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Original Article

Antioxidant Activity and Cytotoxic Effects of *Hypnea musciformis* on MCF7 and MDA-MB-231 Cell Lines

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Abstract

Free radicals impair the cellular and tissue homeostasis, which can cause to cancer. *Hypnea musciformis* is a red seaweed and rich source of secondary antioxidant metabolites. These metabolites probably are effective in cancer treatment. In the present study, the phenolic and flavonoid content of the hydro-methanolic extract of *Hypnea musciformis* were determined respectively by the Folin-Ciocalteu and aluminum-chloride assay. The monovalent reducing power and total radical scavenging activity of the extract were also evaluated respectively by FRAP and DPPH assay. The cytotoxic effects of the extract were evaluated by MTT assay on MDA-MB-231 and MCF7 cell lines. Morphological changes of the cell lines were also examined by invert microscope. Statistical analysis was performed using t-test in GraphPad Prism 8.0.2 software. The results of the study indicate that the phenolic and flavonoid contents of the extract respectively were 14.05± 2.39 µgGAE/mg and 39.08± 8.78 µgQE/mg. The monovalent reducing power of the extract was 243.32± 27.86 µmol Fe^{2+}/g and its total radical scavenging activity (in 1000 µg/mL) was 18.75± 1.51 %. The cytotoxic effects of the extract on MDA-MB-231 were higher than MCF7 cell line, significantly ($P < 0.05$). The IC50 value of the extract on MDA-MB-231 and MCF7 cells respectively was 634.5 and 826.8 µg/mL, after 72 hours of incubation. The morphological changes of treated cell line include cell granulation, cell contraction and rupture in a concentration and time dependent pattern. The cytotoxic effects of *Hypnea musciformis* extract were not dependent on estrogen, progesterone and HER2 receptors. Therefore, the hydro-methanolic extract of *Hypnea musciformis* probably is a favorable option in drug discovery against the triple-negative tumors.

Keywords: *Hypnea musciformis,* Anti-oxidant, MTT assay, cytotoxicity, Cancer.

1. Introduction

Antioxidant activity disturbs with the constant exposure to free radicals, oxidants and external stimulants. Disruption in cellular and tissue homeostasis lead to oxidative stress induction and damage to large biomolecules such as DNA and proteins, uncontrolled cell growth, metastasis and cancer induction [1, 2]. Abortion, smoking, overweight, stress, anxiety and use of preservatives in various industries are the most

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important factors can cause to breast cancer by oxidative stress. Breast cancer is a heterogeneous tumor with widespread clinical manifestations and is the most common non-cutaneous malignancy in the United States. About 40,000 people in the United States and 400,000 worldwide die each year due to breast cancer. In recent years, research has led to a good understanding of its pathogenicity, but there is still no definitive cure for it [3]. A tumor lacks estrogen receptor, progesterone receptor, and HER2 epidermal growth factor receptor is called a triple-negative tumor. These tumors are very resistant to common treatments and their disease does not have a good prognosis. Surgery, chemotherapy, immunotherapy, radiotherapy or hormone therapy are the usual treatments for breast cancer. These procedures are costly and have severe side effects. Therefore, efficient drugs with fewer side effects are critical; especially in triple-negative tumors [4-6].

According to recent studies, the plant derived secondary metabolites have significant inhibitory effects on the cancer cell migration, tissue angiogenesis and cell proliferation. The tendency to use medicinal plants is increasing, due to the side effects of usual treatments [7]. Seaweeds are the traditional products, contain multiple effective secondary metabolites in free radical inhibition and treatment of cancer. *Hypnea musciformis* as a red seaweed belongs to the *Cystocloniaceae* family. These seaweeds are a rich source of minerals, amino acids, vitamins and anti-cancer secondary metabolites [8]. *Hypnea musciformis* is in the diet of some people, especially in East Asia. *Hypnea musciformis* was also cultivated for its kappa carrageenan [9].

