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# Anticancer Activity of a scarcely investigated Red Sea Brown Alga *Hormophysa cuneiformis* against HL60, A549, HCT116 and B16 Cell Lines

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**ABSTRACT**

Discovering new effective anticancer drugs from natural source have been a field of concern for many researchers. Many researches had reported seaweeds to fill this need. Therefore, this study was performed to screen the anticancer activity of six seaweed species (*Actinotrichiafragilis*, *Cystoseiramyrica*, *Hormophysa cuneiformis*, *Laurencia papillosa*, *Sargassum cinereum*, and *Turbinaria turbinata*) collected from the Red Sea, Hurghada, Egypt. The inhibition of cell viability of the 80 % methanol extract of the six species have been tested on four cancer cell lines (HL60, A549, HCT116 and B16)with MTT assay. The results revealed that *H. cuneiformis* was the best extract in suppressing HL60, A549, HCT116 with IC50 143.9, 40.97 and 92.6 µg/ml respectively, while the IC50 for B16 was higher than 200 µg/ml. In order to study the anticancer mechanism for the extract, apoptotic body formation test and cell cycle analysis were performed using Hoechst 33342 stain and flow cytometer respectively upon HL60, A549, HCT116 cells. The results showed that *H. cuneiformis* extract caused DNA damages and rising in the apoptotic body formations for the three cancer cell lines in a dose-dependent manner. Analysis of cell cycle exposed that *H. cuneiformis* extract could induce cell cycle arrest, which was noticed from increasing the sub-G1 and G2/M phases and decreasing of G0/G1 phase. Thus, the extract suggested anticancer mechanism is through the induction of apoptotic body formation in the cancer cells and arresting in the cell cycle. In conclusion, the extract of *H. cuneiformis* could be a potential anticancer drug that requires *in vivo* study for more confirmation. Furthermore, this study about *H. cuneiformis*

## INTRODUCTION

Nowadays, Cancer has become one of the most concerning diseases that include unusual cell growth with the possibility to invade or spread to different regions of the body. About 90–95% cases of cancers are caused by environmental factors and oxidative stress and the other 5–10% acquired by inherited genetics (Anand *et al*., 2008).The world health organization reported Lung, liver, colorectal, prostate and stomach cancer to be the most common types of killing cancer in men, while in women colorectal, lung, breast,

cervix and thyroid cancer are the most common killing types (WHO, 2018). With regard to cancer cells resistance to antitumor medications; discovery of new effective anticancer drugs with minor harmful effects have been a field of concern for many researchers. There has been an excessive attention to natural compounds gained from marine source to be investigated for their therapeutic possessions. Various algal metabolites have been demonstrated powerful cytotoxicity effect against cancer cells. These metabolites have played a vital role in discovery of new pharmaceutical compounds from algae for anticancer medications (Moussavou *et al*., 2014). Marine algae develop defense and competitive strategies to survive in their environment. These strategies have given rise to the synthesis of significant bio-products from different metabolic pathways. Focusing upon bio-products, recent patterns in drug investigation from natural means suggested that seaweeds are promising for many novel biochemically substances (Burja *et al.*, 2001 and Blunt *et al*., 2005).Numerous investigators have stated that crude seaweeds or their organic extracts possess anti-proliferative activity against human cancer cell lines *in vitro*, along with inhibiting activity for tumors developing in mice *in vivo*. Many reports have informed that compounds extracted from seaweeds such as bromophenols, fucoidan, flavonoid, laminarin, carotene and steroids, may have anticancer activity (Kim *et al*., 2010; Namvar *et al*., 2013; Ryu *et al*. 2013; Moussavou *et al*., 2014). In Red Sea, seaweeds constitute an important component of the reef dwellers (El Shafay *et al*., 2016). They have been the subject of taxonomic works since the 18$^{th}$ century, yet the available information on the seaweed anticancer potentiality in Red Sea is scarce. Therefore this study was conducted to estimate the anticancer activity for some of the abundant seaweed in the Red Sea.

