Unveiling the Biomedical Potential of *Hypnea valentiae*: Isolation and Characterization of Phytochemicals with Anticancer Activity

Data availability Statement

Data available within the article or its supplementary materials.

Disclosure Statements

There is no conflict of Interest.

Abstract

Biologically active chemicals with anticancer properties have been discovered in marine Rhodophyceae seaweed *Hypnea valentiae*, coastal red algae species, was obtained from the coasts of Porbandar and Kuchhdi, Gujarat, India. In this present study, marine red algae *Hypnea valentiae* extract is used for the analysis of physicochemical, phytochemical Chromosomal Aberrations and MTT anti-cancer activity. Despite tremendous advancements in medicine, infectious diseases continue to pose a significant threat to public health. Various synthetic medications have developed resistance to infectious diseases in recent years. As a result, medicinal plants contain a variety of phytochemicals that can be used to treat a variety of oxidative stress-related diseases. The Proanthocyanidin content of *Hypnea valentiae* (0.11mg/g) and physicochemical parameter like of moisture in the seaweed was 88.98 %, the amount of ash was 16.95 % and Carbonated ash accounted for 26.67%. The physicochemical parameters like different ash values and moisture content are plant-specific and they help to ensure the purity of the drug and also prevent adulteration. MTT assay against HeLa cell line human cervical cancer was used to estimate cell viability and outcomes showed excellent results against HeLa cell lines Human cervical cancer cell lines. In our experiment on Chromosomal Aberrations Seaweed *H. valentinea* cannot damage normal chromosomes and cannot affect any part of chromosomes or chromatin. Based on these results, it is concluded that the marine macro seaweed extracts from the Gujarat coast have cytotoxic against cancer cells and have potential, which could be considered for identifying novel natural drugs and future applications in medicine from the marine resources.

Keywords: Seaweeds, *Hypnea valentiae*, Anticancer (HeLa Cell Line), Phytochemical, Physicochemical, Proanthocyanidin and Chromosomal Aberrations.

1. INTRODUCTION

Hypnea, a red algal genus, is widely distributed on tropical and subtropical shores (Nauer F. *et al.*, 2014). The ethanolic extract of *H. pannosa* has been reported to exhibit potent analgesic and anti-emetic effects due to the presence of phytochemicals such as sterols and sesquiterpenes (Mazhar F *et al.*, 2011). Seaweeds contain carotenoids, polysaccharides, and polyphenols, which offer health benefits to consumers and can be applied in foodstuffs, pharmaceuticals, and

cosmetic products (Nagaoka, M *et al.*, 1999). Phlorotannins, a group of polyphenol compounds found in seaweeds that function as polymers of phloroglucinol, have strong antioxidant properties and greater free radical scavenging ability compared to other polyphenols found in terrestrial plants (Ahn *et al.*, 2007).

A study conducted by Shareef Khan *et al.* (2012) evaluated the nutritional value of several *Hypnea* species and concluded that they could be utilized as functional food ingredients due to their high content of fatty acids and essential amino acids. In another study, Bast *et al.* (2014) employed morphological and molecular techniques to confirm the introduction of an Indo-Pacific *Hypnea* species to the Mediterranean region and conducted a comparative morphological and molecular analysis of *H. valentiae* from the Indian subcontinent.

Hypnea valentiae, a prevalent rhodophyceae seaweed, has been shown to possess pharmaceutical properties, including anti-bacterial (Anandhan and Sornakumari, 2011), antioxidant, and anti-tumor activity (Chandramohan and Divya 2017). The current study aimed to evaluate the therapeutic potential of *Hypnea valentiae* as an herbal medicine for cancer cells and to assess its impact on normal chromosomes.

2. MATERIAL AND METHOD

2.1 Seaweeds Collection and Physicochemical Study

Samples of *Hypnea valentiae* were collected from the sea-coasts of Porbandar (21°38’08.2” N 69°36’09.9” E) and Kuchhdi (21° 40’47.0” N 69°31’58.8” E) in Gujarat, India. The algae were thoroughly washed with tap water, air-dried, finely ground, and stored in a sealed container for further use.

Loss on drying

Two grams of finely powdered seaweed were transferred into a silicon crucible and subjected to high temperature in an oven. The resulting weight loss, measured in mg/g, was recorded.

