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Modulation of cell cycle and proliferation in endometrial cancer cells by Chlorella pyrenoidosa – *in vitro* studies

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Abstract

Introduction and Objective. Endometrial cancer involves uncontrolled cell proliferation and cell cycle dysregulation. Natural compounds, such as microalgae-derived extracts, are being studied as potential chemopreventive agents. The aim of the study is to assess the effects of Chlorella pyrenoidosa water extract on proliferation, cytoskeletal marker expression, and cell cycle progression in human endometrial cancer cells.

Materials and Method. Endometrial adenocarcinoma cell lines (HEC-1-B, KLE) and patient-derived EDC cells were treated with chlorella extract at 50 to 1,000 µg/mL for 48 hours. Morphological changes were evaluated by May-Grünwald-Giemsa staining. Ki-67 and cytokeratin 7 expression were measured by flow cytometry and cell cycle distribution was analyzed using propidium iodide staining.

Results. Treatment with C. pyrenoidosa extract induced morphological alterations and reduced cell density in all examined models. Ki-67 expression decreased, indicating reduced proliferative activity, with the strongest effect noted in HEC-1-B cells. Cytokeratin 7 expression was also reduced. Cell cycle analysis showed accumulation of EDC cells in the G0/G1 phase, whereas HEC-1-B cells demonstrated an increased S-phase fraction.

Conclusions. C.pyrenoidosa water extract exerted antiproliferative effects in human endometrial cancer cells, associated with reduced proliferative activity, decreased cytokeratin 7 expression and altered cell cycle distribution. The obtained results indicate that chlorella extract may modulate key cellular features related to tumour cell growth and phenotype maintenance, supporting its relevance as a natural compound with chemopreventive potential in endometrial cancer models.

Key words

flow cytometry, chemoprevention, Chlorella pyrenoidosa, endometrial cancer, Chlorella Growth Factor

INTRODUCTION

Endometrial cancer is the most common malignancy of the female reproductive tract in developed countries and represents a growing global health concern [1, 2]. Its incidence has increased in recent decades, primarily due to population ageing and higher rates of metabolic disorders such as obesity and diabetes [1, 2]. Standard treatments include surgical tumour removal, often combined with radiotherapy, chemotherapy, or hormonal therapy, depending on clinical stage and histopathology [3]. However, these treatments are often limited by tumour heterogeneity, therapeutic resistance, and uncontrolled cancer cell proliferation [1].

Unrestricted cell proliferation is a key biological feature of malignant transformation. In normal tissues, cell cycle progression is tightly regulated to control growth and division. Disruption of these mechanisms leads to abnormal cell cycle progression, excessive division, and tumour development [4, 5]. Many anticancer strategies target cell cycle regulation to suppress proliferation and eliminate malignant cells. Ki-67 is a widely used molecular marker of cellular proliferation. This nuclear protein is present in actively dividing cells throughout the G1, S, G2, and M phases, but absent in resting cells [6]. Due to this property, Ki-67 serves as a marker of tumour growth and proliferative activity in many malignancies, including endometrial carcinoma. High Ki-67 expression is often linked to increased tumour aggressiveness and poor prognosis [6, 7]. In addition to proliferation markers, cytoskeletal proteins characteristic of epithelial cells are analyzed to verify the cellular phenotype of cancer models. Cytokeratins are a diverse family of intermediate filament proteins that provide structural stability to epithelial cells. Cytokeratin 7, in particular, is frequently expressed in epithelial tumours of the female reproductive tract [8].

In recent years, attention has been focused on natural products as potential sources of chemopreventive and anticancer compounds. Due to their biological activity and relatively low toxicity to normal tissues, these compounds are increasingly studied as complementary strategies for cancer prevention and treatment [9, 10]. Among natural sources, microalgae are particularly promising. Studies have shown that extracts from various Chlorella species inhibit proliferation and induce cell death in several cancer cell models [11, 12, 13].