## MATERIALS AND METHODS

### 1. Chemicals and reagents

A549 cell line (human lung carcinoma), HCT 116 cell line (Human colon carcinoma), HL60 (promyelocytic leukemia cells), and B16 cell line (mouse melanoma cells)were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco/ BRL (Burlington, ON, Canada). 3-(4, 5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals and reagents used in this investigation were of analytical grade.

### 2. Sample collection and preparation

Six seaweeds (*Actinotrichia fragilis*, *Cystoseira myrica*, *Hormophysa cuneiformis*, *Laurencia papillosa*, *Sargassum cinereum*, and *Turbinaria turbinata*) were collected from the intertidal and subtidal zones from Hurghada reefs during late December, 2012. Fresh samples were washed in seawater to remove encrusting material, thoroughly washed with fresh water to remove excess salt and air dried in shade then grinded. Each grinded algal sample was mixed with 80% methanol, putted in a shaking incubator at 25$^{o}$C overnight and then the extract was collected. The process was repeated three times or till the methanol extract became clear and it was combined together. The extract were then filtered through Whatman No:4 filter paper and evaporated to dryness under reduced pressure vacuum. The crude extract was dissolved in dimethyl sulfoxide (DMSO) to

prepare a 50 mg/mL stock solution. Stock was diluted to get the desired concentrations using Dulbecco's Phosphate Buffer saline (DPBS).

**3. Evaluation of anticancer activity:**

**3.1. Inhibition of cancer cells proliferation by seaweed extracts against HL60, A549, HCT116 and B16 using MTT assay**

The cell growth inhibitory activity on cancer cell lines were evaluated by the colorimetric MTT assay (Mosmann, 1983) using different concentrations of methanol extracts (50,100,200µg/ml). HL-60 suspended cancer cell line and A549, HCT116 and B16 attached cancer cell lines were seeded in separate 96-well plates at $1\times10^5$cells/ml conc. After 24hs of incubation, the cells were treated with samples at different concentrations and incubated for additional 24hrs. After 24hrs MTT was treated and the plates were incubated for 3hrs. After incubation, the plate of the suspended cells HL-60 was 10min centrifuged at 2000rpm and the supernatant was removed. For the other attached cells, MTT was simply removed. The formazan crystals formed inside different wells were re-suspended using DMSO and the quantity of purple crystals was evaluated by assessing the absorbance at 540nm.

**3.2. Apoptotic body formation observed under a fluorescent microscope using Hoechst 33342 stain**

The nuclear profile of the cells was examined through Hoechst 33342 stain (cell permeable DNA dye), which become motivated via UV light and radiates blue fluorescence at 460 − 490nm. The stain is capable of binding to the DNA preferentially. The stained nuclei reflect a viable cell with healthy DNA. Furthermore, the existence of fragmented DNA and condensed chromatin were visualized with more florescent and considered as apoptosis (Lizard *et al*., 1995). Each cancer cell line was seeded in 24-well plates at a $1 \times 10^5$ cells/ml concentration. The cells were later treated by different concentrations of *H. cuneiformis* extract and incubated for an extra 24 hs. After incubation Hoechst dye was putted into the culture media at a final concentration of 10µg/ml and plates were further kept for 15min at 37˚C. Stained cells were detected under a fluorescent microscope provided with a Cool SNAP-Pro color digital camera in order to determine the apoptotic bodies.

**3.3. Cell cycle analysis using flow cytometer**

In an attempt to survey the proportion for apoptotic cells, a cell cycle analysis was performed for the studied cancer cells. It was performed according to Nicoletti *et al.* (1991). Cancer cells were seeded on 6-well plates with $2 \times 10^5$ cells/ml concentration and treated with *H. cuneiformis* methanol extract at different concentrations. The cells were harvested after 24hs, fixed in1ml of 70% ethyl alc. for 30min at 4˚C, rinsed twice via 2mM EDTA in PBS (spin at 2000 rpm for 5min per each wash), then was incubated in the dark with 1ml propidium iodide (PI) solution composed of 2mM EDTA PBS, 100µg of (PI) and 100µg RNaseA for 30 min at 37˚C. The effect of the extract on the cell cycle was calculated by determination of the modifications occurred in the cell distribution percentage at each cell cycle stage, which was evaluated by histograms produced from the detecting computer program. The FACS flow cytometer was used to perform cell cycle analysis (Becton–Dickinson, SanJose, CA, USA) (Wang *et al.*, 1999).