Determination of total ash

The algal material was incinerated in a muffle furnace at 500°C for five hours to eliminate all carbon. The resulting colorless ash was then estimated in mg/g of air-dried seaweed.

Determination of acid-insoluble ash

The entire ash in the crucible was treated with 25 ml of HCL. The insoluble residue was collected on ash-free filter paper and subjected to heating in a muffle furnace at 5000 C for 2 hours. The weight of the residue was determined and expressed as mg/g of air-dried seaweed material.

Determination of water-soluble ash

The total ash in the crucible was mixed with water and boiled for 5 minutes. The weight of insoluble elements was subtracted from the initial total ash weight. The remaining weight was considered as water-soluble ash and was expressed as mg/g of air-dried material.

Determination of carbonated ash

As previously mentioned, total ash was determined by adding 0.1 N Na2CO3 to the crucible containing the complete ash, which was then covered and kept at room temperature before being placed in a Muffle furnace set to 5000C.

Determination of Proanthocyanidins

According to previous studies (Zilic *et al.*, 2011), the presence of proanthocyanidins in seaweed extracts was determined using the butanol-HCl test. To prepare the ferric reagent, 0.5 ml of seaweed extract was mixed with 3.0 ml of butanol-HCl reagent (95:5 v/v) and 2.0 M HCl containing 2% ferric ammonium sulfate in a 96-well plate. The mixture was boiled for 60 minutes in a water bath and the absorbance was measured at 550 nm using a digital spectrophotometer (Evolution 201 UV-visible spectrophotometer, Thermo Scientific). The amount of proanthocyanidins was calculated based on the leucocyanidin equivalent using the formula (A550 nm x 78.26 x dilution factor)/(% dry weight), as described by Porter *et al.* (1985).

2.2 MTT assay [HeLa cell – cervical cancer cell]

MTT (3- [4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

In this study, the MTT test was conducted with modifications based on the methods described by Labieniec *et al.* (2003) and Lapshina *et al.* (2005). The HeLa cell lines (human cervical cancer cell lines) were obtained from the National Repository of Animal Cell Culture (NCCS), Pune, India.

Cell cultivation

The HeLa cells were cultured in Dulbecco's minimal essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). The culture was maintained in a CO_2 incubator at 37 degrees Celsius with 5% CO_2.

Seeding of HeLa cells

To remove confluent cells, trypsin solution was applied to Corning flasks containing 70-80% cells, which was later removed. The cells were counted using a hemacytometer and resuspended in D-MEM growing medium. The cell density of 1x10^5 cells/ml was used, and a cell suspension of 1000 µl was seeded onto HiMedia Tissue Culture Plates labeled with 12 wells. Three sets of replicas were produced for each chemical concentration. The tubes were then incubated for 24 hours at 37°C with 5% CO_2 using the Laby instrument from India.

Assay procedure

After incubating the cells for 24 hours at 37°C and 5% CO_2, and exposed to D-MEM growing medium containing the test drug. Throughout the next 24 hours, examined eleven different concentrations of each chemical. Afterward, MTT solution was added (5 mg/ml final concentration) to the wells and incubated for an hour at 37°C with 5% CO_2. Following two washes using saline water, the excess dye was removed from the well plate, and DMSO (200 µl) was added to each well. the color was measured at a wavelength of 562 nm (Elisa reader Lilac interrupts to add a thought.). The concentration and duration of the test formulation were determined based on early metabolic stress indicators measured by MTT. The MTT assay used Mitomycin-c (MMC) as a positive control.

***2.3 In vitro* genotoxicity testing: Analysis of Chromosomal Aberrations**

Lymphocyte Culture and exposure:

In Hungerford's (1965) study, modifications were made to the lymphocyte culture protocol. The culture medium RPMI 1640, Lectin (0.01 ml), and Heparin (0.05 ml) were added to a 5 ml mixture, and the vials were incubated at 37°C for 72 hours. After 72 hours, seaweed extract was introduced to the incubation, and the vials were kept in the incubator for another 96 hours. Demecolcine (0.1 ml) was then provided, and a solution was injected during the final two hours of incubation to prevent cells from entering metaphase. The cells were resuscitated for 20-25 minutes with a pre-warmed hypotonic solution (KCl, 0.075M) and then fixed in a cooled methanol/acetic acid (3:1, v/v) solution. Cell suspensions were made after numerous washing of Carnoy's fixative, and slides were created from these suspensions. The slides were dried on hot plates at 50-60°C and immediately labeled and coded after ensuring that the chromosomes were evenly distributed.

