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RESEARCH ARTICLE

Inhibitory effect of *Sargassum latifolium* extract on hypoxia pathway in colon cancer cells

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ABSTRACT

Sargassum latifolium, (Turner) C. Agarth, 1820, is an edible brown alga that was collected from red seashores in Egypt. Colon cancer is a lethal disease world-wide. Hypoxia is a key player in progressive colon tumor growth and stemness. This work was planned to extract water-soluble polysaccharide from *S. latifolium*, to separate its fractions (SL1, SL2, SL3, and SL4) and hence to investigate their anti-hypoxia characteristics in colon cancer HCT-116 cells. Algal fractions cytotoxicity was assayed by MTT; DNA staining was used to analyze apoptosis and necrosis; total hypoxia status was assessed by pimonidazole, HIF-1α and HIF-1β were estimated by ELISA, and hsa-miRNA-21-5p and hsa-miRNA-210-3p were analyzed by qPCR. The results indicated that SL1 and SL4 are cytotoxic agents against HCT-116 cells through enhancing apoptosis. SL1and SL4 were potent inhibitor of total cell hypoxia ($p < 0.001$). Both fractions significantly suppressed the expression of miR-21 ($p < 0.01$) and miR-210 ($p < 0.001$), and the concentration of HIF-1α protein ($p < 0.01$ and $p < 0.001$, respectively), while only SL1 inhibited HIF-1β protein concentration ($p < 0.05$). Taken together *S. latifolium* polysaccharide extract fractions SL1 and SL4 exhibited anti-hypoxic property in HCT-116 cells through mechanistic role in the expression of hypoxia regulators miRNA-21 and miRNA-210, and accordingly in HIF-1α and HIF-1β biosynthesis.

Keywords: *Sargassum latifolium*; colon cancer HCT-116 cells; hypoxia; HIF-1α, HIF-1β, miRNA-21; miRNA-210

INTRODUCTION

Hypoxia triggers the pathophysiology of a variety of human disorders including cancer, chronic lung disease, and ischemic cardiovascular disease (Semenza, 2003). In tumor microenvironment (TME), irregular rapid cell growth limits the availability of oxygen and hence results in a hypoxia status in all the solid tumors including colon cancers (Shao et al., 2018). In this status, a dramatic inhibition in the oxygen level (2–9%) into a hypoxic level (<2%) arises. The tumor cell adapted itself to hypoxia status into an aggressive and therapy-resistant phenotype. Hypoxia evokes modified gene expression and proteomics, which in turn disturbs the physiological functions of tumor cells including slow cell division in hypoxic regions to escape anti-cancer drugs that are targeting the rapidly dividing cells (Roma-Rodrigues et al., 2019; Birner et al., 2000). In hypoxic regions of TME, oxygen gradients stimulate the plasticity and heterogeneity of tumors towards metastatic and aggressive phenotype. The main player in hypoxia status is the overexpressed hypoxia-inducible factor 1α (HIF-1α), as a member of HIFs family that executed cellular hypoxia-associated consequences (Huang et al., 2017; McKeown, 2014). As a recent therapeutic approach, hypoxia inhibition, especially HIF-1α inhibitors, is confirmed as a strategy treat solid cancers via targeting and controlling the hypoxia-stimulated cell-resistance (Jing et al., 2019).