### 4. Statistical analysis

All the measurements were made in triplicate and all the values were represented as mean ± SD. The statistical analyses were performed using SPSS statistical package (SPSSInc., Version 11.5). Significant differences among treatments were tested by analysis of variance. Least significant differences among means were calculated using Duncan test at the $P \leq 0.05$ probability level.

## RESULTS AND DISCUSSION

Cancer is considered to be one the chief health problems distributed worldwide. According to the world health organization (WHO), cancer is considered as the second death causing globally that estimated with 9.6 million loss in 2018 (WHO, 2018) Several ways have been developed for curing cancer; however the existing treatments have a number of undesired adverse side effects, which may be reduced by natural compounds (Anand *et al*., 2008). Certain seaweeds have been noticed for having anticancer effect in a form of crude or purified compounds from brown and red algae (Namvar *et al*., 2013). In the present study the 80% methanol extract of six algal species with different concentrations were tested for its growth inhibition activity against HL60 (A), A549 (B), HCT116 (C) and B16 (D) cancer cell lines with MTT assay (Fig.1 A, B, C, and D).

Among the tested species, *H. cuneiformis* extract was the highest in cancer growth inhibition dose-dependently compared to the control to reach more than 50 % inhibition at a concentration of 200µg/ml except for B16 that did not reach 50% inhibition. The IC50 value for growth inhibition upon HL60, A549, and HCT116 was approximately 143.9, 40.97 and 92.6µg/ml respectively (Table 1). *A. fragilis* demonstrated good inhibitory activity against HL60 with $IC_{50}$ 153.9 ±2.3 µg/ml, while did not show any activity against the other cancer cells. The rest of the studied species did not show an inhibitory activity on the tested cancer cells. The current results disagreed with Moo-Puc *et al*. (2009), El-Saharty *et al*. (2018), and Rocha *et al*. (2018) that showed a cancer cell inhibition activity for *C. myrica*, *S. cinereum*, and *T. turbinate*. The difference in activity may be due to difference in geographical and environmental factors. Thus, the methanolic extract of *H. cuneiformis* was chosen to define the mechanism of anticancer action against HL60, A549, and HCT116 cancer cell lines.

**Table 1: The 50 % inhibition concentrations ($IC_{50}$) of Cancer cell growth by methanolic extract of *H. cuneiformis* against different tumor cells. Experiment was performed in triplicate and the data are expressed as mean ±SD**

| $IC_{50}$ of *H. cuneiformis* for cancer cells in µg/ml | | | |
|---|---|---|---|
| **HL60** | **A549** | **HCT116** | **B16** |
| **114.4 ± 0.6** | **40.97 ± 2.3** | **92.6± 0.1** | **> 200** |