Analysis of Chromosomal Aberrations:

Following the drying process, the slides were treated with 2% Giemsa stain for 10 minutes. Subsequently, 100 metaphase chromosomes with good spreading were examined on each plate for the detection of chromosomal and chromatid abnormalities.

3. RESULTS AND DISCUSSION

3.1 Chromosomal Aberration

The outcomes of genotoxicity tests of the *H. valentiae* extracts were assessed using chromosome aberration assay given in table 1. These results indicate that the cell's mitotic index and the level of DNA damage are approached to various chromosome aberrations were found to be more common in the Mitomycin-C than in the extract ($p > 0.05$), with the most common form being chromatid and chromosomal gap.

Table 1: Chromosome aberration study by using *H. valentiae* extract

Sr.	Concentration (PPM)	Chromosomal aberration per 50 metaphases		
		Chromosomal Type aberration (G/B/I/D)	Chromatid Type Aberration (B'/G')	Total Aberrations
1	*H. valentiae* 10 µl (200 ppm)	0	1	1
2	*H. valentiae* 50 µl (1000 ppm)	0	1	1
3	Mitomycin C (50 µl)	34	48	82
4	Negative control	0	1	1

Chromosomal aberrations such as chromosomal gap (G), chromatid gap (G'), chromosomal break (B), chromatid break (B'), chromosome interchange (I), and chromosomal deletion (D) were compared between the *H. valentiae* extract and Mitomycin-C. The results, as shown in Table 1, indicate that Mitomycin-C induced more chromosomal aberrations, including chromatid and chromosomal gaps, compared to the extract ($p > 0.05$). The synthetic drug induced a total of 82 chromosomal aberrations, including chromatid interchange and deletion, in 50 metaphases,

whereas the *H. valentiae* extract caused only a single aberration in chromatid, which was similar to the negative control and not harmful to chromosomes.

The genotoxicity or chromosomal damage activity of seaweed extract was found to be negligible as depicted in Figures 3 (A) and 3(B). The negative control for Chromosomal Aberrations showed no observed chromosomal or chromatid aberration. However, aberrations in chromosomes were observed in all cultures treated with Mitomycin C, with chromosome exchange and chromatid breaks being the most prevalent. In Figures 1and 2, the study of chromosomal aberrations was conducted using *H. valentiae* extract at doses of 10 µl and 50 µl, respectively, and Figure 3(b) showed the observation of chromosome interchange. Figure 4 shows the use of Mitomycin-C as a positive control at a dose of 50 µl.

The DNA in the genome is constantly exposed to various agents that can cause damage, both from external sources and those that originate within the body. Identifying these agents and other protective measures against them is crucial. One such agent is the alkylating agent MMC, which can cause mutations by cross-linking DNA and leading to base substitutions, chromatin breaks, chromosome breaks, and deletions (Poersch *et al.,* 2007). These agents are often used to assess increased sensitivity to DNA cross-linking agents in studies using different cytogenetic endpoints (Liou *et al.,* 2002). Additionally, some antitumor agents, such as MMC, can activate apoptotic cell death via ROS-dependent mechanisms, indicating the potential use of ROS as an antitumor treatment (Fang *et al.,* 2007). In our study, we evaluated the Chromosomal Aberrations effect of *H. valentinea* extract against MMC. Our findings suggest that *H. valentinea* extract does not cause any harm to normal chromosomes or affect any part of chromosomes or chromatin.