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Our previous research was the first to demonstrate that a water extract of *Chlorella pyrenoidosa* exhibits promising chemo-preventive activity against human endometrial cancer cells. The extract reduced metabolic activity and DNA synthesis, decreased cancer cell migration, and activated cell death pathways [14]. However, it remains unclear whether the antiproliferative effects of *C. pyrenoidosa* extract are related to changes in cell-cycle progression, or in the expression of key proliferation markers.

OBJECTIVE

The aim of the study is to further investigate the biological activity of *C. pyrenoidosa* extract in human endometrial cancer cells, focusing on mechanisms related to cell proliferation. Morphological changes induced by the extract were evaluated using May-Grünwald-Giemsa staining. The study also analyses Ki-67 and cytokeratin 7 expression, as well as cell cycle progression. The results obtained may provide new insights into the anticancer activity of *C. pyrenoidosa* and its potential role in the chemo-prevention of endometrial cancer.

MATERIALS AND METHOD

Reagents. Unless otherwise specified, chemicals used in the study were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Cell culture media and supplements were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies used for flow cytometry were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Chlorella extract. Dried chlorella (*Chlorella pyrenoidosa*) was purchased from Green Ways International (Prague, Czech Republic). The water extract was prepared as described previously [14]. Powdered chlorella biomass was suspended in sterile distilled water and extracted for 24 h under constant agitation at room temperature. The suspension was centrifuged to remove insoluble material, and the supernatant was filtered through a microbiological filter. The resulting filtrate was freeze-dried to obtain a dry extract. The lyophilized extract was stored at –20 °C until further use. A stock solution was prepared by dissolving the lyophilizate in phosphate-buffered saline (PBS). Working solutions of the extract were prepared by dilution of the stock solution in cell culture medium immediately before the experiments.

Quantification of sugars, proteins, and nucleic acids in Chlorella extract. Freeze-dried chlorella extract was resuspended in double-distilled water at 1 mg/mL and analysed for chemical composition. Protein content was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, with bovine serum albumin serving as the standard. Total sugar content was determined by the phenol–sulfuric acid method. Samples were mixed with 5% phenol and concentrated sulfuric acid, incubated at room temperature for 20 min, and absorbance was measured at 490 nm using glucose as the standard. Nucleic acid concentration was assessed spectrophotometrically by measuring absorbance at 260 nm. All absorption measurements were performed using a microplate reader (BioTek, Winooski, VT, USA).

Commercial cell lines. The human endometrial adenocarcinoma cell lines HEC-1-B and KLE were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEC-1-B cells were grown in Eagle's Minimum Essential Medium, while KLE cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham. Both media were supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

Non-commercial cell line. Human endometrial adenocarcinoma-derived EDC cells were obtained from a tumour fragment collected from a patient diagnosed with endometrial adenocarcinoma. Immediately after surgical removal, the tumour specimen was placed in PBS supplemented with an antibiotic–antimycotic solution and transported to the laboratory. The tissue sample was cleaned of blood clots and connective tissue, then cut into small fragments. The tissue was then incubated in digestion medium containing collagenase and DNase with constant agitation. After enzymatic digestion, the suspension was centrifuged, and the obtained cell pellet was resuspended in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin(100 µg/mL) and streptomycin (100 U/mL). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2. For experimental procedures, cells at early passages were used.

The experimental protocol was approved by the Bioethics Committee at the Lublin Medical University, Lublin, Poland (Resolution No. KE-0254/35/02/2023).

