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Cymodocea rotundata Methanolic Extract, Red Sea Seagrass, Induces Anti-proliferative and Cell Cycle Arrest in Human Breast Cancer and Liver Cancer *in vitro*

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ABSTRACT

Background: Cancer is a disorder when abnormal body cells proliferate out of control and invade other organ sites.

Objective: Our goal is to study how the marine sea grass ***Cymodocea rotundata* (CR)** suppresses breast and liver cancer growth by inducing cell cycle arrest *in vitro*.

Materials and methods: Using high performance liquid chromatography (HPLC), the phenolic chemicals in CR were identified. The anti-proliferative effects on MCF-7, HepG-2 and normal cell of HSF were evaluated using the MTT test. Using flow cytometry, the cell cycle arrest processes were examined in both cancerous cell lines. Lastly, utilizing quantitative RT-PCR, the expression level of BCL-2, survivin, CDC-2, and CC2D1A and P53 genes was examined.

Results: HepG-2 and MCF-7 cell growth was concentration-dependently inhibited by the seagrass extract, while normal cells HSF was not adversely affected. The S phase cell cycle arrest was indicated by a marked drop in the G0/G1 phase and an increase in S phase cells. A quantitative real-time RT-PCR study for CR seagrass on HepG-2 concluded that CR extract showed a significant decrease in the expression levels of the genes BCL-2, Survivin. Additionally, compared to control cells, it also showed a significant increase in the expression of the CC2D1A, CDC-2 and p53 gene. Moreover, BCL-2, survivin, and CDC-2 expression levels were markedly increased, however CC2D1A and p53 expression levels were markedly decreased in MCF-7. **Conclusion:** CR may prove to be a unique adjuvant in the treatment of liver and breast cancer.

Keywords: Liver cancer, Cytotoxicity, Seagrass, Cell cycle arrest, Breast cancer.

INTRODUCTION

Cancer is a permanent disruption of cellular homeostasis. It is a diverse, complex metabolic illness [(1)]. With approximately 906,000 cases and 830,000 deaths from cancer in 2020, primary liver cancer ranked as the second most common cause of cancer-related deaths as well as the seventh most frequently diagnosed disease. Adverse reactions and cancer recurrence are among the drawbacks of conventional liver cancer treatment, such as trans-arterial chemoembolization (TACE) and sorafenib [(2)]. The most prevalent cancer in women and the main cause of death is breast cancer. In 2022, there were 670 000 global mortalities from breast cancer and 2.4 million new cases of the disease between women. Every country in the globe has females at any age after puberty who develop breast cancer, but the incidence rises with age. Roughly, 0.5–1% of breast cancers occur in men [(3)].

63% of anti-cancer medications came from natural sources [(4)]. Programmed cell death, or apoptosis, is a useful tactic for cancer treatment and prevention. The process of apoptosis is one that is highly impacted by the existence of functional p53. Many anticancer medications target the Bcl-2 protein family because of its important role in the process of apoptosis. The anti-apoptotic properties of Bcl-2 protein inhibit apoptosis and accelerate the growth of breast cancer [(5)]. As a member of the inhibitor of apoptosis protein family (IAP), survivin promotes cancer cell survival and growth [(6)]. It was discovered that CDC-2 controls the G2/M, G0/G1, and S phases [(7)]. The function of CC2D1A, also known as Akt kinase-interacting protein 1 (Aki1), in cancer biology is still not completely understood. Nonetheless, its function as a tumor suppressor is well-established [(8)].

As a result, it is imperative to continually monitor nature for novel drugs with the fewest negative effects. Approximately 71% of the earth's surface is covered in water, with oceans and seas making up 97% of this water [(9)]. The marine environment is home to an extensive variety of species, providing an abundant supply of medicines with distinct physicochemical properties from both terrestrial and aquatic plants. Numerous chemically distinct compounds with a marine origin have been studied and/or created as novel nutraceuticals. One such compound is plitidepsin, which has been shown to be useful in treating a variety of malignancies, including bladder, small cell and non-small cell lung, and melanoma [(10,11)]. Phlorizin from seagrass was isolated and its properties were studied for the first time against hepatocellular cancer in a mouse model. Phlorizin that had been isolated showed promise as an anticancer agent against HepG2 cell lines [(12,13)].

In the present study, we were looking at ***Cymodocea rotundata* (CR)** (Ehrenberg and Hemprich) Asch. (CR, family Cymodoceaceae) for the first time on MCF-7 and HepG-2 cell line. Additionally, we investigated the pro-apoptotic effect of ***Cymodocea rotundata*** (Ehrenberg and

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Hemprich) Asch. (CR) on expression levels of BCL-2, survivin, CDC2, P53 and CC2D1A genes. Metabolite profiling of the alcoholic ***Cymodocea rotundata*** was conducted using HPLC spectroscopic techniques, in order to cover their primary and secondary metabolites as well speculate its relationship to the activity.

