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RESEARCH ARTICLE

PHYTOCHEMISTRY PROFILE AND IN-VITRO CYTOTOXICITY INVESTIGATION OF RED ALGAE *GRACILLARIA SP.* AGAINST HT-29 COLON CANCER CELLS

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ABSTRACT

Background: Cancer encompasses a heterogeneous group of non-communicable diseases characterized by uncontrolled cellular proliferation that can arise in virtually any organ. Globally, the burden is projected to reach approximately 19.3 million new cases and 10.0 million deaths by 2030. Among these malignancies, colorectal cancer which originating in the colon or rectum, remains one of the most prevalent forms. Varying treatments for colorectal cancer ranging from surgery to chemotherapy are used, which are expensive with low efficacy. Active components from organic materials i.e., marine algae could be utilized as alternative treatment for cancer. One of these marine algae is the red algae species of *Gracilaria* sp., that is known as a producer of biologically active phytochemicals. **Objective:** To investigate the phytochemical profile and in vitro cytotoxic properties of the red algae *Gracilaria* sp. against HT-29 colon cancer cells. **Methods:** Cleaned and dried *Gracilaria sp.* biomass was finely pulverized into powder. Sequential multistage maceration was then performed using solvents of increasing polarity of n-hexane, ethyl acetate, and ethanol, yielding the corresponding n-hexane, ethyl acetate, and ethanolic extracts, respectively. These extracts were subsequently subjected to phytochemical screening and thin-layer chromatography (TLC) to characterize their secondary metabolite profiles, followed by evaluation of their cytotoxic activity against HT-29 colon cancer cells using the MTT assay. **Results:** Seaweed *Gracilaria* sp. contains secondary metabolites of flavonoids, alkaloids, and triterpenoids. All extracts exhibited measurable cytotoxic effects against HT-29 colon cancer cells. The ethanolic extract demonstrated the highest activity, with an IC_{50} value of 53.32 µg/mL. In contrast, the ethyl acetate and n-hexane extracts displayed comparatively weak cytotoxicity, with IC_{50} values of 107.58 µg/mL and 180.65 µg/mL, respectively. **Conclusion:** Red algae of *Glacilaria* sp. should be further investigated as a new candidate of anti-colon cancer agent.

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INTRODUCTION

Cancer encompasses a wide range of diseases that can develop in any organ due to the abnormal growth of cells. According to WHO's 2020 report, the most frequently diagnosed cancers are lung cancer in men and breast cancer in women. Globally, the incidence of this disease continues to rise, presenting an ever-growing challenge for the medical community (1). Over the years, advancements in early detection systems have led to an increase in cancer survival rates. However, 18.1 million new cases and 9.6 million cancer-related fatalities were reported in 2018, according to Globocan data, suggesting that the mortality rate is still higher than 40%. (2). One type of cancer that is common is the colorectal cancer or the colon cancer that occurs in the colon or rectum (3). This cancer develops from non –cancerous abnormal growths known as polyp in the mucosal epithelial cells. Colorectal cancer ranks among the most prevalent malignancies globally, constituting the third most common cancer in men and the second in women. It is also a leading cause of cancer-related mortality, ranking third in terms of deaths and fourth in overall incidence worldwide. In 2018, approximately 1.8 million new cases were reported, with nearly 1 million associated deaths (2,4). As the technology and information within the medical world advances, means to research on cancer is available. With this advancement, treatment options for cancer are also evolving. Nowadays, there are multiple means of treatment depending on the types, these methods include surgery, immunotherapy, chemotherapy/radiation therapy, and targeted therapy among