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Seaweeds are macroalgae that occur in shallow marine shores. They are predominant nutritious plants worldwide, due to their low-fat content and their enrichment bioactive constituents (*e.g.,* sulphated polysaccharides, laminarin, terpenoids, mannitol, vitamins, fucoidan and alginic acid) (Kim et al., 1997). Besides being traditional food, they are used in food industry as a gelling and thickening factor (Jimenez-Escrig and Sanchez-Muniz, 2000; Nagai and Yukimoto, 2003). Seaweeds are widely participated in the folk medicine recipes for cancer, gallstone, arteriosclerosis, renal trouble, ulcers, heart disease, and asthma (Burtin, 2003). Brown algae are a seaweeds-family that characterized by high content of cell-wall polysaccharides (Kumar and jain, 2014; Suryaningrum and Samsudin. 2020), which provides a battery of medical applications including antitumor, antiangiogenic, anticoagulant, antiviral, antioxidant, antimetastatic, antiinvasive and antiproliferative activities (reviewed in Asker et al., 2007). As an edible safe additive, brown algae are extensively used as food supplement (Burtin, 2003; Kuda et al., 2005). One of the brown Algae, the tropical and sub-tropical family sargassum are wide-spread and it is comprised of >150 species (Dar et al., 2007). Many sargassum algae afford a plenty of polysaccharides that showed active biological functions (*e.g.*, *S. fulvellum*, *S. stenophyllum*, *S. latifolium, and S. horner*; reviewed in Mayer et al, 2007). For example, the polysaccharide extract of *S. dentifolium* was found to enhance apoptosis and histone acetylation. The extract fractions showed significant antioxidant, anti-mutagenic, and anti-genotoxic activities (Gamal-Eldeen et al., 2013).

In previous work, Gamal-Eldeen et al.; (2009) described that polysaccharide fractions of *S. latifolium* showed strong tumor anti-initiating and tumor anti-promoting activities. Similarly, *S. asperifolium* polysaccharide extracts exhibited anti-progression function in leukemia, in addition to anti-inflammatory activity and anti-progression effect in HepG2 cells (Raafat et al., 2014). It had been reported that *S. latifolium* extract showed antioxidant activity that inhibited the environmental heat stress-induced toxicity (Ellamie et al., 2020), but to our knowledge, there are lack of published studies that investigating the effect of *S. latifolium* extract of hypoxia. In continuation of this work, the objective of this study is to explore the hypoxia-inhibitory activity of *S. latifolium* polysaccharide-extracts fractions in colon cancer cells, as a promising approach to inhibit hypoxia-induced drug resistance.

MATERIALS AND METHODS

Extraction *S. latifolium* polysaccharides

S. latifolium was collected in November 2018, from Hurghada city, Red Sea governorate, Egypt. After many washes by distilled water, alga was dried and grounded in electric mill and sieved (2 mm mesh). Algal water-soluble polysaccharides were extracted using the protocol of Zhuang *et al.*; (1995), with some alterations (Gamal-Eldeen et al., 2009), as demonstrated in the scheme in Fig.1. The powdered algal fractions SL1, SL2, SL3, and SL4 were submitted for in vitro investigations in HCT-116 cells to explore their anti-hypoxic properties.

Cell culture

Human colon carcinoma HCT-116 cells (ATCC, VA, USA) were cultured in supplemented Mc Coy's medium. Extract