MATERIALS AND METHODS

Seagrass material:

In 20 April 2018, ***Cymodocea rotundata*** (Ehrenberg and Hemprich) was obtained from Wadi El Gemal National Park in Egypt, which is located near the Red Bughdadi Torfa. Dr. Amgad ElShaffai meticulously gathered and analyzed the samples. The Wadi El Gemal National Park herbarium now houses the voucher specimens of the grass under specimen number WGAE0011. A portion of the Red Sea coastal plain—approximately 70 km of coastline, including the regions designated for ecotourism development—as well as mountains that roughly stretch from 24°39' 54.35" N in the north to 35°06' 34.68" N in the south are included. The gathering of new plant material in the wild was done while adhering to the SeagrassNet protocol and standards for sample design, identification, and collection of seagrasses. Seawater was used to thoroughly wash the samples and epiphytes were carefully removed. Clean fresh samples were kept for further analysis.

Extraction of *Cymodocea rotundata*

Using an electric blender, a new sample of ***Cymodocea rotundata*** was mixed with Methanol (MeOH), heated for 20 minutes, and then left to soak overnight [14]. after being filtered, the extracts were combined, and the mixture was evaporated at 45°C under low pressure to create a crude MeOH extract. The entire extraction was achieved by repeating this process. The unrefined extract was stored for further analysis.

Qualitative and quantitative HPLC analysis

The HPLC analysis was carried out using an Agilent 1260 series **(Agilent, Santa Clara, USA)**. For the separation, a Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d., 5 µm) was employed. The mobile phase consisted of 0.05% trifluoroacetic acid in acetonitrile (B) and water (A) at a flow rate of 0.9 ml/min. The following was the sequential programming of the mobile phase in a linear gradient: min (82% A); min 0–1 (82% A); min 1–11 (75% A); min 11–18 (60% A); min 18–22 (82% A); min 22–24 (82% A). At 280 nm, the multi-wavelength detector was observed. Five microliters were injected into each of the sample solutions. At 40 °C, the column temperature was kept constant.

Maintaining of cell lines

The American Type Culture Collection (ATCC) provided HSF human skin fibroblast, HepG-2 human hepatocellular carcinoma, MCF-7 human adenocarcinoma breast cancer cells, which were cultured according to the proper methods. Both cell lines were grown at 37°C in a humid incubator augmented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin sulphate in Dulbecco's Modified Eagle Medium (Lonza, Belgium) with 5% CO_2. Following trypsinization with 0.025% trypsin in 0.02% EDTA, the cells were gathered and put through two DPBS washes. When the density approached 80%, the cells were split up for sub-culturing. When the cell growth was in the logarithmic phase, the analyses were performed.

Cytotoxicity assay

Using the MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma-Aldrich Corp, St Louis, MO, USA), the effects of an ***Cymodocea rotundata*** MeOH extract on the proliferation of HepG-2, MCF-7 and HSF normal cell line were investigated [15]. Essentially, the extract was diluted in steps of 0, 25, 50, 100, 200, 400, 800, and 1000 µg/mL on seeded cells in a 96-well plate with a flat bottom for a duration of 48 hours. As a reference cytotoxic agent with 100% inhibition, doxorubicin (Dox, Mr=543.5) was used at doses of 0.37, 0.75, 1.5, 3, and 6 µg/mL. Dimethyl sulfoxide (DMSO) from Sigma-Aldrich Corp. (St Louis, MO, USA) was the solvent used to dissolve the assessed crude extracts, and the final concentration on the cells was less than 0.2%. The density of light (570 nm) was measured by a micro-plate reader and DMSO was utilized as a blank. The formula used to calculate cell viability inhibition (%) was:

$$1- \frac{\text{OD570 treated}}{\text{OD570 untreated}} *100$$

Cell cycle analysis

Trypsinization was used to harvest cells (1×10^5) after they were exposed for 48 hours to crude extracts of **CR** on HepG-2 and MCF-7 cell lines at an IC_{50} values and doxorubicin (10 µM) as a reference drug. The cells were then washed twice with ice-cold PBS at pH 7.4 [16]. Cells were kept for an hour at 4 °C in 60% ethanol. Following two PBS washes (pH 7.4) and fixation, cells were re-suspended in PBS (1 mL) containing 50 µg/mL RNAase A and 10 µg/mL PI (propidium iodide). After 20 minutes of dark incubation at 37°C, the DNA content of the cells was determined by flow cytometry analysis using a FL2 (ex/em 535/617 nm) signal detector (ACEA NovocyteTM flow-cytometer, ACEA Biosciences Inc., San Diego, CA, USA). Ultimately, 12,000 instances had been collected for every sample. ACEA Biosciences Inc., San Diego, CA, USA, computed the cell cycle distribution using Novo ExpressTM software.

