreover, the average IC_{50} of *Holothuria atra* extract against HeLa cells was reported to be 9.14 ± 0.8 µg/mL [24], which is similar to the IC_{50} value of the n-hexane fraction of *S. vastus*. This suggests that the anticancer activity of *S. vastus* is comparable to that of *H. atra*, a related sea cucumber species, but with the ethyl acetate fraction of *S. vastus* showing even more potent cytotoxicity against HeLa cells.

These findings reinforce the idea that saponins and other bioactive compounds found in *S. vastus* could serve as promising candidates for the development of new anticancer drugs. The higher activity of the ethyl acetate fraction suggests that polar solvents may be more efficient at extracting bioactive compounds with strong cytotoxic properties, which warrants further investigation into the chemical profile of this fraction and its potential therapeutic applications.

Doxorubicin, a well-known chemotherapeutic agent, produces a stronger IC_{50} value against both MCF-7 and HeLa cells, which signifies its potent anticancer activity. However, despite its effectiveness, the use of doxorubicin in cancer treatment is restricted due to its immunosuppressive properties and harmful effects on normal tissues, particularly the heart. The cardiotoxicity associated with doxorubicin can lead to heart failure and other serious side effects, limiting its clinical use. In an effort to mitigate these risks, reducing the doxorubicin dosage has been suggested, but this comes at the cost of decreased efficacy, which can complicate the treatment process. Therefore, finding alternative sources of anticancer agents that are both effective and less toxic to normal tissues is of great importance in the field of cancer therapy [25].

The marine environment presents a vast and largely untapped source of bioactive compounds with a wide range of potential therapeutic benefits. Marine organisms, such as sea cucumbers, corals, and sponges, offer countless and diverse resources for the development of novel medications with anticancer, anti-inflammatory, antifungal, antiviral, antibiotic, anti-obesity, and immunoprotective properties. These natural products are seen as promising candidates to combat serious disorders and provide new avenues for treating diseases that are difficult to address with conventional pharmaceutical options [26]. This highlights the critical role of marine biodiversity in drug discovery and underscores the need for further research into marine-derived compounds as alternatives to traditional therapies.

In the case of *Stichopus vastus*, the ethyl acetate fraction has shown significant anticancer potential, making it an excellent candidate for further exploration. Future analyses will focus on isolating bioactive compounds from the ethyl acetate fraction of *S. vastus* to identify the specific compounds responsible for its cytotoxicity against cancer cells. By isolating and characterizing these compounds, we can better understand their mechanisms of action and potentially develop new, more targeted anticancer therapies with fewer side effects than existing treatments.

CONCLUSION

From the results obtained, it can be concluded that the ethyl acetate fraction of *Stichopus vastus* exhibited significantly higher cytotoxic activity against MCF-7 and HeLa cells compared to the other fractions tested. The ethyl acetate fraction is likely to contain a higher concentration of these active compounds, making it more effective in inhibiting cancer cell growth and proliferation. This highlights the importance of further exploring the chemical profile of *S. vastus* and other marine organisms to identify and isolate the specific bioactive compounds responsible for their therapeutic properties. Further studies on the mechanisms of action of these compounds will be crucial in understanding their full potential in cancer treatment.

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