others. These methods, although still being renewed over time, are often expensive and do not perform optimally. An example for this is the cornerstone drug used in systemic treatment of colorectal cancer, Fluorouracil only shows 20% chance of the patient's tumour being reduced in size by 50% or more (5). Another problem of cancer medicines nowadays is the fact that they are relatively expensive. This is why the modern medical world is still searching for a solution to this cancer problem. Some ongoing research shows interest in an effort to utilize chemicals found in herbal substances. One of these organic materials is the marine algae species *Gracilaria sp.*, commonly found in tropical and subtropical regions. It lives in shallow seas and is often cultivated in brackish water because, as a euryhaline organism, it can tolerate wide variations in salinity (6). This red alga is widely recognized as a rich source of bioactive phytochemicals, including polyphenols, carotenoids, xanthophylls, chlorophyll, fucoidan, fucoxanthin, phycocyanins, phycobilins, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, alkaloids, terpenoids, halogenated compounds, and a diverse array of polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, and galactosylglycerol (7-9). Because of its content, *Gracilaria* sp. can be a source of anticancer agent that can be tested for their effect on colon cancer cells. This study is aimed to elucidate the phytochemical profile and cytotoxic potential of *Gracilaria* sp. collected from Bekasi, West Java, Indonesia. In this investigation, the algal biomass was washed, dried, and finely ground into powder, followed by extraction using three organic solvents of varying polarity. In this investigation, the algal biomass was washed, dried, and finely ground into powder, followed by extraction using three organic solvents of varying polarity: n-hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar).Extracts of algae *Gracilaria* sp. were then used in phytochemistry test, thin layer chromatography analysis and cytotoxic activity investigation on HT-29 colon cancer cells through MTT assay.

MATERIAL AND METHODS

Materials: *Gracilaria* sp. (Figure 1) was collected from Bekasi, West Java, Indonesia. The HT-29 colon cancer cell line was obtained from the culture collection of the Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia. The taxonomic classification of *Gracilaria sp*. is presented in Table 1(8,9).

Figure 1. ***Glacilaria sp.***

Table 1. Taxonomy of *Glacilaria sp.*

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Biliphyta
Division	Rhodophyta – red algae, algues rouges
Subdivision	Eurhodophytina
Class	Florideophyceae
Subclass	Rhodymeniophycidae
Order	Gracilariales
Family	Gracilariaceae
Genus	Gracilaria Grev.

Methods

Extraction of *Glacilaria* sp: This research starts with the extraction of the sample, *Glacilaria* sp. The sample of *Glacilaria* sp. was washed, dried, and pulverised into a fine powder. Extraction was performed using three solvents of differing polarity, namely n-hexane, ethyl acetate, and ethanol, via sequential multistage maceration. The resulting extracts were subsequently subjected to thin-layer chromatography (TLC) and phytochemical screening to elucidate their secondary metabolite profiles, followed by evaluation of their cytotoxic activity against HT-29 colon cancer cells using the MTT assay.

Thin-layer chromatography (TLC): A Thin-layer chromatography is to be done in order to separate different bioactive compounds found in the extract. The extract will be placed onto a TLC plate in spots. The TLC plate will then be lowered into a chromatography tank filled with solvents (polar) as the mobile phase. The resulting travelling metabolites will be compared to a control after the retention factor (Rf) has been calculated (10). Silica plates will be cut out in 6 cm x 7 cm rectangles. Next, a straight line will be drawn in 1 cm at the bottom marking the bottom line and another line is drawn within 0.3 cm from the top to mark the end point (solvent front). Three dots for three different extracts are plotted in the straight line, and other dots will represent the positive control substances. Following sample application onto the designated dots, the silica plate was placed in a developing chamber containing the mobile phase, consisting of a 4 mL mixture of methanol and chloroform (10:1, v/v). The solvent front was allowed to ascend to the marked limit. After drying, the plate was visualized under UV illumination at 254 nm and 366 nm to detect phytochemical constituents. The retention factor (Rf) for each spot was subsequently calculated as the ratio of the distance traveled by the sample to that of the solvent front.

Qualitative Phytochemistry Test (11): Phytochemistry test is a screening process to determine if there are the following chemicals; alkaloid, flavonoids, saponins, tannins, glycosides, triterpenoid and steroid.

Alkaloids Screening: First, mix 1 mL of the diluted extract with 2 drops of NH_4OH and 5mL of $CHCl_3$. The solution is then heated. Five milliliters of 2 N HCl was added, and the solution was subsequently divided into three separate test tubes. The first tube served as the blank, while the second and third tubes were treated with three drops of Dragendorff's reagent and Mayer's reagent, respectively. If the tubes with dragendroff and Meyer reagents turns yellowish/orange it is a positive for alkaloids.

Flavonoids Screening: Mix 1ml the diluted extract with a 0.5 mL of HCl and 1 spatula of Mg powder. Positive results in yellow, red, or green colour.

Saponins Screening: Dilute 1 mg of the extract with 5 mL of aquadest. The mixture is to be mixed thoroughly for 10 seconds. The presence of saponins is indicated by the formation of a stable foam layer approximately 2 cm in height that persists for at least 10 minutes. Continued stability of the foam following the addition of a single drop of 2 N HCl further confirms a positive result for saponins.