The present study was performed for the first time to evaluate the cytotoxic effects of *Hypnea musciformis* extract on MCF7 and MDA-MB-231 breast cancer cell lines. The phenolic and flavonoid content, Monovalent reducing power and Total radical scavenging activity of *Hypnea musciformis* extract were also determined. The aim of the present study was to evaluate the pharmacological capacity of *Hypnea musciformis* extract in regard to anti- oxidant and anti-breast cancer activities.

2. Materials and Methods

2.1. Collection and Preparation of the Seaweed Extract

The red seaweed specimens of *Hypnea musciformis* were collected from the shores of the Persian Gulf (Bandar Abbas, Iran) in autumn 2017. The samples were collected and identified in the Department of Marine Biology, Faculty of Marine Science and Technology, Hormozgan

University, Iran. The identification of the algae was conducted according to checklist of the marine macro algae of Iran [10]. The voucher specimen of the algae was deposited in the herbarium of Fasa Medicinal Plants Research Center (FMPRC), Fasa University of Medical Sciences, Fasa, Iran. The Voucher number of FMPRC-100-7 was assigned to the collected sample of *Hypnea musciformis*.

The alga sample was dried in a dark environment with 10 to 15% humidity and powdered by a home grinder. Hydro- methanolic extract was obtained by maceration method [11]. Algae powder (100 g) was immersed in methanol (70:30 v/v) at room temperature for one week in darkness. The excess solvent was evaporated at 50 °C and the concentrated extract was dried in 40 °C. The stock solution of the extract (100 mg/mL) was prepared by dimethylsulfoxide (DMSO) and was sterilized with a 0.22 µm filter. The solution was diluted (1:100 v/v) to prepare working solutions of 1 mg/mL with distilled water and DMEM for biochemical and cell culture experiments, respectively [11].

2.2. Measurement of Phenolic Content

To measure the phenolic content of hydro-methanolic extract of *Hypnea musciformis* alga, the Folin-Ciocalteu assay was used. 500 µL of Folin-Ciocalteu reagent (10% v/v) was added to 100 µL of alga extract at a concentration of 1 mg/mL and incubated for 5 minutes at room temperature and darkness. Then 400 µL of sodium carbonate (7.5% w/v) was added to the sample and the resulting solution was kept at room temperature and darkness for 60 minutes. Finally, the absorbance of the samples was measured by a Synergy HTX multi-mode reader at 765 nm wavelength. Gallic acid was also used as the standard and the phenolic content of the extract was reported in microgram Gallic Acid Equivalent (GAE) per milligram of dry weight (µgGAE/mg) [12, 13]. Measurement of phenolic content of the *Hypnea musciformis* seaweed extract was repeated three times in duplicate.

2.3. Evaluation of Flavonoid Content

To measure the Flavonoid content of the *Hypnea musciformis* hydro- methanolic extract, the aluminum-chloride assay was used. 50 µL of aluminum chloride (10% w/v) and 50 µL of sodium nitrite (5% w/v) was added to 200 µL of the *Hypnea musciformis* extract at a concentration of 1 mg/mL. The resulting solution was incubated for 6 minutes at room temperature and darkness; and 700 µL of sodium hydroxide (4% w/v) was added. The total volume of the solution was equal to 1 ml and incubated for 15 minutes at room temperature and darkness. Finally, the absorption of the solution was read at 510 nm using a Synergy HTX multi-mode reader. Quercetin was used as the standard and the flavonoid content of the extract was reported in micrograms Quercetin Equivalent (QE) per milligram of dry weight (µgQE/mg) [14]. Measurement of flavonoid content was repeated three times in duplicate.