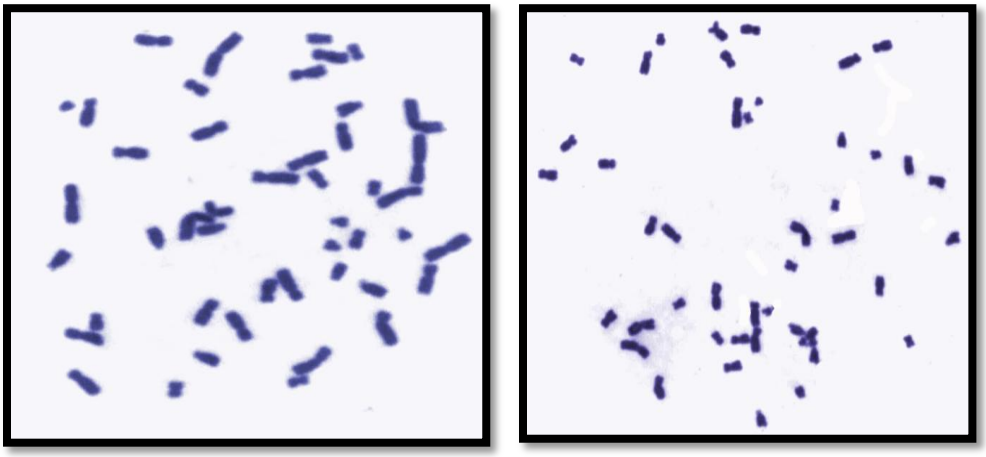

Figure 1 Study of Chromosomal Aberrations by using *H. valentiae* extract at dose 10 µl

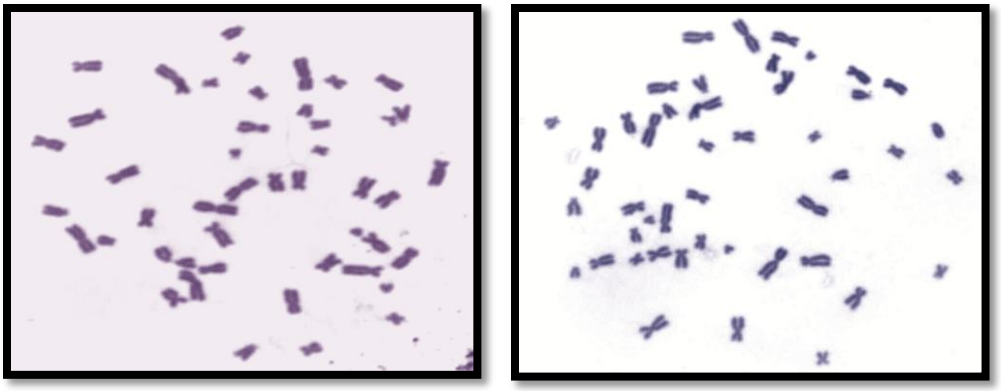

Figure 2. Study of Chromosomal Aberrations by using *H. valentiae* extract at dose 50 µl