Tannin Screening: An aliquot of 1 mL of 10% $FeCl_3$ solution was combined with 1 mL of the extract. A positive reaction is indicated by a color change to greenish-black or dark blue.

Glycosides Screening: An aliquot of 1 mL of the extract was evaporated to dryness using a hot water bath. Subsequently, 1 mL of sodium acetate hydrate and 2 mL of concentrated H_2SO_4 were added. The development of a blue or green coloration was considered indicative of a positive result for glycosides.

Triterpenoids and Steroids Screening: The presence of triterpenoids and steroids was evaluated using the Liebermann–Burchard reaction. Briefly, two mL of extract was dissolved in chloroform, treated with acetic anhydride (0.5 mL), and carefully layered with 2 mL of concentrated sulfuric acid. The appearance of reddish to violet coloration was considered indicative of triterpenoids, whereas blue-green coloration suggested the presence of steroids.

MTT Assay (12,13)

Preparing MTT Solution: MTT was dissolved in phosphate-buffered saline (PBS, pH 7.4) to obtain a final concentration of 5 mg/mL. The resulting solution was sterilized by filtration through a 0.22 µm membrane filter, transferred into a sterile, light-protected container, and stored at 4 °C in the dark.

Preparing Cell Line: Cultured cells were seeded into 96-well plates and allowed to adhere for 24 hours. Each well was subsequently treated with varying concentrations of the extract and incubated for an additional 48 hours.

Performing MTT Assay: Using a micropipette, the culture medium was carefully aspirated from each well of the 96-well plate. An MTT stock solution (5 mg/mL in PBS) was prepared, followed by the preparation of an MTT working solution through a 1:10 dilution in serum-free medium. Subsequently, 100 µL of the MTT working solution was added to each well, while selected wells were maintained as controls without MTT. The plates were then incubated for an additional 2 hours to allow cellular uptake and reduction of MTT. After incubation, the plates were gently agitated on a plate shaker for 2 minutes. The supernatant was then removed, and 200 µL of DMSO was added to each well to solubilize the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell cytotoxicity was subsequently calculated based on the relative absorbance (abs.) values using the appropriate formula, as follows.

Cell cytotoxicity (%)=100-((abs.control-abs. solution tested)/(abs. control) x 100)

Data Analysis: Data analysis is completed using Microsoft Excel. Data that is evaluated are the phytochemical components of the *Gracilaria* sp., and its cytotoxic activity against HT-29 cancer cells. Phytochemical profileis shown as present or absent (+ or -) values, whereas phytochemical component is described as retention factor (Rf) value of the spot on TLC chromatogram. The linear-regression and R^2 of the cytotoxic activity are calculated using Microsoft Excel.

RESULTS

Phytochemistry Profile of of Red Algae *Gracillaria* sp: The products of sequential multistage maceration of *Gracilaria* sp. were collected in separate containers according to the extraction solvent and appropriately labeled as EE (ethanolic extract), NHE (n-hexane extract), and EAE (ethyl acetate extract).Phytochemistry test to detect flavonoid, saponin, tannins, alkaloid, glycosides, and triterpenoids/steroids are then conducted. All the extracts show positive results for flavonoids, as shown in Table 2. Other than flavonoid, all three extracts also show a positive result for alkaloids. The ethanolic extract (EE) shows positive results for triterpenoids and saponin. No extracts show positive result on tannins and only ethanolic extract shows a positive result for glycoside.

Thin layer chromatography (TLC) analysis of*Glacilaria* sp. The TLC profiles of all three *Gracilaria* sp. extracts are presented in Figure 2. Separation was carried out on silica gel plates using a methanol–chloroform mixture (10:1, v/v) as the mobile phase. Visualization was performed under UV illumination at 254 and 366 nm. The chromatographic outcomes were evaluated based on retention factor (Rf) values. Among the extracts, the ethanolic extract (EE) exhibited the fewest detectable phytochemical constituents, with only three spots observed, whereas both the n-hexane extract (NHE) and ethyl acetate extract (EAE) displayed four components each. The corresponding Rf values are summarized in Table 3.