2.4. Monovalent Reducing Power

In the Ferric Reducing Antioxidant Power (FRAP) assay, the antioxidant activity of The *Hypnea musciformis* extract to reduce Fe^{3+} ions (contained in the Fe-TPTZ complex) and converting to Fe^{2+} ions is measured. According to the previous study [15], 50 µl of the seaweed extract (1 mg/mL) was added to the FRAP working solution (1.5 ml) and mixed. After 10 minutes of incubation at 37 ° C, the absorption of the sample was read at 593 nm. FeSO4 serial dilutions were also used as the standard and the antioxidant activity of the *Hypnea musciformis* extract was reported in µmol Fe^{2+}/g [16, 17]. All measurements were repeated three times in duplicate.

2.5. Total Radical Scavenging Activity

In the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) clearance assay, the total radical scavenging capacity of the *Hypnea musciformis* extract is determined. DPPH radical has a purple color that turns yellow after reduction by antioxidants and its colorlessness indicates the antioxidant activity of the extract. Different concentrations of the extract (100, 200, 500 and 1000 µg/mL) were prepared using ethanol 70%. The prepared extracts (40 µL) were added to 160 µL of the DPPH radicals (0.3 mM), mixed and incubated for 30 minutes at room temperature and darkness. Finally, the absorption of the samples was measured in 517 nm by the Synergy HTX multi-mode reader. All measurements were repeated three times in duplicate. Ascorbic acid, a potent antioxidant, was used for comparing the results. The control group was the Ethanol 70% (v/v) that used in dilution of the extracts. The percentage of the antioxidant activity was calculated using the following equation [18]:

Antioxidant power= [(Optical absorption of the control group - Optical absorption of the experimental group) / Optical absorption of the control group] × 100

2.6. Cell Culture

Two invasive breast ductal carcinoma cell lines, human MDA-MB-231 (triple negative) and MCF7 were purchased from the National Cell Bank of Pasteur Institute of Iran. Cells were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (Gibco, USA) and 1% penicillin streptomycin (Bio idea, Iran) at 37°C in a humidified 5% CO2 incubator. Cells after 70% confluency were used for subsequent experiments. For evaluation of cytotoxic effects, the cell lines were cultured in 96-well plates. 150µl of the cell suspension containing 10,000 of MCF7 and MDA-MB-231 breast cancer cells was distributed in the plate and incubated for 24 hours [19].

2.7. Cytotoxic Effects of Hypnea musciformis

MTT colorimetric assay was used to evaluate the cytotoxic effects of *Hypnea musciformis*. MTT assay is based on the reducing power of MTT by mitochondria of the cells which shows

the rate of the cell survival in treatment with various drugs. MTT assay is widely used in research on anti-cancer drugs. For this purpose, the 96-well plates after 24 hours of incubation were treated with seaweed extract concentrations of 200, 400, 600 and 1000 µg/mL according to the anticancer compounds classification criteria, and incubated in three groups of 24, 48 and 72 hours [20, 21].

To evaluate the cytotoxic effects of the seaweed extract, 20 µL of MTT solution (0.5 mg/mL) was added to the treated cells (1:10 v/v) in darkness and the plates were incubated for 4 hours. Next, the supernatant was carefully removed and the formed Formazan crystals were completely dissolved by adding 200 µl of DMSO. Finally, the samples absorption was measured by a Synergy HTX multi-mode reader at 540 nm wavelength. Cell viability in exposure to the extract was calculated by dividing the optical absorption of the treated cells by the optical absorption of the control group. The results were reported as the cell viability percentage. The cytotoxic effect of the *Hypnea musciformis* extract was calculated by the reduction rate in cell viability of the cancer cell line. Finally, IC50 value of the cytotoxic effects of the extract was calculated by linear regression. IC50 indicates the minimum concentration of the extract that can inhibit the viability of MCF7 and MDA- MB-231 cell lines by 50 % [22, 23]. All measurements were repeated three times in duplicate.

2.8. Evaluation of Morphological Changes

In evaluation of morphological changes, MCF7 and MDA-MB-231 cell lines were treated with concentrations of 200, 400, 600 and 1000 µg/mL of *Hypnea musciformis* extracts. the morphological changes of treated cells were evaluated by inverted microscope after 24, 48 and 72 hours of incubation [24]. All measurements were repeated three times in duplicate.