Morphological changes examination – MGG staining. To evaluate changes in cell morphology, May-Grünwald-Giemsa staining was used. Human endometrial adenocarcinoma cell lines HEC-1-B and KLE, and human endometrial adenocarcinoma-derived EDC cells were seeded onto sterile culture dishes and incubated overnight to allow attachment. The following day, the culture medium was removed, and cells were treated with *C. pyrenoidosa* extract at concentrations of 50–1000 µg/mL, prepared in culture medium supplemented with 10% FBS. Cells cultured in medium without the extract were used as a control. After 48 h of incubation, the cells were stained according to the May–Grünwald–Giemsa method. In the first step, the culture medium was replaced with May–Grünwald solution. After 3 min of incubation, the dye solution was diluted twice with distilled water, and incubation was continued for the next 3 min. Subsequently, the May–Grünwald solution was replaced with diluted Giemsa solution (1:20 in distilled water), and staining was continued for 30 min. After removing excess dye and drying the preparations, the dishes were examined under a light microscope (MW 50, OPTA-TECH, Warsaw, Poland). Micrographs were captured using Capture software version 2.2.1 (OPTA-TECH, Warsaw, Poland).

Expression of Ki-67 and Cytokeratin 7 – flow cytometry staining. Expression of proliferation markers Ki-67 and cytokeratin 7 was analysed using flow cytometry. After treatment with Chlorella extract, cells were harvested by trypsinisation, washed twice with phosphate-buffered

saline (PBS), and centrifuged. The obtained cell pellet was resuspended in PBS and prepared for intracellular staining. Cells were first fixed with BD Cytofix fixation buffer (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at room temperature. After fixation, the cells were permeabilised using BD Phosflow Perm Buffer III (BD Biosciences, Franklin Lakes, NJ, USA) for 5 min at room temperature. Subsequently, the cells were washed twice with PBS. To prevent nonspecific antibody binding, cells were incubated in a blocking buffer containing 3% FBS in PBS for 30 min at room temperature. Following the blocking step, cells were incubated with fluorochrome-conjugated antibodies against Ki-67 and cytokeratin 7 diluted in staining buffer according to the manufacturer's instructions. Cells were incubated with the antibody solution for 1 h at room temperature in the dark. After incubation, the cells were washed twice with PBS to remove unbound antibodies. The stained cells were then resuspended in PBS for cytometric analysis. Samples were analysed using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed using BD Accuri C6 Plus software. For each sample, a minimum of 30,000 events was collected and analysed to determine the percentage of positive cells and fluorescence intensity.

Cell cycle progression – flow cytometry. Cell cycle progression was analysed by flow cytometry based on DNA content measurements. After 48 h of treatment, cells were harvested by trypsinisation, washed with phosphate-buffered saline (PBS), and centrifuged. The obtained cell pellet was fixed with 80% cold ethanol and incubated at –20 °C for 24 h. Following fixation, the cells were centrifuged and washed with PBS to remove residual ethanol. Subsequently, the cells were stained with PI/RNase Staining Buffer (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instructions.

Samples were analyzed using the BD Accuri C6 Plus Flow Cytometer (BD Biosciences, San Jose, CA, USA). During data analysis, the initial gating step involved selection of the cell population based on forward scatter (FSC) and side scatter (SSC) parameters to exclude debris and non-cellular particles. Subsequently, singlet cells were identified to eliminate doublets and cell aggregates. For the selected singlet population, DNA content analysis was performed by generating histograms of propidium iodide (PI) fluorescence intensity. Based on DNA content, the proportions of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined using BD Accuri C6 Plus software.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Differences between experimental groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test, which was used to compare treated groups with the control. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Component of *Chlorella pyrenoidosa* extract. Spectrophotometric techniques were employed to determine the total contents of proteins, sugars, and nucleic acids. The results indicated that the chlorella extract consisted of nucleic acids (24.43%), proteins (26.86%), and sugars (42.32%).

Morphological alterations in endometrial cancer cells induced by *Chlorella pyrenoidosa* extract. Morphological and growth-pattern changes in human endometrial cancer cells were evaluated after 48 h of treatment with *C. pyrenoidosa* extract, using May-Grünwald-Giemsa staining. Alterations were assessed by light microscopy (Fig. 1).