Gene expression analysis

Doxorubicin, as a positive control, and **CR** extract at IC_{50} values were used to treat cells. RNA was isolated from treated HepG-2 and MCF-7 cells using Qiazol buffer **(Qiagen, USA)** [17]. The RNA was subsequently cleaned

using an RNA-easy mini Kit **(Qiagen, USA)**. After that, RNA was reverse transcribed using the easy-script first strand cDNA synthesis supermix (Transbiotech, China). A qPCR supermix (China) manufactured by Transbiotech was utilized to measure the copy numbers of β-actin, BCL-2, survivin, CDC-2, and CC2D1A and P53 genes. We used the housekeeping gene beta actin to get the copy counts down to 100,000.

Table 1 contains a primer sequence list (S1). The following were the settings for the RT and subsequent PCR cycling: qPCR: 40 cycles were completed at 94°C for 30 seconds, 94°C for 5 seconds, and 60°C for 30 seconds. RT: 85°C for 5 seconds, 42°C for 10 minutes. To measure gene expression, the MiniOpticonTM Bio-Rad Real Time Thermal Cycler was utilized.

Table 1: Primers used for gene expression analyses.

Gene	Primer forward (5'-3')	Primer reverse (5'-3')
β-actin	CCTTCCTGGGCATGGAG TCCT	GGAGCAATGATCTTGA TCTTC
BCL-2	CCTGGTGGACAACATCG CC	AATCAAACAGAGGCC G CATGC
Survivin	AGGACGGCCCTTCTTGG AGG	CTTTTTATGTTCCTCTA TGGGGTC
CDC2	CAAATATAGTCAGTCTT CAGGATG	CCTGTAGGATTTGGTA TAAATAAC
CC2D1A	GTGGATGTCGCTGAATT GCC	GTGGATGTCGCTGAAT TGCC
P53	AGA GTC TAT AGG CCC ACC CC	GCT CGA CGC TAG GAT CTG AC

Ethical approval

All experimental protocols were approved by the Projects Research Committee at National Research Centre, Cairo, Egypt (Approval number, 12060107, 2019-2022, 19-267).

Statistical analysis

Three individual experiments' means were computed and displayed along with their standard deviation (mean ± SD). All of the data were processed using Graph Prism v5, and the posttest findings of the one-way analysis of variance (ANOVA) were examined using Dunnett's multiple comparison tests. *$P < 0.05$* was used to indicate a statistically significant result for each test.

RESULT

HPLC analysis

HPLC analysis was carried out to establish the bioactive markers in the methanolic extract of **CR.** The chromatography in **figure 1** and **Table 2** indicated the presence of fifteen active constituents out of nineteen standard markers were tested by HPLC analysis**.** The data showed the presence of hydroxybenzoic acids; gallic acid, ellagic acid, methyl gallate and hydroxycinnamic acids; chlorogenic acid, coumaric acid, ferulic acid, vanillic acid, syringic acid, cinnamic acid, and flavonoids; rutin, daidzein, kaempferol, hesperetin and quercetin. Gallic acid was the most abundant biomarkers detected at 3.58 minutes with concentration of 947.34 µg/g of extract.

Table (2): Polyphenol compounds detected (µg/ml) in *Cymodocea rotundata* (Ehrenberg and Hemprich) Asch. extract using HPLC investigation

Authentic samples	Retention time (min)		Concentration (µg/g)
	Phenolic acids		
	Hydroxybenzoic acid		
Gallic acid	**3.58**	+++	947.34
Methyl gallate	**5.48**	-	0.00
Syringic acid	**6.46**	+	26.78
Ellagic acid	**7.47**	+	65.14
Rosmarinic acid	**12.06**	++	161.40
Pyro catechol	**6.61**	-	0.00
Vanillin	**9.06**	+	4.62
	Hydroxycinnamic acid		
Caffeic acid	**5.81**	++	194.09
Cinnamic acid	**19.53**	+	0.98
Ferulic acid	**9.73**	+	6.14
Chlorogenic acid	**4.31**	+	48.50
Coumaric acid	**8.68**	+	27.99
	Flavonoids/ Isoflavone		
Rutin	**7.00**	++	214.22
Naringenin	**10.37**	-	0.00
Catechin	**4.47**	-	0.00
Daidzein	**15.27**	+	4.00
Querecetin	**17.36**	+	41.04
Kaempferol	**20.51**	+	3.24