2.9. Statistical Analysis

Statistical analysis of data was performed using t-test in GraphPad Prism 8.0.2 software. Significance level was considered less than 0.05 ($P<0.05$) and the data were expressed as Mean± SD. IC50 value of total radical scavenging activity and cytotoxic effects were calculated respectively by four parametric logistic regression and linear regression.

Results and Discussion

The phenolic content of the *Hypnea musciformis* extract by the Folin- Ciocalteu assay was 14.05± 2.39 µgGAE/mg and its flavonoid content by aluminum chloride assay was 39.08± 8.78 µgEQ/mg. The monovalent reducing power of the *Hypnea musciformis* extract was also 243.32± 27.86 µmol Fe^{2+}/g by FRAP assay. According to the Table 1, the total radical scavenging activity percentage of the *Hypnea musciformis* extract on concentrations of 100, 200, 500 and 1000 µg/mL were respectively evaluated as 8.88± 2.42, 11.32± 2.20, 14.18± 1.94 and 18.75± 1.51 %. The total radical scavenging

activity of the extract at different concentrations was fewer significantly (P< 0.0001) than ascorbic acid (Figure 1). The IC50 value of the total radical scavenging activity were >1000 and 30.99 µg/mL respectively for *Hypnea musciformis* extract and ascorbic acid.

The highest cytotoxic effects of *Hypnea musciformis* extract were shown in 1000 µg/mL concentration of the extract after 72 hours of incubation. The cytotoxic effect of the *Hypnea musciformis* extract was time and dose dependant. As the cytotoxic effects of the extract on MDA-MB-231 cell line increased from 7.76 % to 89.07 % in concentration of 200 µg/mL compared to 1000 µg/mL, respectively. Besides, the cytotoxic effects of the extract on MCF7 cell line increased from 14.89 % to 63.72 % in concentration of 200 µg/mL compared to 1000 µg/mL, respectively.

After 24 hours of incubation, the cytotoxic effect of 600 µg/mL concentration of the *Hypnea musciformis* extract on MDA-MB-231 cell line (22.94 %) was significantly (P=0.009) higher than MCF7 (7.41 %). After 48 hours of incubation, the cytotoxic effect of 1000 µg/mL concentration of the extract on MDA-MB-231 cell line (63.10 %) was also significantly (P=0.03) higher than MCF7 (48.01 %). The cytotoxic effects of the extract were intensified by incubation time. After 72 hours of incubation, the cytotoxic effects of 400, 600 and 1000 µg/mL concentration of the extract on MDA-MB-231 cell line respectively were 24.53 % (P=0.02), 44.16 % (P=0.03) and 89.07 % (P<0.0001) which was significantly higher than MCF7 (16.67 %, 34.12 % and 63.72 %, respectively).

Morphological changes of MCF7 and MDA-MB-231 cell lines in treatment with *Hypnea musciformis* extract were consistent with the cytotoxic effects and had the concentration and time dependent pattern. As shown in Fig.2, the cell membranes of MCF7 and MDA-MB-231 cells treated with *Hypnea musciformis* extract were ruptured. Cell debris and cell granules were also increased clearly. These morphological changes were more severe in MDA-MB-231 cells as a triple negative cell line compared to MCF7 cell line. The highest amount of morphological changes such as cell granulation, cell contraction and rupture were observed in MDA-MB-231 cells treated with 1000 µg/mL concentration of the extract after 72 hours of incubation (Figure 2).