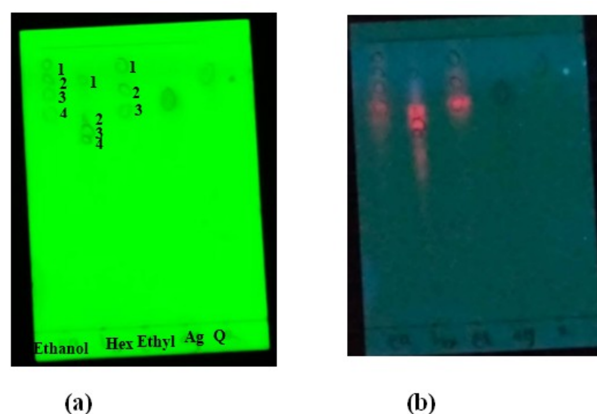

Figure 2. TLC analysis of *Gracilaria* sp. under UV light at 254 nm (a) and 366 nm (b). Ethyl: ethylacetate extract; Hex: n-hexane extract; Ethanol: ethanol extract; Ag: gallic acid and Q: Quercetin

Investigating cytotoxicity of *Glacilaria* sp. extracts on HT-29 colon cancer cells: The cytotoxic activity of *Gracilaria* sp. extracts were evaluated against the HT-29 human colon carcinoma cell line using the MTT assay. IC_{50} values were determined by substituting Y = 50 into the corresponding linear regression equation (y = ax + b), derived from plotting extract concentration (X-axis) against percentage inhibition (Y-axis). Each extract was tested in triplicate, and the mean values were used for regression analysis. For the ethanolic extract (EE), the regression equation obtained was y = 33.996x + 8.7083 (R^2 = 0.9022), as illustrated in Figure 3. The ethyl

Table 2. Phytochemistry profile of *Gracilaria* sp

	Saponin	Flavonoid	Tannin	Glycoside	Alkaloid	Triterpenoids/Steroids
EE	+	+	-	-	+	Triterpenoids
EAE	-	+	-	-	+	Triterpenoids
NHE	-	+	-	-	+	-

Table 3. Retention factor (Rf) values of TLC analysis from *Gracilaria* sp. Extracts

	Rf value	Phytochemical Components
EE	0.95(1), 0.88(2), 0.83(3), 0.76(4)	4
NHE	0.88(1), 0.74(2), 0.69(3), 0.67(4)	4
EAE	0.95(1), 0.88(2), 0.83(3)	3

EE: Ethanol extract, EAE: Ethyl acetate extract, NHE: N-Hexane extract

acetate extract (EAE) yielded a regression equation of y = 47.057x + 45.608 (R^2 = 0.9363), presented in Figure 4. Meanwhile, the n-hexane extract (NHE) showed a regression equation of y = 45.097x + 51.777 (R^2 = 0.9657), as depicted in Figure 5.

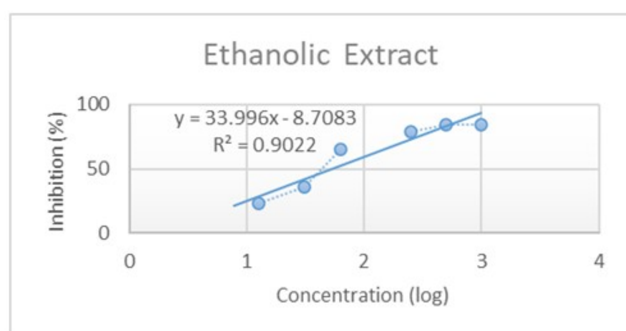

Figure 3. Linear-regression of ethanolic extract (EE) of *Gracilaria* sp. on HT-29 cells

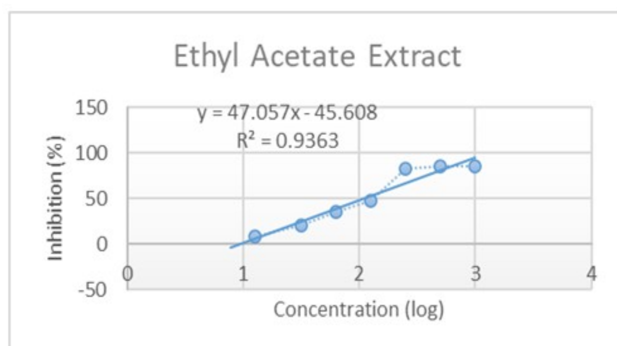

Figure 4. Linear-regression of ethyl acetate extract (EAE) of *Gracilaria* sp. on HT-29 cells