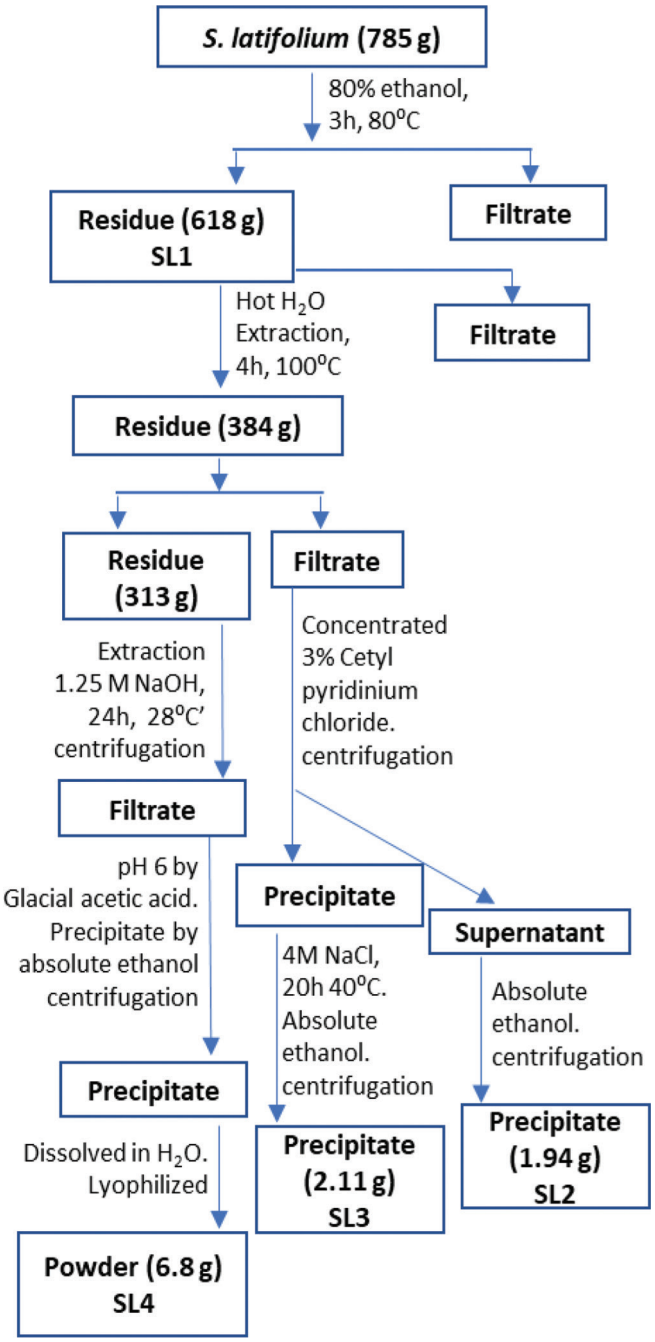

Fig 1. A scheme for *S. latifolium* extraction and fractionation process.

dilutions were endotoxin-free using Pyrogent® Ultra gel clot assay. The results of all experiments (n=8) were presented as (mean ± S.D.). The materials for cell culture and chemicals were obtained from Sigma–Aldrich (VA, USA).

Cell viability and mode of cell death

The viability of HCT-116 cells was examined using metabolic cytotoxicity MTT assay (Hansen et al. 1989), after 48 h of HCT-116 cells co-culture with *S. latifolium* fractions. The results were presented as % of control cells; mean ± standard deviation and the half-maximal inhibitory concentration (IC_{50}) was calculated. The mode of cell death was explored in HCT-116 cells after being co-cultured with IC_{50} of extract fractions, or celastrol for 48 h, and then DNA dual staining with ethidium bromide (EB)/acridine orange (AO) (100 μg/ml; V/V in PBS) (Baskić et al., 2006).

Monitoring of hypoxia in HCT-116 cells

Tracing the alterations in the hypoxia status of HCT-116 cells in absence and presence of 30% of IC_{50} of extract fractions for 6, 12, 24 and 48 h was assayed by pimonidazole, a hypoxia sensitive reagent by microplate fluorometer.

Estimation of HIF-1α and HIF-1β proteins

HCT-116 cells (5×10^4 cells per well) were co-cultured with 30% of IC_{50} of fractions for 48h. Human HIF-1α ELISA Fluorescent Kit (#ab229433; Abcam, Germany) and Human ARNT/HIF-1 beta Colorimetric ELISA Kit (#LS-F9594; LifeSpan Biosciences, USA) were used to estimate HIF-1α and HIF-1β proteins, respectively, in the cell lysates.

Estimation of miR-210 and miR-21 expression

HCT-116 cells (1×10^6 cells) were co-cultured 30% of IC_{50} of the extract fractions for 48 h. The relative miRNA expression was calculated via ΔΔCt method (Livak and Schmittgen 2001), and normalized to U6 expression of control cells. The following kits were purchased from Qiagen, Germany and used: miRNeasy RNA extraction kit (#217004, Qiagen, Germany), miScript RT II kit (#218161), miScript Sybr green PCR kit (#218073), hsa-miR-21 primer (#MS00009079), hsa-miR-210 primer (#MS00003801), and U6 (#600750).

Statistical analysis

The results were presented as mean ± SD; n=8. Graphpad Prism software V6 was used. Data were analyzed with one-way analysis of variance (ANOVA)/Dunnett's multiple comparisons test. $p < 0.05$ was significant.