According to Table 2, the IC50 value of cytotoxic effects of *Hypnea musciformis* extract on MDA-MB-231 cell line after 24, 48 and 72 hours of incubation respectively were >1000, 790.3 and 634.5 µg/mL. The IC50 values of the extract on MCF7 cell line were also >1000, >1000 and 826.8 µg/mL, respectively. According to the classification criteria of Baharum et al. (about the anti-cancer compound), *Hypnea musciformis* was considered as a "weakly active" compound (14). According to these criteria, the cytotoxic effects of the traditional medicinal products are divided into four categories according to the IC50 value. Medicinal products with an IC50 value of 0-20, 20-100, 100-1000 and >1000 µg/mL are classified respectively as very

active, relatively active, weakly active and inactive cytotoxic compounds.

Consistent with the present study, the weak cytotoxic effects of *Hypnea flagelliformis* native to the Persian Gulf was shown on MDA-MB-231, MCF7 and T-47D breast cancer cell lines in the study of Erfani et al. [25]. In the study of Mosaddegh et al, The IC50 value of different species of the *Hypneaceae* family such as *Hypnea boergeseni*, *Hypnea charoides* and *Hypnea valentiae* were more than 100 µg/mL, considered as a weak active anti-cancer compound [26]. In our previous study, Hypnea musciformis had higher cytotoxic effects than Ulva lactuca on MDA-MB-468 cells. The IC50 value of cytotoxic effect of Hypnea musciformis on MDA-MB-468 was 905 and 701.2 µg/mL respectively after 48 and 72 hours of incubation and classified as a weak active compounds [27].

Unlike the present study, some studies have shown higher cytotoxic and antioxidant effects. This dissimilarity may have been due to the differences of extraction methods and geographical conditions. For example, the study of Guedes et al. showed that the cytotoxic effects of the chloroform and dichloromethane extracts of *Hypnea musciformis* were considerable on K562 cell line. The IC50 value of the extracts respectively were 17.4± 1.1 and 3.8± 0.2 µg/mL and classified as very active anti-cancer compounds. The methanolic extract of *Hypnea musciformis* also had high cytotoxic effects on NCI- H292 (human lung mucoepidermoid carcinoma) and HEp-2 (laryngeal epidermoid carcinoma) cell lines with IC50 value of 40.2± 3.1 and 48.3± 3.9 µg/mL, respectively [28].

The cytotoxic and antioxidant effects of *Hypnea musciformis* appear to be directly related to type of extraction, cancer cell line and geographical conditions. As mentioned earlier, normal cells exposed to free radicals undergo physiological changes such as growth signal production, reduction of inhibitory signals, angiogenesis, and cancer incidence under oxidative stress [29]. Therefore, improving the oxidative stress and strengthening the antioxidant system is one of the effective strategies in the prevention and treatment of cancer. In the present study, the anti-oxidant activity of the *Hypnea musciformis* extract was not considerable, which is probably due to the relatively low phenolic and flavonoid content. Therefore, the total extract of *Hypnea musciformis* probably has a little effect on the reduction of oxidative stress. The cytotoxic effect of the *Hypnea musciformis* total extract was relatively weak on MDA-MB-231 and MCF7 cell lines. The *Hypnea musciformis* total extract was in the range of weakly active anticancer compounds; but its cytotoxic effects were independent of estrogen, progesterone and epidermal growth factor HER2 receptors. The cytotoxic effect of the extract on MDA-MB-231 triple negative cell line was higher compared to MCF7 cell line.

Therefore, it is appeared that the *Hypnea musciformis* extract is a favorable option in drug discovery of anti-cancer compounds. More effective seaweed compound probably be

introduced on the triple negative cancer cells, if in the future studies focus on isolation of the active compounds of seaweed fractions. In the study of Chakraborty et al., was shown that the aryl monoterpenoids extraction from the *Hypnea musciformis* ethyl acetate fraction lead to the intensification of anti-oxidant activity [30]. Aryl monoterpenoids extraction may be effective in the suppression of cancer cells proliferation; due to the direct relationship between the antioxidant and anti-cancer effects.