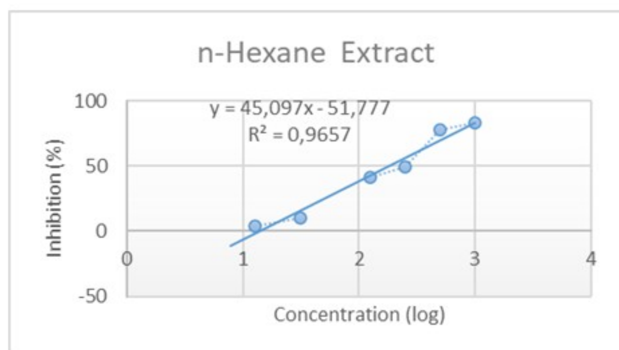

Figure 5. Linear-regression of n-hexane extract (NHE) of *Gracilaria* sp. on HT-29 cells

The cytotoxic activity of *Gracilaria* sp. extracts against the HT-29 human colon cancer cell line is summarized in terms of IC_{50} values (Table 4). Among the tested extracts, the ethanolic extract (EE) exhibited the lowest IC_{50} (53.32 µg/mL), followed by the ethyl acetate extract (EAE) with a moderate IC_{50} of 107.58 µg/mL, while the n-hexane extract (NHE) showed the highest IC_{50} at 180.65 µg/mL. Doxorubicin, employed as the positive control in the MTT assay, demonstrated a markedly lower IC_{50} value of 1.62 µg/mL (Figure 6).

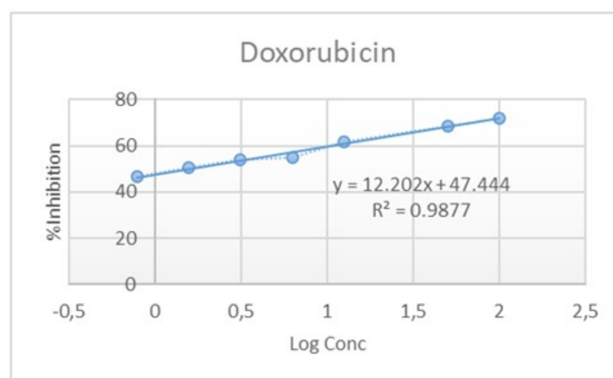

Figure 6. Linear-regression of doxorubicin on HT-29 cells

Table 4. Result of cytotoxicity investigation of *Gracilaria* sp. extracts on HT-29 cells

Extract/compound	IC50 (µg/mL)
Doxorubicin	1.62
EE	53.3234
EAE	107.5842
NHE	180.6534

EE: Ethanol extract, EAE: Ethyl acetate extract, NHE: N-Hexane extract

Table 5. Cytotoxicity classification based on IC50 value by Atjanasuppat *et al.* (2009)

IC_{50} value	Classification
< 20 µg/mL	active
20-100 µg/mL	moderate
100-1000 µg/mL	weak
> 1000 µg/mL	inactive

DISCUSSIONS

Phytochemistry Profile of *Gracilaria* sp: Phytochemical screening was conducted on *Gracilaria* sp. extracts to characterize their bioactive constituents, several of which are

known to confer beneficial effects in humans. The analysis encompassed major classes of secondary metabolites, including tannins, saponins, flavonoids, alkaloids, triterpenoids, steroids, and glycosides. Notably, terpenoids have been reported to exhibit diverse pharmacological activities, including cytotoxic effects against various cancer cell lines, as highlighted by Victor Kuete *et al*. (13). Flavonoids on the same hand also have an array of pharmacological properties. Panche *et al*. in 2016 stated that flavonoids have the capability to modulate important cellular enzyme function. They are also anti-inflammatory, antioxidant, anti-carcinogenic, and anti-mutagenic (14). Tannins, akin to flavonoids, are recognized for their potent antioxidant capacity and exhibit a broad spectrum of pharmacological activities, including anti-asthmatic, anti-inflammatory, antiviral, anticancer, anticarcinogenic, antimicrobial, anti-allergic, and antihypertensive effects, as well as protective roles in maintaining cardiovascular health (15). Furthermore, previous research suggests that glycosides exhibit analgesic properties, cardiotonic, anti-inflammatory, antibacterial, antiviral, anticancer, and antifungal effects (16). Lastly, alkaloids show a promising anti-angiogenic and anti-proliferative effects they also exhibit promising apoptotic abilities and anti-neoplastic towards some specific cancer cell lines (17).