Hesperetin	**20.83**	+	9.17

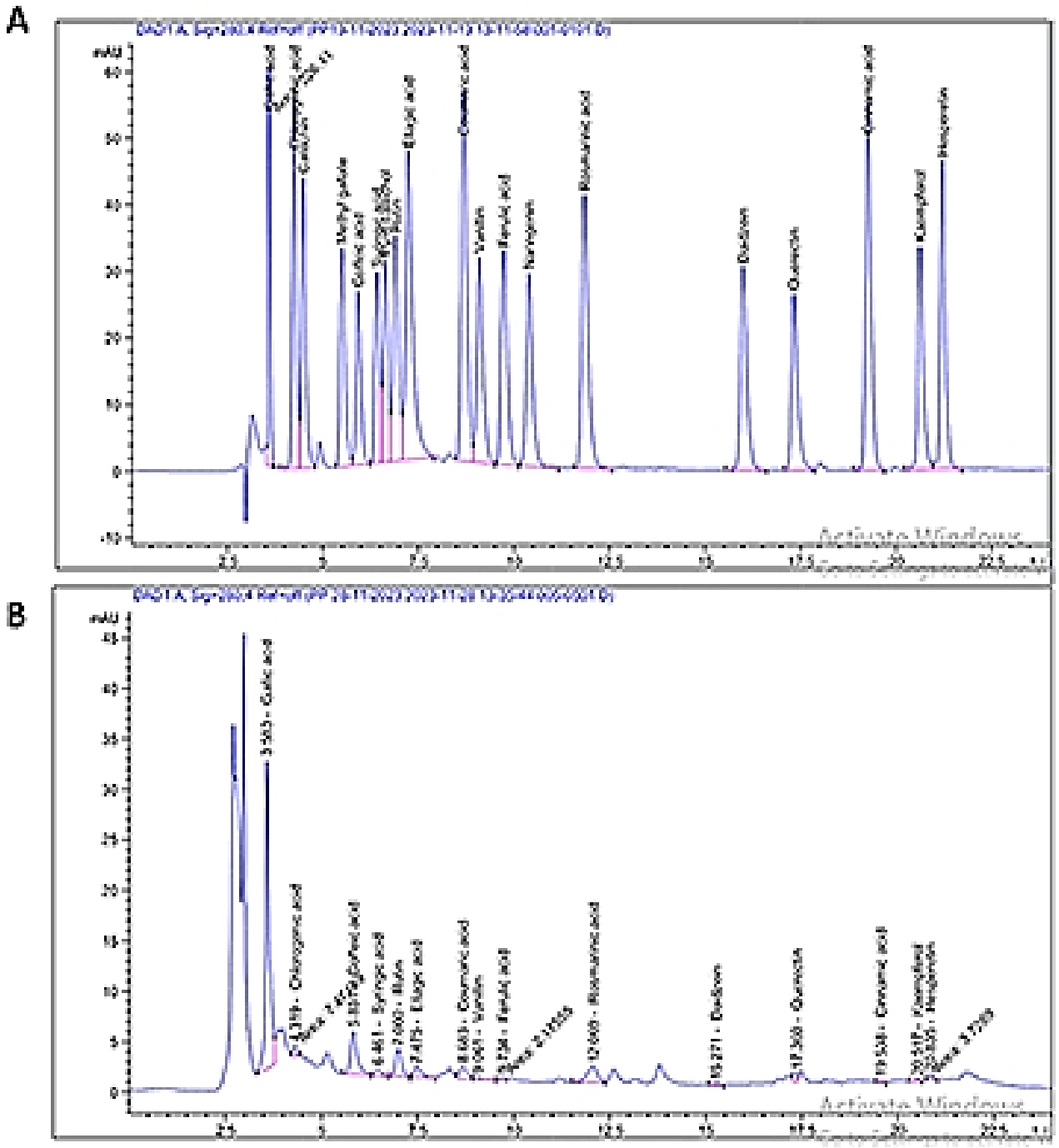

Fig. (1): HPLC chromatographic analysis for (A) Mixture of phenolic standard compounds and (B) Methanolic crude extract of ***Cymodocea rotundata*** (Ehrenberg and Hemprich) Asch.

Anticancer activity against MCF-7, HepG2 and HSF

Cytotoxicity of the MOH crude extract of CR on three different cell lines; MCF-7, HepG-2 and HSF normal cell line. The data in **Fig. (2 a)** show that CR extract induced cytotoxic impact on both cancer cell lines, MCF-7 and HepG-2, dose dependently. The percentage of growth inhibition on HepG-2 and MCF-7 reaches up to 92.76 and 62.1 %, respectively. DMSO, the solvent, had no apparent impact on the viability of HepG-2, MCF-7 and HSF cells after 48 hours. **Table 3** displays the half maximum inhibitory concentration (IC_{50}) values for the **CR** extract and the positive control, doxorubicin, for both treatments **(Fig. 2 b).** The results indicate that **CR** extract did not have any toxicity on normal skin fibroblast cell line showing that it is safe and only possess cytotoxicity against cancerous cell lines.