RESULTS AND DISCUSSION

Colon cancer is a major diagnosed cancer in men and women and it is the third leading deaths cause in cancerous patients (Vadde et al., 2017). Hypoxia is participated in proliferation, apoptosis, and differentiation, and it is also fundamental in developing aggressive tumor phenotype (Vaupel and Mayer, 2007). Due to the key role of hypoxia in drug resistance, cell death resistance, invasiveness, angiogenesis, metastasis, genome instability, metabolism alteration and hypoxia may be considered as the potential therapeutic target (Wilson and Hay, 2011). Recent studies indicated that hypoxia is a key player in progressive colon tumor growth and stemness (Nagaraju et al., 2015). Therefore, recent therapeutic strategies for colon cancer are focusing on finding new inhibitors of HIFs (Yu et al, 2017), since HIFs play a crucial role in the maintenance of stemness in colon cancer via regulating genes and transcription factors intricate the differentiation processes and self-renewal of colorectal cancer stem cells (Santoyo-Ramos et al., 2014).

The cytotoxic effect, of *S. latifolium* extract fractions, was explored in colon cancer cells, in comparison with celastrol, anticancer drug, after co-culture with HCT-116 cells for 48 h. The results indicated that the fractions resulted in variable cytotoxic profiles, where they depressed the cell viability with the calculated IC_{50} of 62.59, 110.20, 162.79 and 51.15 μg/ml for SL1, SL2, SL3, and SL4, respectively, (Fig. 2a). Celastrol-treated cells showed a concentration-dependent cytotoxicity with IC_{50} 5.93 μg/ml. Dual DNA-staining by AO/EB was carried out to detect cell death mode in HCT-116 cells. After 48h of cell-coculture with IC_{50} of SL1 and SL4, the most cytotoxic fractions, the overall cells were harvested and assayed. The results revealed that the treatment with celastrol led to dramatic elevation in the population of early apoptotic cells (36.67%; $p < 0.001$) and late apoptotic/necrotic cells (13.01%; $p < 0.05$), compared to the control 6.64% and 3.71%, respectively (Fig. 2b). Whereas, in fractions-treated cells, a remarkable increment in the early apoptotic cells war detected in SL4-treated cells (37.92%; $p < 0.001$) while in SL1-treated cells there were high percentages of both early apoptotic cells (21.22%; $p < 0.001$) and late apoptotic/necrotic cells (20.77%; $p < 0.001$, respectively), as shown in Fig. 2b.

In normoxic status, ubiquitin-dependent machinery by O_2-dependent prolyl hydroxylation targets α-subunit and degrades HIF-1α (Mylonis et al. 2019). Subsequently, HIF-1α remains in low concentration that averts the development of transcriptional functional complex (HIF-1α/HIF-1β). Whereas, in severe hypoxic status, HIF-1α subunit is the active part of this complex, HIF-1α concentration is quickly increased after degradation (Mylonis et al. 2019). HIF-1α accumulates in cytoplasm before it heterodimerize with β subunit in the nucleus and binds hypoxia-response elements of targeted genes,

a process that stimulates expression of hypoxia-regulated target genes (Semenza, 2003).

The time-dependent alterations in the hypoxia status before and after treatment with *S. latifolium* extract fractions were explored by pimonidazole. The adducts fluorescence intensity was qualitatively estimated by microplate fluorescence reader, to assess hypoxia status. Cells were seeded with 30% of IC_{50} of *S. latifolium* extract fractions for 6, 12, 24, and 48 h. The experiment indicated that SL3 treatment resulted in a non-significant decrease in cell hypoxia ($p > 0.05$), while SL2 was a mild inhibitor of hypoxia at 48h interval ($p < 0.05$), Fig. 3a. In the other hand, the treatment with SL1 and SL4 led to dramatic time-dependent inhibition in the cell hypoxia, especially after 24 h and 48 h ($p < 0.001$), Fig. 3a, where the high maximal inhibitory time for hypoxia (t_{50}) for SL1 and SL4 was 19.25 h and 24.61 h, respectively, whereas t_{50} for SL2 and SL3 was 51.14 h and 98.15 h, respectively (Fig. 3a).