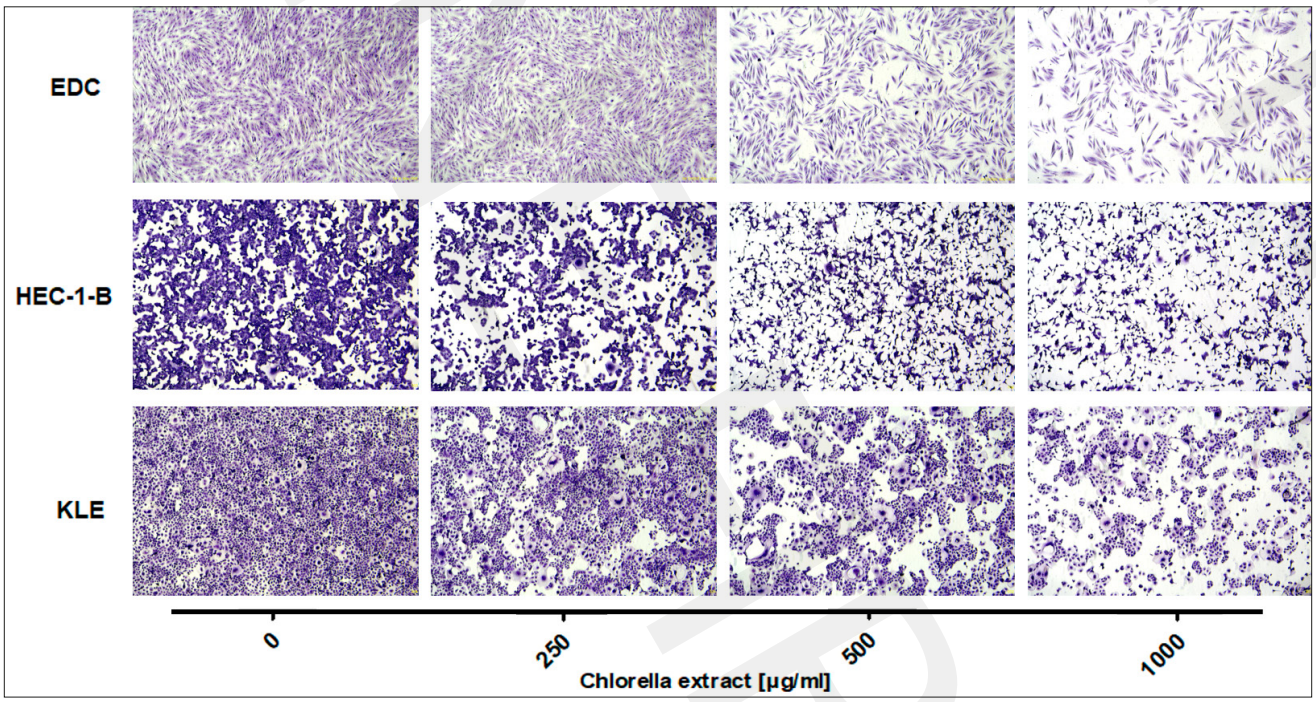

Figure 1. Morphological changes in human endometrial adenocarcinoma-derived EDC Cells and human endometrial adenocarcinoma cell lines HEC-1-B and KLE after treatment with C. pyrenoidosa extract. Cells were exposed to a culture medium alone (control), or chlorella extract at concentrations 250, 500 and 1,000 µg/mL. After 48 h of incubation, cells were stained using the May–Grünwald–Giemsa method and observed under a light microscope. Representative micrographs illustrating morphological alterations induced by C. pyrenoidosa extract are presented