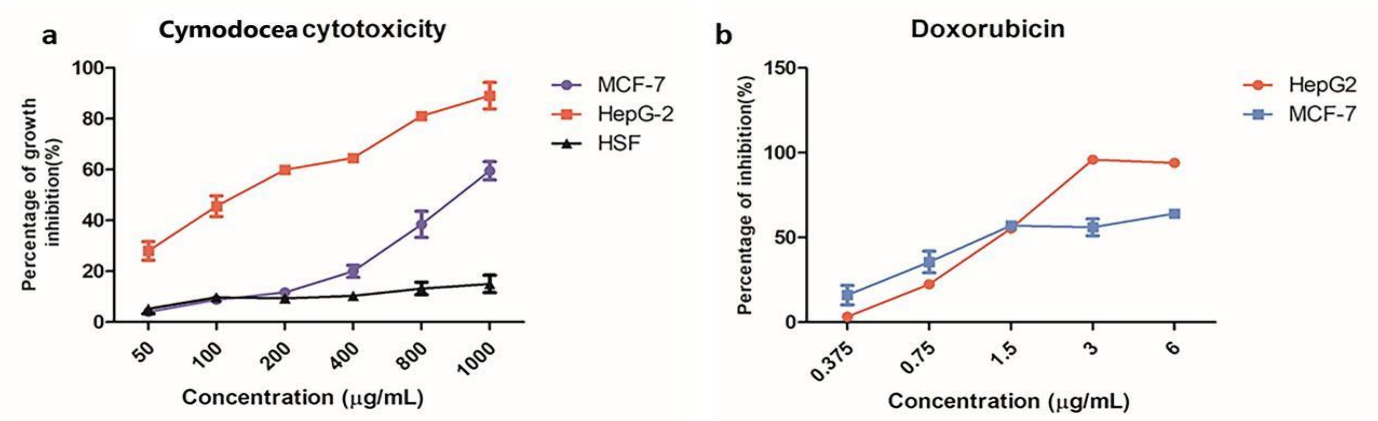

Fig. (2): Cytotoxic effects of (a) ***Cymodocea rotundata*** (Ehrenberg and Hemprich) Asch extract and (b) Doxorubicin on **HepG-2, MCF-7 and HSF** cells at 48 h. The cytotoxic effect significantly increased dose dependently without any significant effect on HSF normal cell.

Table (3): IC $_{50}$ (µg/ml) of *Cymodocea rotundata* and Doxorubicin on HepG-2 and MCF-7 cell lines after treatment for 48 h.

Sample	MCF-7 (IC_{50} (µg/ml)	HepG-2 IC_{50} (µg/ml)
Cymodocea rotundata	883	106.32
Doxorubicin	1.2	0.6

Cell Cycle arrest

The mechanism by which CR crude methanolic impeded the proliferation of HepG2 and MCF-7 cells was established using a cell cycle study. In MCF-7 treated cells, the cells percentage in the G0/G1 phase decreased while in the S phase and G2/M phase the cell percentage significantly increased in response to **CR** treatment, according to the data in **Fig. 3 a**. Following a 48-hour treatment with extract, the proportion of G1 phase cells deceased from 89% to 70%. On the other hand, the percentage of cells in S phase rose from 5.2 % to 11 % and the percentage of G2/M increased from 3.8 to 14.2% indicating that CR induced cell cycle arrest at S phase as well as G1/M phase. On the contrary, CR treated HepG-2 cells showed a significant increase in S phase cell percentage from 7.9 to 14.7% referring to the induction of S phase cell cycle arrest in HepG-2 treated cells.