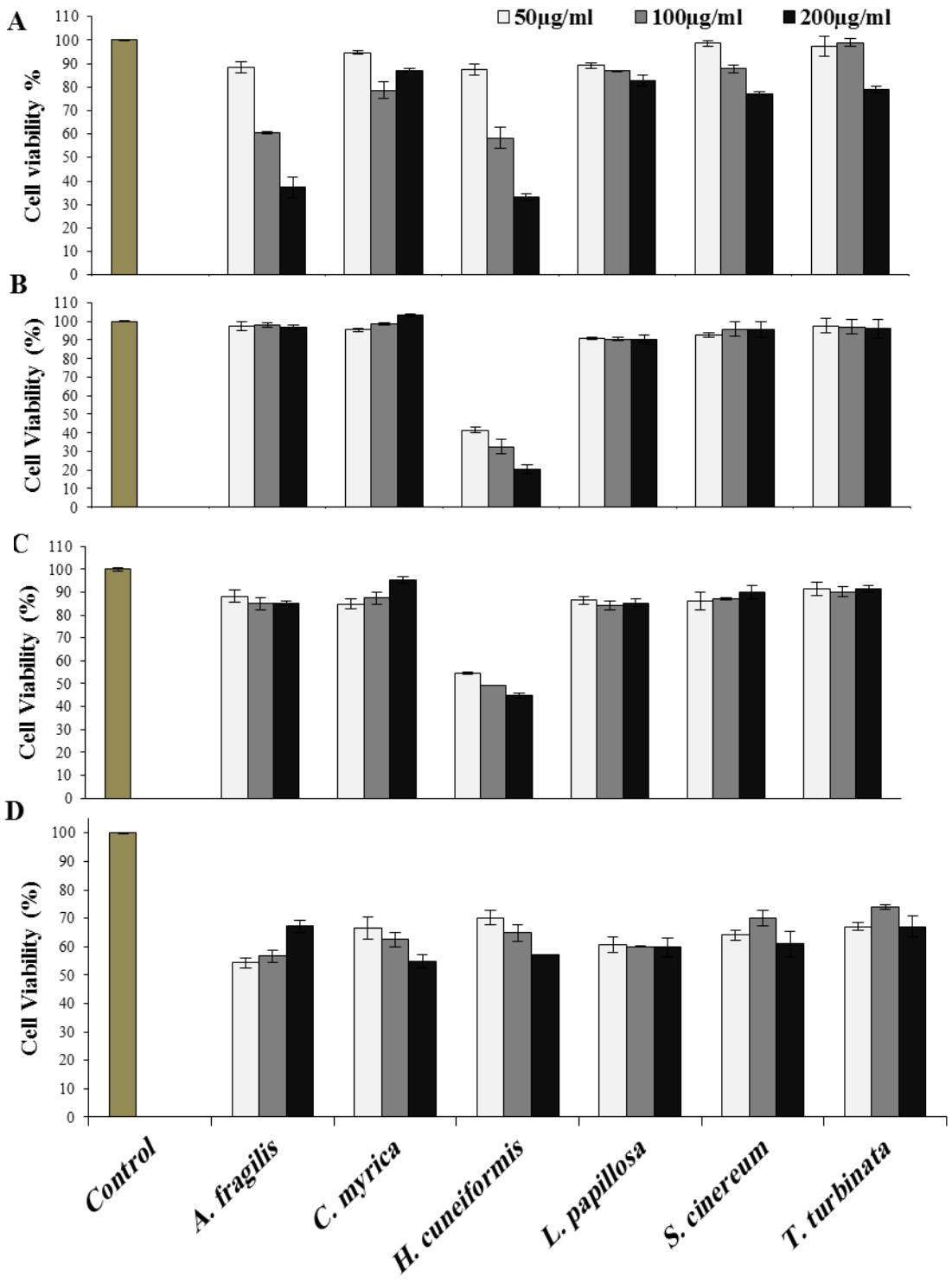



**Fig. 1:** The growth inhibitory effect of the algal methanol extracts at different concentrations on HL60 (A), A549 (B), HCT116 (C) and B16 (D) cell lines. Experiment was performed in triplicate and the data are expressed as mean ±SD.

A possible mechanism of the anticancer activity for *H. cuneiformis* is the induction of programmed cell death (apoptosis), which characterized by cell reduction in size, fragmented DNA, condensed chromatin and apoptotic body formation (Kroemer *et al*., 1995). The methanolic extract of *H. cuneiformis* at 50, 100, and 200µg/ml concentrations was incubated with cancer cells for 24hrs and the cells were stained with Hoechst 33342 staining and the induction of apoptosis in the cancer cells was detected via fluorescent microscopy (Fig.2 A, B, and C).