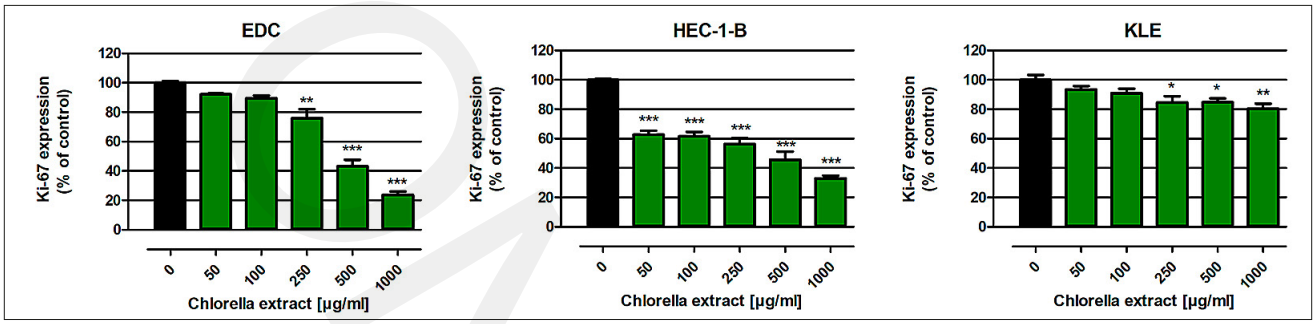

Figure 2. Effect of C. pyrenoidosa extract on Ki-67 expression in human endometrial adenocarcinoma-derived EDC cells and the HEC-1-B and KLE cell lines. Cells were treated with either culture medium alone (control) or chlorella extract at 50, 100, 250, 500, or 1,000 µg/mL. After 48 h of incubation, Ki-67 expression was measured by flow cytometry. Results are shown as mean ± SEM from three measurements.
Statistically significant differences compared to the control are indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). One-way ANOVA test; *post-hoc* test: Dunnett

Microscopic analysis demonstrated that exposure to Chlorella extract significantly altered cell morphology and reduced cell density. Control cells exhibited typical epithelial morphology, forming dense monolayers with uniform shapes and distinct nuclei. In treated cultures, cell numbers decreased, indicating inhibited proliferation. Increasing extract concentrations further reduced cell density, with the most pronounced effect observed at 1,000 µg/mL.

Effect of *Chlorella pyrenoidosa* extract on Ki-67 expression in endometrial cancer cells. The effect of *C. pyrenoidosa* extract on Ki-67 expression, a proliferation marker, in human endometrial cancer cells was evaluated by flow cytometry after 48 h of treatment. The results, expressed as a percentage of the control, showed that the tested extract decreased Ki-67 expression in all analysed cell lines. However, the extent of this effect varied across the models investigated (Fig. 2).

As shown in Figure 2, Chlorella extract at concentrations from 250 µg/mL to 1,000 µg/mL reduced Ki-67 expression. A reduction in proliferative activity was observed at the highest tested concentrations, with 75.86% of control at 250 µg/mL, 43.22% at 500 µg/mL, and 23.58% at 1,000 µg/mL.

A markedly stronger response to chlorella treatment was observed in HEC-1-B cells. In this cell line, Ki-67 expression decreased significantly across the entire range of analyzed concentrations. The percentage of Ki-67-positive cells was reduced to 62.77% of control at 50 µg/mL and 61.53% at 100 µg/mL, while further inhibition was noted at higher concentrations of the extract, reaching 56.43% of control at 250 µg/mL, 50.93% at 500 µg/mL, and 34.00% at 1,000 µg/mL.

In contrast, KLE cells exhibited a weaker response to chlorella extract. Significant inhibition of Ki-67 expression in KLE cells was observed at 250 µg/mL, 500 µg/mL, and 1,000 µg/mL of the extract, reaching 84.64% of control at 250 µg/mL, 84.78% at 500 µg/mL, and 80.36% at 1,000 µg/mL.

Effect of *Chlorella pyrenoidosa* extract on cytokeratin 7 expression in endometrial cancer cells. The effect of Chlorella extract on cytokeratin 7 expression in human endometrial cancer cells was evaluated using flow cytometry after 48 h of treatment. The obtained results, presented as the percentage of control, demonstrated that the tested extract decreased the expression of this epithelial marker in the analyzed cell lines, although the magnitude of this effect differed between the investigated models (Fig. 3).