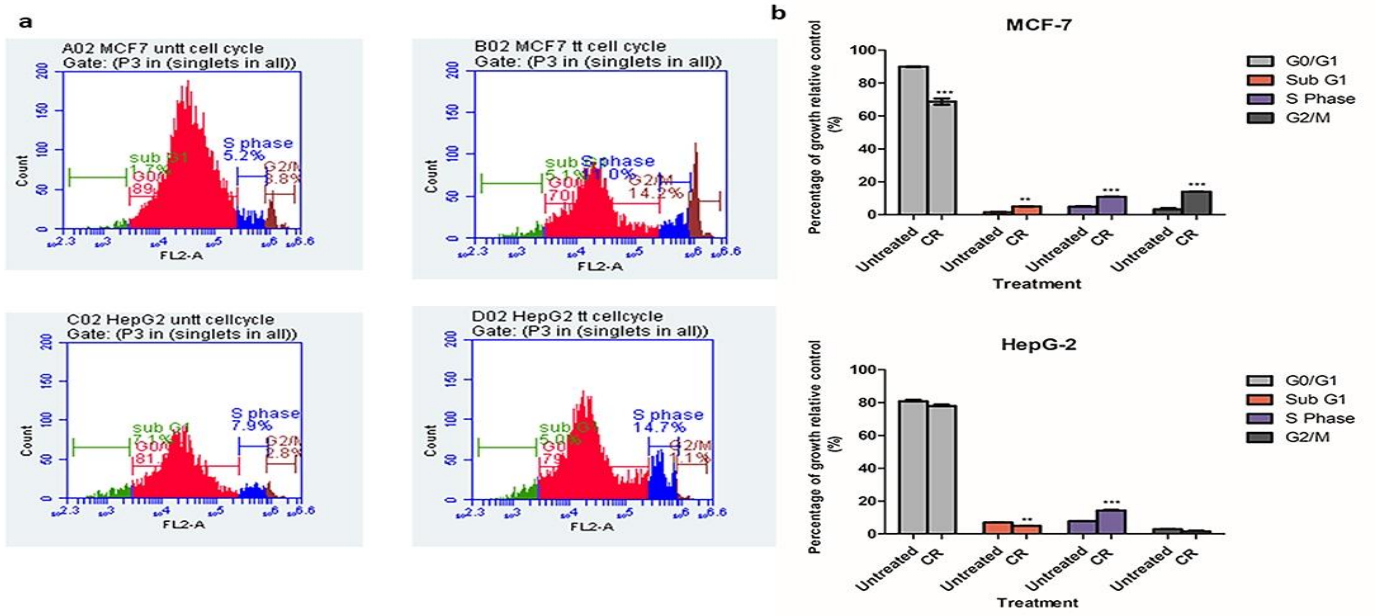

Fig. (3): (a) ***Cymodocea rotundata*** was used to treat the MCF-7 and HepG-2 cell line to investigate cell cycle using flow cytometry analysis. (b) The percentage of cell populations in MCF-7 and HepG-2 treated cells was presented in bar graph and the mean ± SD is used to express the data (%). The percentage of cells in the S phase and G2/M phase cell cycle in the treated sample was significantly higher *(*** P<0.001, ** P<0.01)* than in the negative control group (untreated cells).

Gene expression analysis

The effects of doxorubicin and ***Cymodocea rotundata***, are shown in **Fig. 4 and 5**, on MCF-7 and HepG-2 cell lines, respectively. The expression levels of the BCL-2, survivin, CDC2, CC2D1A, and p53 genes were assessed by comparing them to negative control cells and normalizing their expression to that of β-Actin. Following the treatment with CR methanolic extract, the expression level of BCL-2, survivin, CDC-2 was dramatically elevated while the expression level of CC2D1A and p53 was significantly diminished **Fig. 4.**