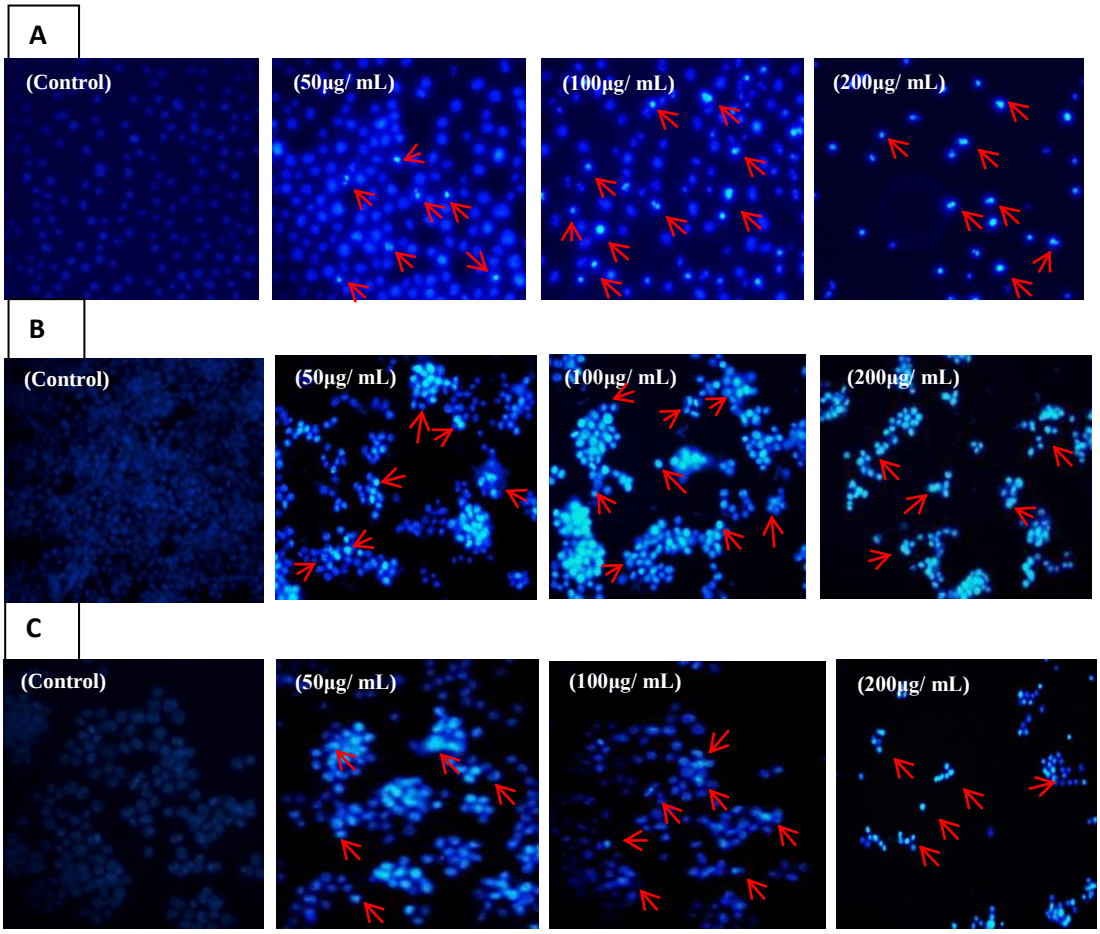



**Fig. 2:** Apoptotic body formation was observed under a fluorescent microscope after Hoechest 33342 staining in HL60 (A), A549 (B) and HCT116 (C) with *H. cuneiformis* ethanol extract treated in different concentrations. Arrows refer to DNA condensation and apoptotic body formation in the cells.

The results indicated that the control represent cell nuclei with healthy DNA. Though, *H. cuneiformis* extract treatments at different conc. displayed a damaged DNA and an elevation in the production of apoptotic bodies within the three cancer cell lines. These results were dose-dependent, which were associated with cell growth inhibition.