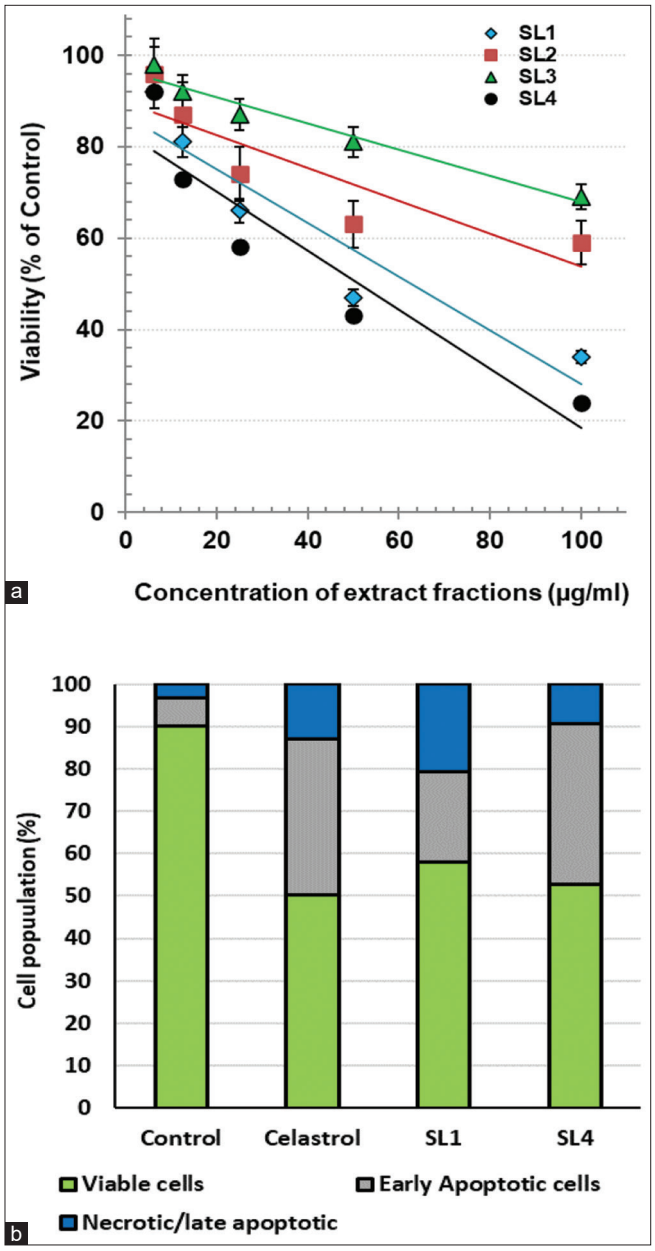

Fig 2. a. The viability of HCT-116 cells after 48 h of incubation with *S. latifolium* extract fractions (SL1, SL2, SL3, and SL4), as estimated by MTT assay. The viability is presented as % of control (mean ±SD; n=8). b. A segmented-bar histogram for cell population distribution of cell death mode in HCT-116 cells, assayed by EB/AO DNA staining, after being treated with IC_{50} of celastrol or *S. dentifolium* fractions for 48 h, compared to control.