As presented in Figure 3, chlorella extract markedly reduced cytokeratin 7 expression in EDC cells in a concentration-dependent manner. A significant inhibition of cytokeratin 7 expression was observed across the entire concentration range. Exposure of EDC cells to chlorella extract reduced cytokeratin 7 expression to 56.19% of control at 50 µg/mL and 48.67% at 100 µg/mL, while further decreases were detected at higher concentrations, reaching 31.62% of control at 250 µg/mL, 15.04% at 500 µg/mL, and 10.64% at 1,000 µg/mL.

In HEC-1-B cells, the inhibitory effect of chlorella extract on cytokeratin 7 expression was less pronounced. A significant reduction in marker expression was detected at 250 µg/mL, 500 µg/mL, and 1,000 µg/mL of the extract. At these concentrations, cytokeratin 7 expression decreased to 78.16%, 69.92%, and 47.90% of the control level, respectively.

In contrast, KLE cells showed a markedly weaker response to chlorella treatment. A significant decrease in cytokeratin 7 expression was detected only at higher concentrations of the extract, namely 500 µg/mL and 1000 µg/mL, where the

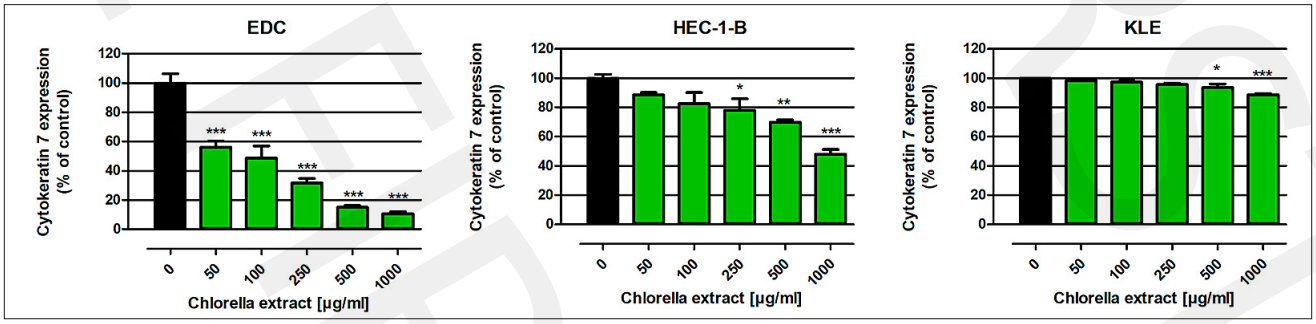

Figure 3. The effect of C. pyrenoidosa extract on cytokeratin 7 expression was evaluated in EDC, HEC-1-B, and KLE human endometrial adenocarcinoma cell lines. Cells were treated with either culture medium (control) or chlorella extract at 50, 100, 250, 500, and 1,000 µg/mL. After 48 h, cytokeratin 7 expression was measured by flow cytometry. Data are shown as mean ± SEM from three replicates.
Statistical significance versus control was determined by one-way ANOVA with Dunnett's post-hoc test at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). One-way ANOVA test; *post-hoc* test: Dunnett