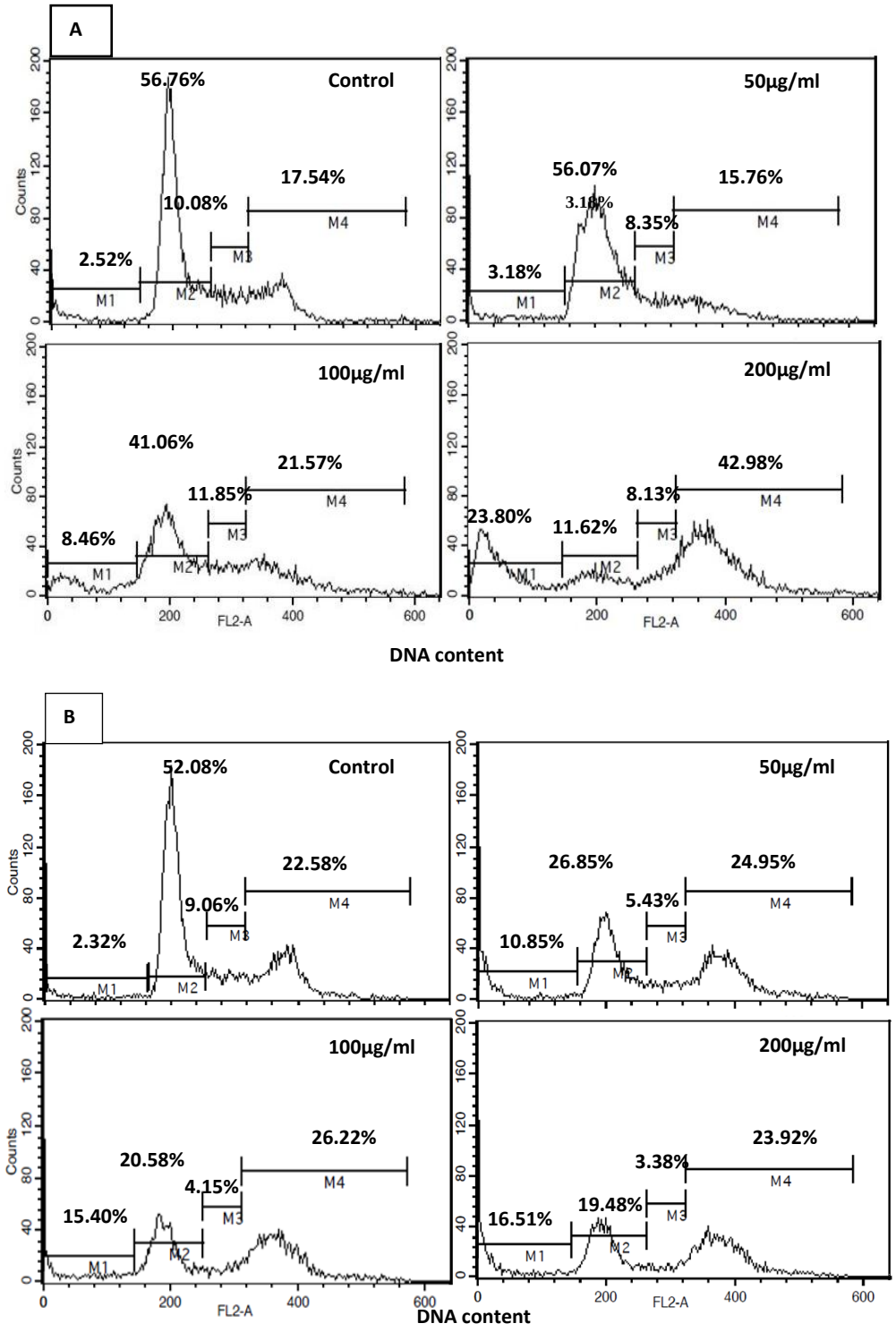
Fluorescence microscopy technique is still one of the finest methods to define apoptosis morphologically (Alabsi *et al*., 2012).