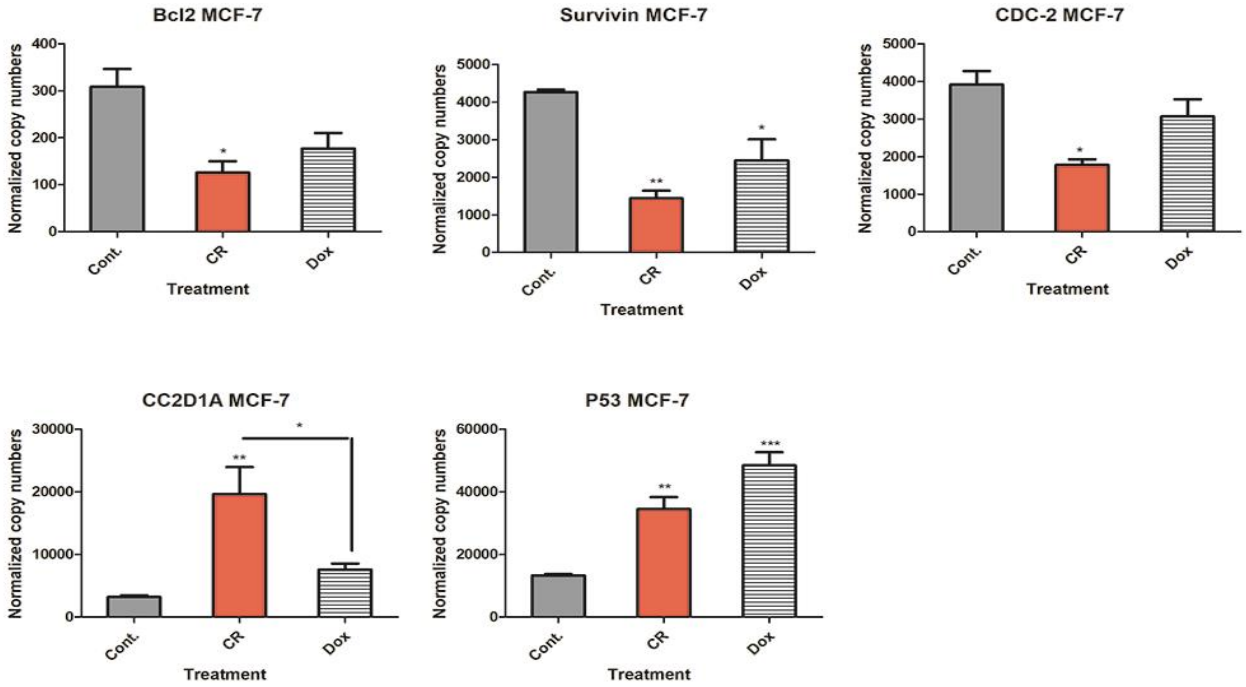

Fig. (4): Analysis of gene expression level (BCL-2, survivin, CDC2, CC2D1A, and p53) in MCF-7 treated with doxorubicin (Dox**)** ***Cymodocea rotundata*** **(CR)**. The mean ± SD was used to express data. ***P<0.01* * *P<0.05,* *** *P<0.001*.

On the other hand, ***Cymodocea rotundata*** and doxorubicin treated HepG-2 cells were used in this investigation. When compared to control cells, CR extract showed a marked decrease in the expression levels of the genes BCL-2, survivin. Additionally, compared to control cells, it also showed a notable elevation in the expression of the CC2D1A, CDC-2 and p53 gene **(Fig. 5).** These results might demonstrate CR extract as potentially useful cell cycle and proliferation regulators. When HepG-2 cells were treated with doxorubicin and ***Cymodocea rotundata***, their levels of CC2D1A gene expression were significantly higher than those of the cells treated as a negative control **(Fig. 5)**.

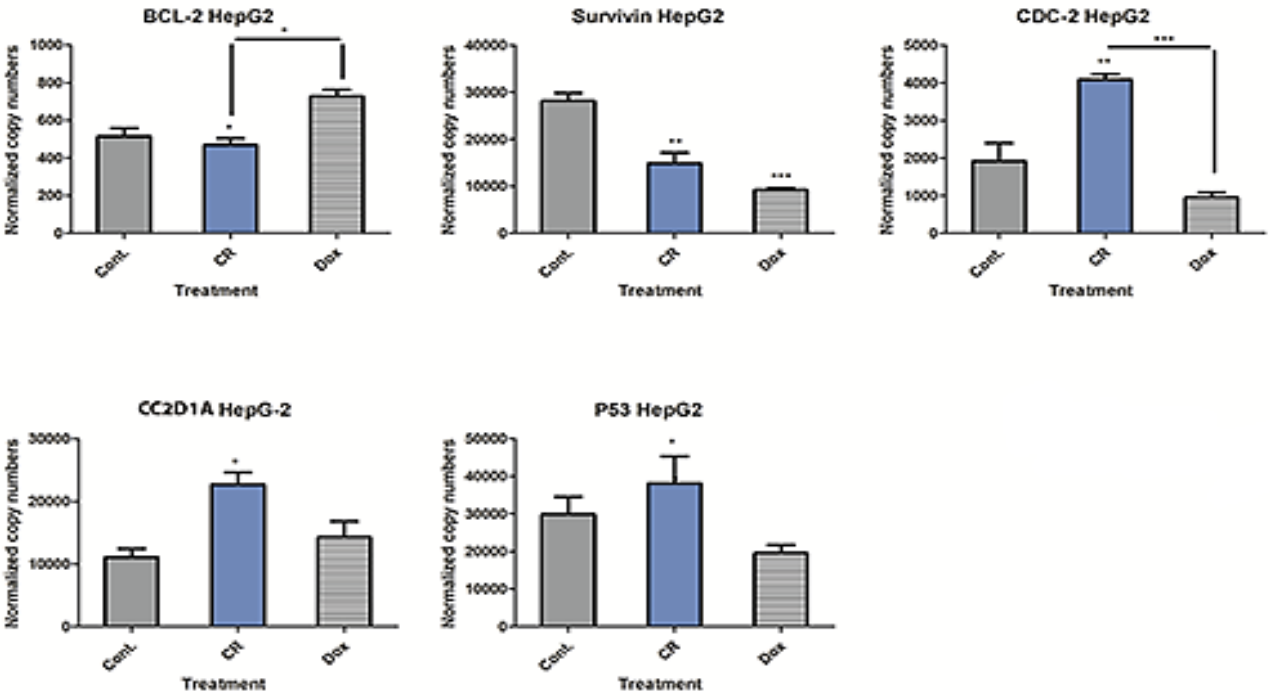

Fig. (5): Analysis of gene expression level (BCL-2, survivin, CDC2, CC2D1A, and p53) in HepG-2 cells administered with doxorubicin (Dox) and ***Cymodocea rotundata***. The mean ± SD is used to express data. * *P<0.05,* ** *P<0.01*.