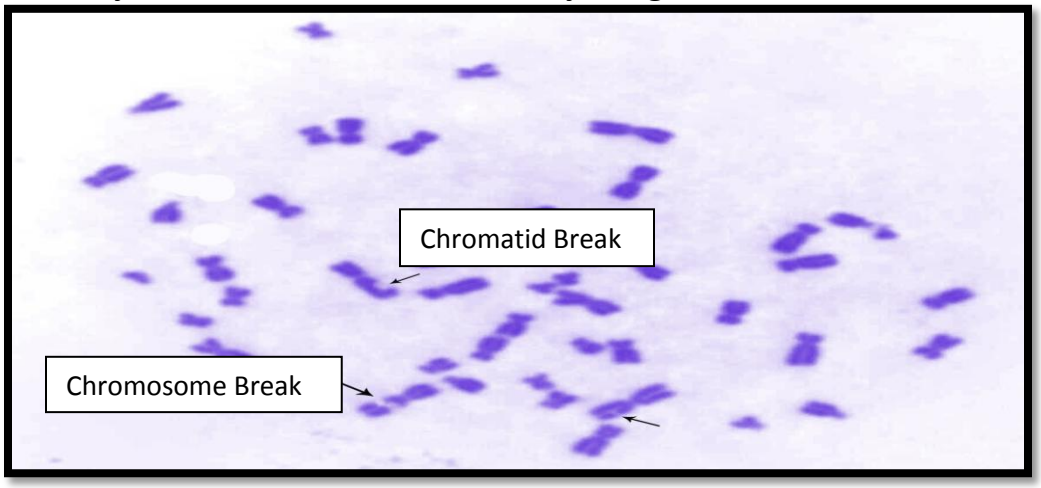

Figure 3(A). Positive Control Mitomycin-C at dose 50 µl

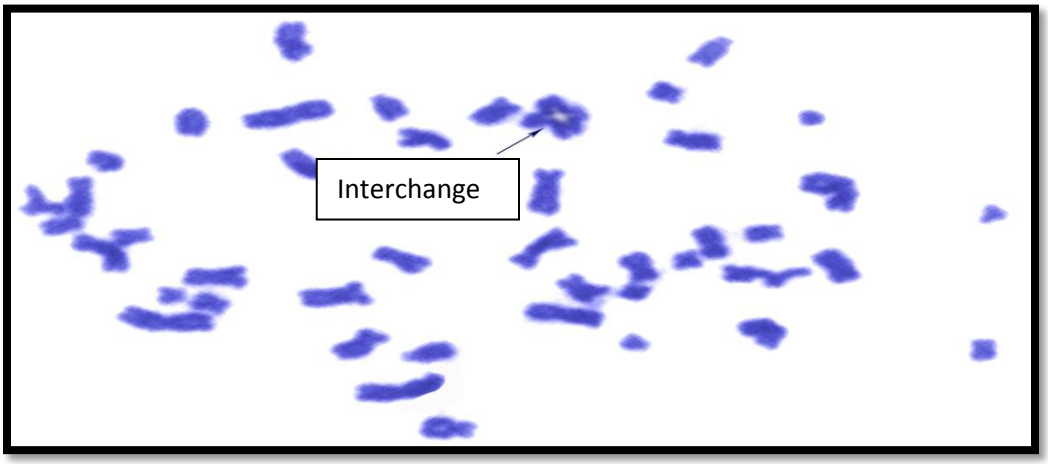

Figure 3(B). Positive Control Mitomycin-C at dose 50 µl

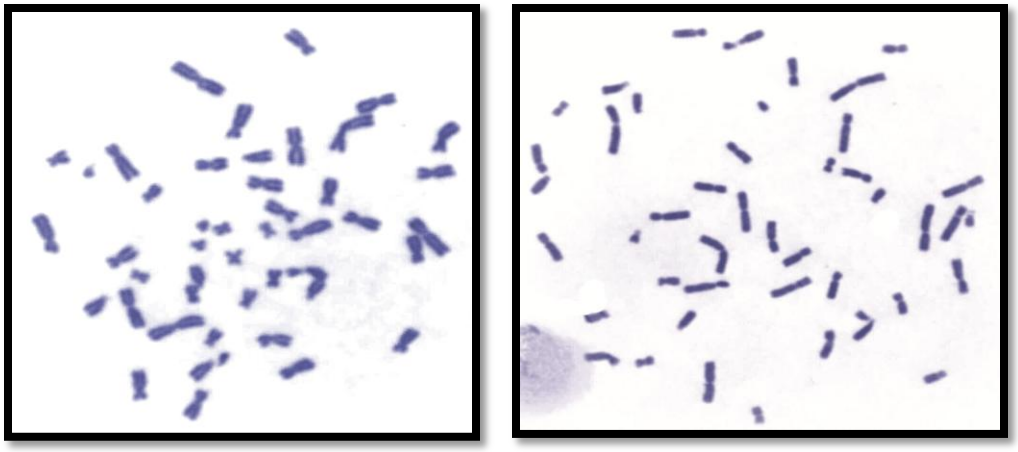

Figure 4. Study of negative Control in Chromosomal Aberrations

3.2 Physicochemical and Phytochemical

Table 2: Physicochemical and Phytochemical parameters of different seaweeds

Crude powder	Moisture %value (w/w)	Total ash %value (w/w)	Acid insoluble %value (w/w)	Water soluble %value (w/w)	Carbonated ash %value (w/w)	Proanthocyanidin content
Hypnea valentiae	88.98	16.95	06.00	13.50	26.67	0.11mg/g

Table 2 displays the physicochemical characteristics of a crude powder obtained from *Hypnea valentiae*, which are summarized as follows: The ash content was found to be 16.95 %, and the moisture content of the seaweed was 88.98%. The total ash content included 13.50% water-soluble ash and 06.00% acid-insoluble ash, while the carbonated ash content was 26.67%. Additionally, the phytochemical analysis revealed a proanthocyanidin content of 0.11 mg/g.

Various methods using different solvents and conditions are available for screening essential bioactive compounds in seaweeds (Ganesan *et al.,* 2008). In this study, we employed methanol extraction for phytochemical screening and detected the presence of the bioactive compound proanthocyanidin, as shown in Table 2. No prior study on the phytochemical screening of *H. valentinea* extracts for proanthocyanidin has been reported in the literature. However, another study by Rafquzzaman *et al.* (2016) identified several other phytochemicals from the red algae *Hypnea musciformis*.