To elucidate whether *H. cuneiformis* possess anticancer effect by only encouraging of apoptosis or that they also induce cell cycle arrest, a cell cycle analysis was performed using flow cytometry. The method is used to distinguish cells depending on their DNA content in different stages in the cell cycle after cell treatment with a fluorescent dye that stains DNA quantitatively. The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate to the DNA content (Prasad and Koch, 2014*).*The quantitative analysis of DNA content of cells during cell cycle is very significant in the study of cell proliferation or cell death (Tao *et al*., 2004). Cell cycle includes several phases (G0, G1, S, G2, and M phase); in which every phase contain different amounts of DNA. The resting stage Gap 0 (G0) phase and Gap 1 (G1) phase has diploid cells (2N). As the cells enter the synthesis (S) phase, it ends up with tetraploid cells (4N). The 4N cells in the G2 phase start preparing for division and entering the mitosis (M) phase. The M phase ends up with two identical diploid (2N) daughter cells, which continue on another division cycle or enter the G0 stage. On the basis of cells DNA content, the M phase cannot be distinguished from the G2 phase and the same for G0 and G1. Therefore, cell cycle is usually divided to three stages based on DNA content G0/G1, S, and G2/M phases. In the present work, a cell cycle analysis was performed to detect the influence of the 80% methanolic extract of *H. cuneiformis* on HL60, A549, and HCT116 cancer cell lines (Fig.3 A, B, and C). According to the results, the treatment with *H. cuneiformis* extract has induced the accumulation of cells in the sub-G1 stage, which reflect an apoptotic body formation that characterized by deficit, fragmentation of DNA and apoptotic body formation. The DNA at sub-G1 becomes less than 2N and the stage can be defined as hypo-diploid (Kajstura *et al*., 2007).

Furthermore, the extract induced arrest of the cell cycle at the $G_2$/M phase. The treatment with the methanolic extract at different concentrations had shifted the population of the three cancer cell lines into the $G_2$/M phase from $G_1$ phase. In HL60, sub-G1 increased from 2.52% in the control to 23.80% at 200 µg/ml and G2/M increased from 17.54% to 42.98%. For A549, the sub-G1 increased from 2.23% in the control to 16.51% at 200 µg/ml and G2/M increased from 22.58% to 23.92%. In HCT116, the sub-G1 increased from 10.44% in the control to 19.64% at 200 µg/ml and G2/M increased from 11.14% to 20.21%. It was also noticed that the S phase was decreased in both A549 and HCT116 in a dose dependent manner. The increase of $G_2$/M phase is considered to be an important checkpoint in the cell cycle in which the cell examines DNA and decides whether going forward with division or not. At G2/M the cells will check if there is any DNA damage and is the DNA had been completely copied during S phase. If there is damage was detected the cells will pause at the G2/M phase in order to repair the damage and complete mitosis, otherwise the cells will undergo apoptosis to eliminate the damage cells that considered an important way to prevent cancer (Kajstura *et al*., 2007).

Thus, according to the results, the methanol extract of *H. cuneiformis* showed an anticancer effect on the studied cancer cell lines through cell cycle arrest at G2/M and the induction of apoptosis. Many marine natural products possess anticancer activity through cell cycle arrest at G2/M and the induction of apoptosis (Prasad and Koch, 2014).Seaweeds reported several compounds with anticancer activity such as fucoidan, polyphenols, flavonoids, laminarin, sterols (Yang *et al*., 2013) Mohamed and Saber

(2019) and Osman *et al*. (2019) considered *H. cuneiformis* to be one of the most untapped brown seaweeds that is why there are scarce results about its composition and bioactivity. For related species *Hormophysa triquetra*, Aravindan *et al*. (2013) showed a similar anticancer behavior and suggested that the activity is due to polyphenols rich extract.