DISCUSSION

One of the recognized polyphenols found in plants, gallic acid is a potent cancer-prevention antioxidant. Research has demonstrated that GA has applications in the treatment of melanoma, colon, breast, and pancreatic cancers [18,19]. Phenolics demonstrate great potential as cytotoxic anti-cancer drugs that target angiogenesis, growth and differentiation, and metastasis, while promoting apoptosis and reducing proliferation. As a result of their potency as medicinal molecules, phenols are frequently used for treating a wide range of ailments, including cancer, diabetes, cardiovascular, and neurological conditions [20].

Based on the HPLC results, **CR** crude extract was found to be rich in polyphenols. Previous study has shown that Gallic acid can cause apoptosis in HL-60 promyelocytic leukaemia [21], human prostate cancer [22], and esophageal cancer [23]. Gallic acid primary mechanisms of action against cancer are inhibition of cellular growth, induction of production of reactive oxygen species (ROS), and cell cycle arrest in the G2/M phase [24]. Fruits, coffee, and vegetables all contain caffeic acid, which has potential antibacterial, anticancer, anti-inflammatory, and antioxidant properties [25]. Its anticancer effect has been attributed to mechanisms such as upregulating the expression of p53 and p21 genes and downregulating the expression of CDK2, which can result in G0/G1 arrest in the cell cycle [26].

On the other hand, rutin is a particular type of flavonoid antioxidant which is mostly present in fruits, vegetables, grains, and a variety of other plant-based foods used by humans [27]. Additionally, previous study has shown that rutin, when combined with medication, can inhibit the growth of several cancer cells by initiating apoptosis and arresting the cell cycle [28].

Our results indicate that extracts CR catalyze both G2/M and S stage cell cycle arrest. The result was in line with the extract's chemical profile, suggesting that the mechanism by which sea grass extract induces anti-proliferative effect is the cell cycle arrest [29]. It is generally known, according to **Yahya *et al.*** [30], that breast cancer MCF-7 cell lines express higher levels of BCL-2, survivin, and CDC2, whereas lower expression levels of P53 genes are present. Furthermore, an elevation in the Bax/Bcl-2 ratio triggers apoptotic processes, such as the activation of caspase3/9 and the ensuing breakdown of intracellular materials. In Hela cells, it was discovered that the knockdown of the survivin protein enhanced cell cycle arrest and suppressed proliferation.

Anticancer drugs can cause G2/M arrest in cancer cells by reducing the levels of the protein Cdc2, as Cdc2 is linked to the progression of the cell cycle from the G2 to the M phase [31]. A significant objective in the treatment of cancer is to prevent this phase. However, in contrast to control cells, ***Cymodocea rotundata*** exhibited an important increase in P53, which is thought to be a crucial transcription factor and tumour suppressor. The cellular

response to DNA damage, cell signal transduction, genomic integrity, apoptosis, and cell cycle arrest are just a few of the numerous biological processes in which P53 is involved. Furthermore, it triggers apoptosis by activating Bax transcription and p21WAF1 [32].

It was revealed that CC2D1A functions as a scaffold protein in the PI3K–PDK1–Akt pathway. Its significance in the biology of cancer is strongly disputed. It has been observed that upon activation by EGFR, CC2D1A forms a complex with EGFR and Akt, which aids in cell survival and proliferation. The silencing of CC2D1A in mouse models of diffuse malignant mesothelioma inhibited the growth of the tumour [33]. According to **Nakamura *et al.*** [34], the CDC2 protein complex is one of the kinases that phosphorylates Aki1 during mitosis. Recently, nevertheless, **Reiff *et al.*** [35] reported that the tumour suppressor gene CC2D1A functions through Notch signaling.

Conclusion

According to the results of our investigation, ***Cymodocea rotundata*** may represent a novel class of adjuvant for treatment of liver and breast cancer because it can cause anti-proliferative and cell cycle arrest in cancer cells without harming healthy cells. This cytotoxic effect was confirmed via studying of the apoptotic related gene expression in the treated cell lines. To confirm its efficacy and safety in trials involving animal and humans, more research in future is necessary.

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- **Conflicts of interest:** There are no conflicts of interest.

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