Sulfated polysaccharides is the other antioxidant and anti-cancer compounds of the *Hypnea musciformis* extract. In the study of Alves et al., it was shown that the sulfated polysaccharides extracted from the *Hypnea musciformis* red seaweed had a significant antioxidant effect on suppression of lipid peroxidation, and significantly inhibited the proliferation of 3T3 and Hela cell lines [31]. Therefore, it is recommended that the cytotoxic effects of aryl monoterpenoids and sulfated polysaccharides be investigated on the proliferation, apoptosis and cell cycle of the triple negative breast cancer cell lines in the future studies. If effective results were achieved, subsequent clinical trials and animal studies are recommended.

The major limitation of the present study was the lack information of cytotoxic effects of the *Hypnea musciformis* total extract on normal cells and its effectiveness on the growth cell cycle of MDA-MB-231 and MCF7 cell line, due to financial problems.

4. Conclusion

The *Hypnea musciformis* seaweed had relatively weak phenolic and flavonoid content and antioxidant activity. Consequently, The *Hypnea musciformis* total extract was in the range of weakly active anti-cancer compounds. The cytotoxic effects of *Hypnea musciformis* extract were not dependent on estrogen, progesterone and HER2 receptors and probably was a favorable option in drug discovery against the triple-negative tumors. Isolation of the active compounds of seaweed fractions in the future studies is recommended.

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Tables:

Table 1. Total radical scavenging activity (%) of different concentrations of *Hypnea musiformis* extract and Ascorbic acid.

CONC (µg/ml)	Hypnea musiformis		Ascorbic acid		P-value
	Mean±*SD*	IC50	Mean±*SD*	IC50	
100	8.88 ± 2.42	>1000	84.10± 4.68	30.99	**< 0.0001**
200	11.32 ± 2.20		92.42± 0.03		**< 0.0001**
500	14.18 ± 1.94		92.62± 0.05		**< 0.0001**
1000	18.75 ± 1.51		93.09± 0.40		**< 0.0001**

P-value indicates the significant differences between the groups by t-test analysis. IC50 value of total radical scavenging activity was calculated by four parametric logistic regression.

Table 2. The viability of MCF7 and MDA-MB 231 cell lines in treatment with *Hypnea musiformis* extract after 24, 48 and 72 hours of incubation.

Time	CONC (µg/ml)	MCF7			MDA-MB-231			P-value
		Viability %		IC50	Viability %		IC50	
		Mean	SD		Mean	SD		
24 h	200	95.28	2.90	> 1000	96.55	1.95	> 1000	0.51
	400	94.55	2.39		91.19	4.67		0.33
	600	92.59	0.61		77.06	4.34		**0.009**
	1000	77.80	5.81		73.25	3.17		0.14
48 h	200	84.03	5.42	> 1000	93.75	4.41	790.3	0.12
	400	80.81	1.65		85.92	1.42		0.08
	600	76.25	1.17		66.44	8.42		0.09
	1000	51.99	11.51		36.90	13.99		**0.03**
72 h	200	85.11	4.46	826.8	92.24	6.88	634.5	0.60
	400	83.33	2.15		75.47	5.41		**0.02**
	600	65.88	6.32		55.84	7.00		**0.03**
	1000	36.28	8.48		10.93	3.11		**<0.0001**

P-value indicates the significant differences between the groups by t-test analysis. IC50 value of cytotoxic effects was calculated by linear regression.

Figures:

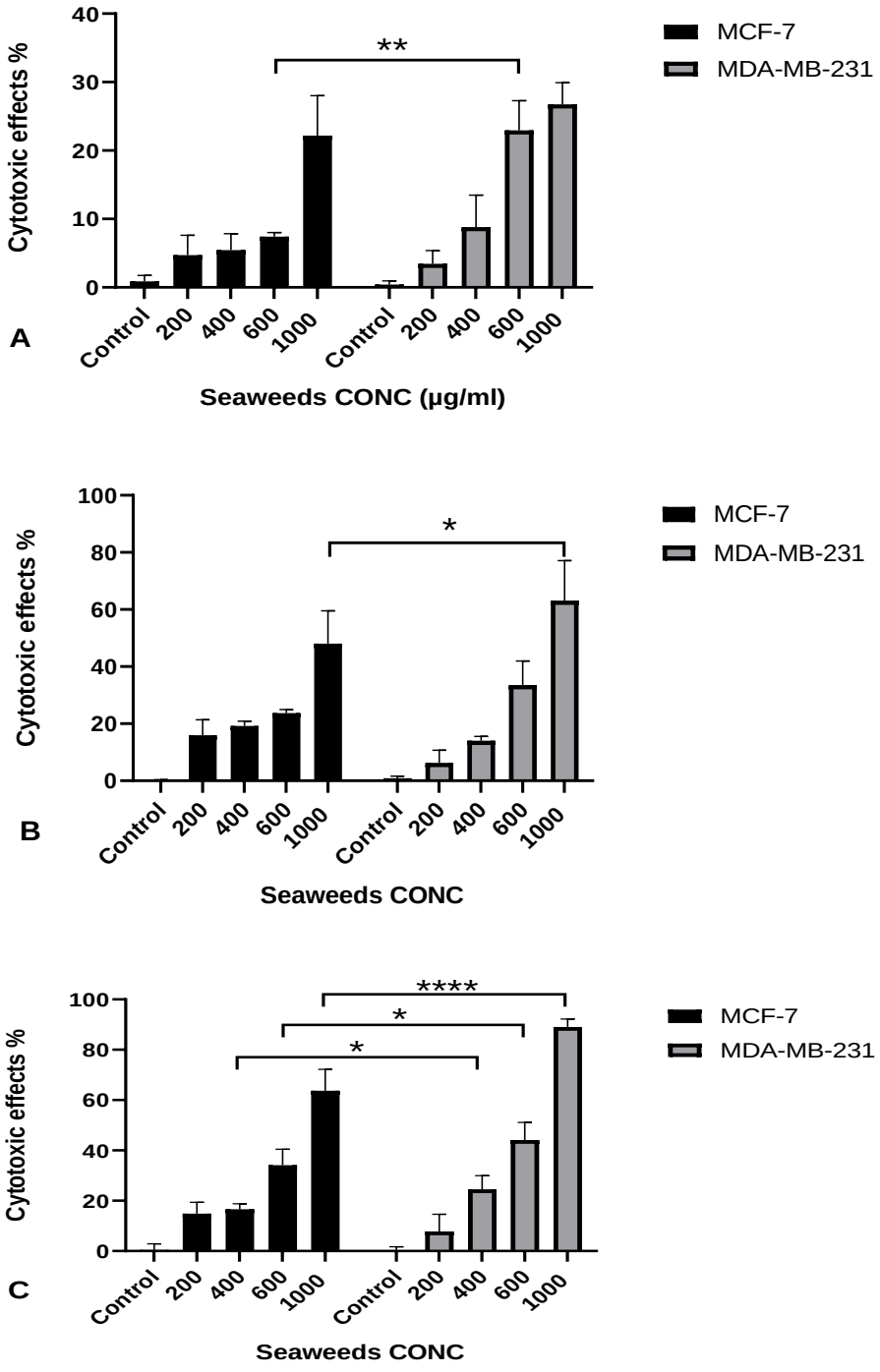

Figure 1. Cytotoxic effects of Hypnea musiformis extract on MCF7 and MDA-MB-231 cell lines after 24 hours (A), 48 hours (B) and 72 hours (C) of incubation. *p<0.05, **p<0.01,***p<0.001 and ****p<0.0001.