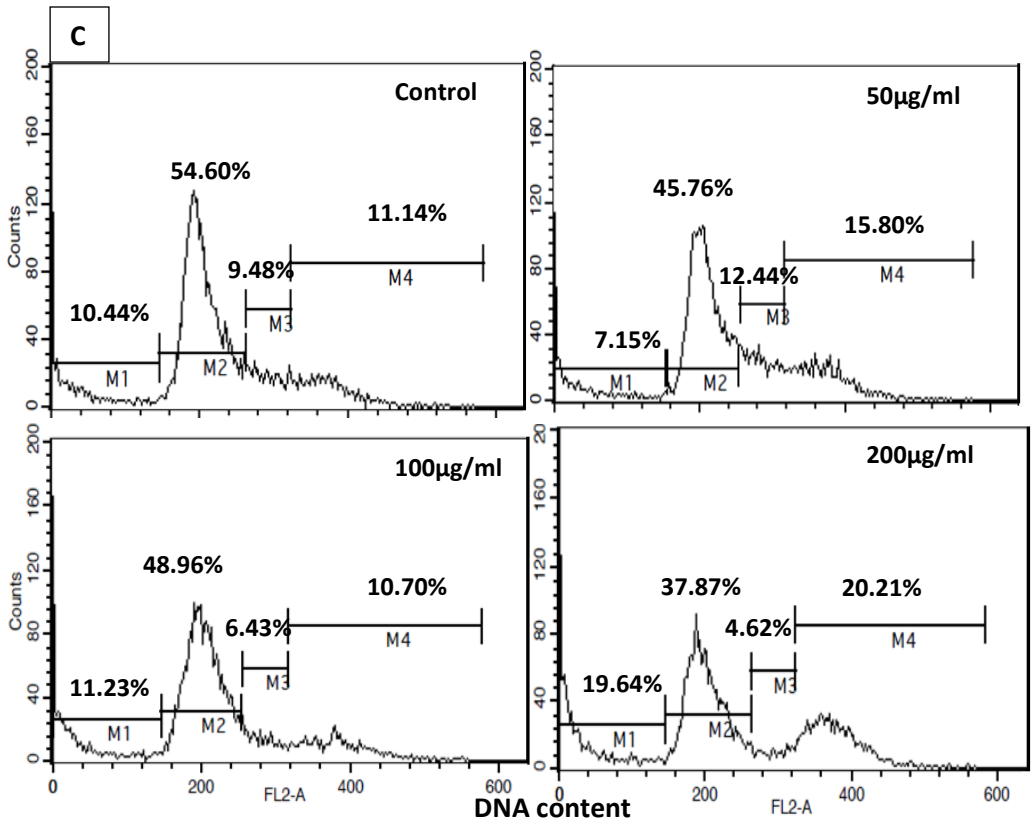



**Fig. 3:** Cell cycle content sensed by flow cytometer for HL60 (A), A549 (B) and HCT116 (C) treated with *H. cuneiformis* methanol extract at different concentrations.M1= Sub G1, M2= G0/G1, M3= S, and M4= G2/M

## CONCLUSION

This study about the anticancer activity of *H. cuneiformis* is considered to be one of the first studies regarding this subject. The study revealed that, *H. cuneiformis* succeeded in suppressing HL60, A549 and HCT116 cancer cell lines in a remarkable way with IC50 less than 200µg/ml. The results of morphology examination for the three cancer cell lines showed that *H. cuneiformis* extract caused DNA damages and raised the the apoptotic body formations in a dose-dependent manner, which were associated with cell growth suppression. Furthermore, the analysis of cell cycle with flow cytometer exposed that *H. cuneiformis* extract anticancer effect was not only through inducing apoptosis but also by induce cell cycle arrest, which was noticed from increasing the sub-G1 and G2/M phases and decreasing of G0/G1 phase. Thus, it could be concluded that the extract anticancer activity was through the induction of apoptotic body formation in the cancer cells and arresting in the cell cycle. Furthermore, the *H. cuneiformis* extract could be used as a potential anticancer drug after checking its bioavailability *in vivo*.

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