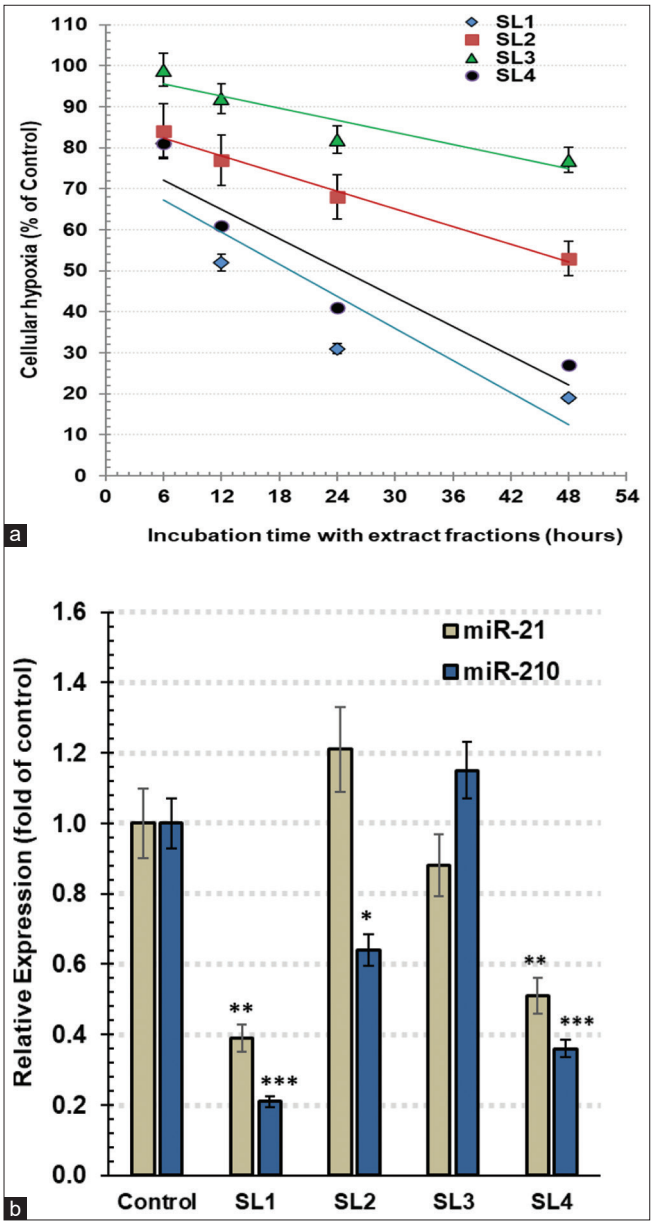

Fig 3. a. Total hypoxia status in HCT-116 cells as assessed by pimonidazole. Cells were treated with 30% of IC_{50} of each fraction for 6, 12, 24, and 48 h (% of control; mean ± SD; n=6), where control was 1284 IFU. b. Relative expression of hsa-miRNA-21-5p and hsa-miRNA-210-3p in HCT-116 cells (mean ± SD; n=6), after treatment for 48 h with 30% of IC_{50} of extract fractions. *$p < 0.05$, **$p < 0.01$, and *** p< 001 compared with the control.

miRNAs are the main players in the upregulation/downregulation of genes that are responsible for balancing the cell proliferation/apoptosis (Wen et al., 2020). Impaired miRNAs expression accelerates of carcinogenesis and cancer progression (Wen et al., 2020). miRNAs act as oncogenes (OncomiRs) or tumor suppressors in carcinogenesis, including colon cancer (Varol et al., 2011). Remarkably expressed OncomiRs enhance neoplastic initiation and tumor progression, while tumor suppressive miRNAs regularly block the tumor development (Varol et al., 2011). miR-210 expression is crucial responder in endothelial cells to the hypoxia status, which in turn affects cell survival, differentiation, and migration (Fasanaro et al., 2008). The effect of *S. latifolium* extract fractions, on the epigenic hypoxia regulators, was investigated via real time qPCR to detect the expression level of miR-210 and miR-21. As a principal hypoxamiR and oncomiR, high miR-210 levels contribute in HIF- 1α stabilization during hypoxia (Puisségur et al., 2011), therefore, we estimated its expression level after the treatment of HCT-116 cells with (30% of IC_{50} of each fraction), for 48 h and found that miR-210 expression was significantly suppressed by SL1 and SL4 reaching 0.21 ± 0.06 ($p < 0.001$) and 0.36 ± 0.04 ($p < 0.001$) fold of the control cells, respectively (Fig. 3b).

In most cancers, miR-21 is overexpressed and plays variable oncogenic functions in colon cancer among other cancers (Selcuklu et al. 2009), additionally, miR-21 is positively-correlated with inhibited apoptosis (Li et al. 2009). In the current study, there was a dramatic suppression in miR-21 expression after SL1 and SL4 treatments down to 0.39 ± 0.05 ($p < 0.01$) and 0.51 ± 0.08 ($p < 0.01$) fold of the control cells, respectively (Fig. 3b). These results suggested SL1 and SL4 as potential inhibitors of hypoxia and its regulators.