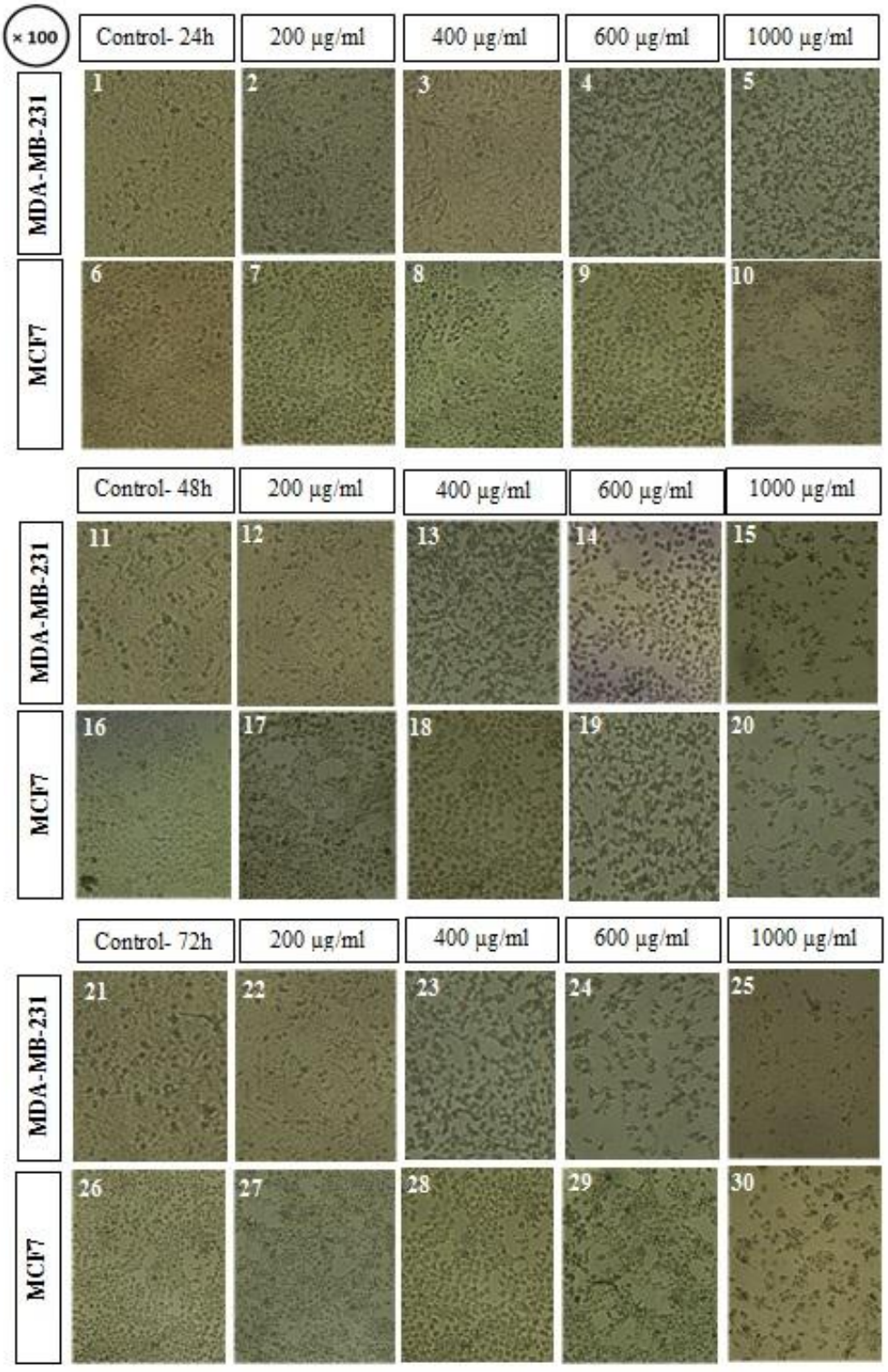

Figure 2. Morphological changes of MDA-MB-231 and MCF7 cell lines in treatment with *Hypnea musiformis* extract after 24 hours (1-10), 48 hours (11-20) and 72 hours (21-30) of incubation.