Our test towards the three types of red algae *Gracilaria* sp. extract is shown in Table 2. For our ethanolic extract (EE) of the red algae, the tests show that it contains saponins, flavonoids, alkaloids, and triterpenoids. As for the ethyl acetate extract (EAE), it contains flavonoids, alkaloids and triterpenoids. On the other hand, the n-hexane extract (NHE) contains only flavonoids and alkaloids. Previous report on the phytochemical contents of *Gracilaria* sp. extracts from Purwaningsih *et al*. (2021) shows a similar result where the ethanol extract (EE) of *Gracilaria* sp. contains alkaloids, phenol hydroquinone, saponin, flavonoid, and triterpenoids (7). However, it differs in the ethyl acetate extract (EAE) of *Gracilaria* sp. where their data suggests that it contains only flavonoids and triterpenoids while their n-hexane extract (NHE) contains alkaloids, saponins, and flavonoids. There are a total of 11 phytochemical components across three extracts of *Gracilaria* sp. as shown by the TLC results in Table 3. N-hexane extract (HE) and ethyl acetate extract have the same amount of noticeable phytochemical components which is 4, while ethanolic extract (EE) has only 3 distinct phytochemical components. All extracts exhibited a common chromatographic spot with an identical Rf value of 0.88, corresponding to that of quercetin, which was employed as the reference standard for flavonoids. This confirms that there is a possibility for all three extracts to contain flavonoids. Other Rf values varies from one extract to the other, this indicates that from the three extracts contains different components.

Cytotoxic Investigation of Red Algae *Gracilaria* sp. against HT-29 Cells

The cytotoxic activity of *Gracilaria sp.* extracts against the HT-29 colon cancer cell line were evaluated using the MTT assay. Positive control, doxorubicin, exhibited a markedly IC_{50} value of 1.62 µg/mL, placing it in the highly active category (IC_{50}<20 µg/mL) (18). Among the tested extracts, the ethanolic extract (EE) exhibited the most pronounced cytotoxic effect, with IC_{50} of 53.32 µg/mL. According to cytotoxicity classification proposed by Atjanasuppat *et al*. in Table 5 (18), this value falls within the range of 20–100 µg/mL, indicating moderate cytotoxic activity. In contrast, the ethyl acetate extract (EAE) and n-hexane extract (NHE) showed IC_{50} values of 107.58 µg/mL and 180.65 µg/mL, respectively, both of which fall into the 100–1000 µg/mL interval and are therefore categorized as weakly cytotoxic. The difference in cytotoxic potency can be rationalized by considering the polarity of the extraction solvents and the corresponding phytochemical profiles. Ethanol, being a polar solvent, is capable of extracting a broader spectrum of bioactive secondary metabolites, including saponin, flavonoids, triterpenoids, and certain alkaloids, which are well-documented for their anti-proliferative and pro-apoptotic activities in cancer cells. Superior cytotoxicity of the ethanolic extract therefore suggests a higher abundance or synergistic interaction of these polar bioactive constituents. Conversely, ethyl acetate, with intermediate polarity, and n-hexane, a non-polar solvent, predominantly extract semi-polar and lipophilic compounds, respectively, which may possess comparatively lower cytotoxic efficacy against HT-29 cells. These findings suggest that *Gracilaria* sp., particularly its ethanolic extract, holds promise as a source of bioactive compounds with anticancer potential. Further fractionation, compound isolation, and mechanistic studies are warranted to identify the specific constituents responsible for the observed cytotoxicity and to elucidate their molecular targets in colorectal cancer cells.

CONCLUSION

Gracilaria sp. was found to contain triterpenoids, alkaloids, saponins, and flavonoids as major secondary metabolites, with a total of 11 phytochemical constituents identified through TLC analysis. The ethanolic extract (EE) exhibited moderate cytotoxic activity, whereas the ethyl acetate (EAE) and n-hexane (NHE) extracts demonstrated comparatively weak cytotoxic effects against the HT-29 colon cancer cell line.

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CONFLICT OF INTEREST: The authors declare that they have no competing interest.

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Glossary of Abbreviations

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TLC: Thin-layer chromatography
IC50: half-maximal inhibitory concentration
HT-29: human colon cancer carcinoma cell line
PBS: Phosphate Buffered Saline
DMSO: Dimethyl sulfoxide
EE: Ethanol Extract
EAE: Ethyl Acetate Extract
NHE: N-Hexane Extract

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