In hypoxia status, HIF-1α disturbance contributed to tumor pathophysiology, including angiogenesis, tumor invasion, motility, apoptosis, cellular metabolism, and cell survival, while HIF-1β subunit occur constitutively within cytoplasm (Semenza, 2003). Subsequently, investigating HIF-1α protein level as a target molecule for *S. latifolium* extract during its suppression total hypoxia. Since miR-210 and by miR-21 regulate the hypoxia key players HIF-1α and HIF-1β, we studied the effect of *S. latifolium* extract fractions on both proteins' concentrations in HCT-116 cells. HIF-1α was found to be basically upregulated in HCT-116 cells. HIF-1α was noticeably upregulated in HCT-116 cells, which indicate of hypoxia status. HIF-1α protein was decreased when cells were treated with SL1 ($p < 0.01$) and SL4 ($p < 0.001$) compared to untreated cells (Fig. 4).

HIF-1β, aryl hydrocarbon receptor nuclear translocator (ARNT), is highly expressed in hypoxic experimental

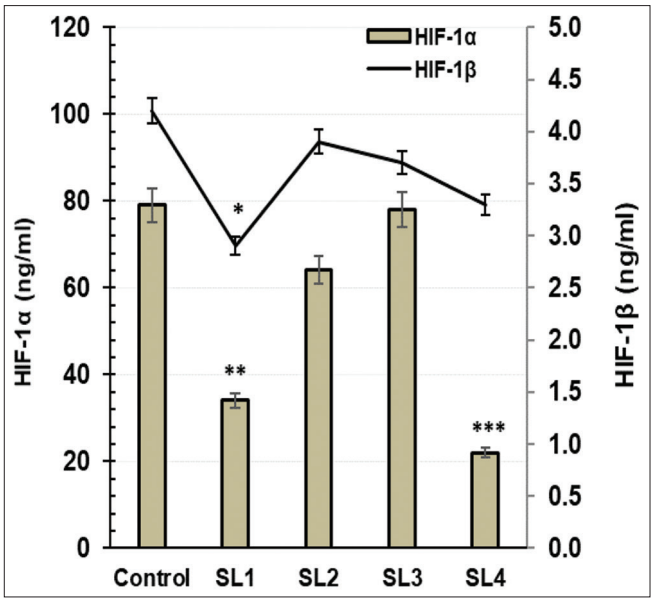

Fig 4. Determination of HIF-1α and HIF-1β proteins in HCT-116 cells, using ELISA. Fraction-treated cells were compared with control cells (ng/ml; n = 6; mean ±SD) *$p < 0.05$, **$p < 0.01$, and *** p< 001 compared with control.

conditions and metastatic tumor cells (Dery et al., 2004; Ercin et al., 2019). Opposing early reports, HIF-1β has a fundamental function in hypoxic response, where recent research stated that HIF-1β concentrations are not constant during hypoxia and it is fluctuated (Ercin et al., 2019; Zagorska and Dulak, 2004). In the current study, HIF-1β concentration displayed non-significant changes in all treatments except in cells treated with SL1 ($p < 0.05$), as shown in Fig.4.

Previously, HIF-1β is found to be essential for full NF-κB activation in response to canonical and non-canonical stimuli (Van Uden et al. 2011), where HIF-1β binds TRAF6 gene and controls its expression, independently from HIF-1α. These results suggested that HIF-1β is one of the NF-κB regulators (D'Ignazio et al. 2020). Consequently, the HIF-1β inhibition by SL1 may provide not only suppressed hypoxia pathway, but also inhibited NF-κB activation.

CONCLUSION

SL1 and SL4 are cytotoxic agents against HCT-116 cells through enhancing apoptosis. SL1and SL4 were potent inhibitor of total cell hypoxia. Both fractions significantly suppressed the expression of miR-21 and miR-210, and the concentration of HIF-1α protein, while only SL1 inhibited HIF-1β protein concentration. Taken together *S. lattifolium* polysaccharide extract fractions SL1 and SL4 exhibited anti-hypoxic property in HCT-116 cells through

mechanistic role in the expression of hypoxia regulators miRNA-21 and miRNA-210, and accordingly in HIF-1α and HIF-1β biosynthesis.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

Gamal-Eldeen: Study concept/design and writing manuscript; Raafat: statistical analysis; El-Daly and Fahmy: ELISA and miRNAs expression; Almehmadi: Cell-based assays; Althobaiti alga extraction.

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