The level of ash in a plant can indicate the presence of inorganic impurities such as silica and sand, making it a crucial parameter for detecting contamination and adulteration (Agboola *et al.,* 2017). In this study, the crude powder of *Hypnea valentiae* was found to have an overall ash content of 16.95%, with carbonated ash accounting for 26.67%. Additionally, the seaweed had a moisture content of 88.98%. These physicochemical parameters, including ash values, moisture

content, and extractive values, are specific to each plant and are important in ensuring drug purity and preventing adulteration.

3.3 MTT assay HeLa cell – cervical cancer cell

Table 3: MTT assays were used to determine the viability after treatment with *H. valentinea* extracts at different doses.

Concentration	*H. valentinea*	
Doses (µl)	**OD**	**Viability**
Positive control	0.1	21.74%
Control	0.46	100%
2	0.46	100.00%
4	0.45	97.83%
10	0.4	86.96%
20	0.38	82.61%
30	0.35	76.09%
50	0.31	67.39%
100	0.28	60.87%
150	0.25	54.35%
200	0.2	43.48%

To confirm the potential toxicity of seaweed extracts on human cervical cancer cells, an in vitro anticancer study was conducted using the MTT assay. The human cervical cancer cell lines were cultured with varying dosages of the algal extract, and the possible harmful effects on the cancer cells were evaluated. Many marine algae have exhibited antitumor properties, making their action against cancer cell lines one of the most significant characteristics of marine algae. The results of the extract cytotoxicity study are presented in Table 3. Our findings revealed that *H. valentinea* had a concentration-dependent inhibitory effect on HeLa cell growth, with viability rates of 100.00%, 97.83%, 86.96%, 82.61%, 76.09%, 67.39%, 60.87%, 54.35%, 43.48%, and 43.48% at concentrations of 2, 4, 10, 20, 30, 50, 100, 150, 200, and 300 µg, respectively. In a previous study (Usoltseva *et al.,* 2018), different cell lines were used and the percentage of cell viability decreased with increased concentration, leading to cell death. Brown algae extracts have exhibited potential cytotoxic and antitumor activity, but Rhodophyceae algae have shown higher results than Phaeohyceae seaweeds. Other seaweeds with reported anticancer activity include those against Hep-2 (liver cancer) and MCF-7 (breast cancer) cell lines (Mary *et al.,* 2012).

4. CONCLUSION

Significant anticancer activity was demonstrated by the extract of *H. valentinea*, which is rich in phytochemicals. This study investigated the anticancer, phytochemical, physicochemical, and chromosomal aberrations properties of the Rhodophyceae algae *H. valentinea*. The results suggest that *H. valentinea* extracts may be a valuable tool in the treatment of cancer and in the development of anticancer agents in medicine. The study showed that *H. valentinea* extracts are effective in extracting polyphenols, including proanthocyanidin, and may have the potential for

use as anticancer herbal agents in drug products, with implications for improving food safety. Furthermore, *H. valentinea* has demonstrated good cytotoxicity results against the HeLa cell line and shows potential as an anticancer drug for future research. Genotoxicity tests conducted on the *H. valentiae* extracts using a chromosome aberration assay showed no adverse effects on the chromosomes of normal cells.

Significance Statement

The discovery of biologically active chemicals with anticancer properties in the marine red algae *Hypnea valentiae* is a significant development in the field of natural medicine. The study conducted on the extract of this seaweed has revealed promising results in terms of physicochemical analysis, phytochemical composition, and anticancer activity. The analysis of phytochemicals has shown the presence of proanthocyanidin in *Hypnea valentiae*. Proanthocyanidin is a potent antioxidant compound known for its protective effects against oxidative stress-related diseases. The MTT assay, which measures cell viability, has demonstrated excellent results against HeLa cell lines, indicating the potential cytotoxic effect of the seaweed extract on human cervical cancer cells. This finding suggests that the *Hypnea valentiae* extract could be a promising candidate for developing novel natural drugs for cancer treatment. Moreover, the experiment on chromosomal aberrations has revealed that the seaweed extract does not cause damage to normal chromosomes or affect any part of chromatin. This is a crucial finding as it indicates the specificity of the extract's cytotoxicity towards cancer cells without harming healthy cells.

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