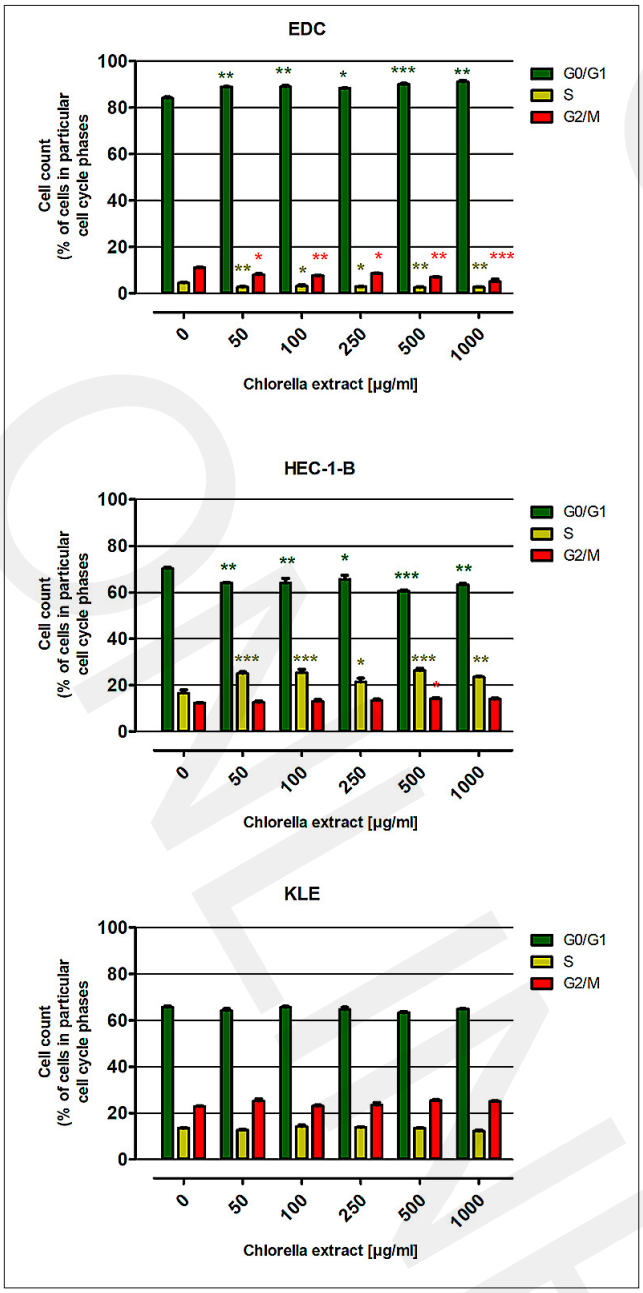

Figure 4. The effect of C. pyrenoidosa extract on cell cycle distribution in human endometrial adenocarcinoma-derived EDC cells and human endometrial adenocarcinoma cell lines HEC-1-B and KLE. Cells were exposed to culture medium (control) or chlorella extract at 50, 100, 250, 500, and 1000 µg/mL. After 48 h, cell cycle distribution was assessed by flow cytometry following propidium iodide staining. Data are expressed as mean ± SEM from three measurements.
Statistically significant differences from the control are indicated at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). One-way ANOVA test; *post-hoc* test: Dunnett

marker level decreased to 93.49% and 88.58% of control, respectively.

Effect of *Chlorella pyrenoidosa* extract on cell cycle distribution in endometrial cancer cells. The influence of *C. pyrenoidosa* extract on cell cycle progression in human endometrial cancer cells was assessed by flow cytometry following 48 h of treatment. The results indicated that the extract modified the distribution of cells across specific cell cycle phases, with the extent and pattern of these alterations varying among the examined cell lines (Fig. 4).

In EDC cells, treatment with Chlorella extract produced a marked shift in cell cycle distribution, characterized by an increased G0/G1 population and a reduced S phase fraction. In control cultures, 84.06% of cells were in the G0/G1 phase, with 4.49% and 11.09% in the S and G2/M phases, respectively. Exposure to the extract increased the G0/G1 population across all tested concentrations, reaching 88.89%, 88.93%, 88.25%, 90.08%, and 91.10% at 50, 100, 250, 500, and 1,000 µg/mL, respectively. The S-phase population decreased to 2.63%-3.14% in treated cultures, while the G2/M phase showed a slight reduction compared with controls. These findings suggest that Chlorella pyrenoidosa extract promotes the accumulation of EDC cells in the G0/G1 phase.

In HEC-1-B cells, chlorella extract induced a different pattern of cell cycle alterations. Control cultures were characterized by 70.28% of cells in the G0/G1 phase, 16.64% in the S phase, and 12.26% in the G2/M phase. Exposure to the extract reduced the proportion of cells in the G0/G1 phase to approximately 60–66%, while the percentage of cells in the S phase increased to 25.11%, 25.35%, 21.36%, 26.35%, and 23.52% at the applied concentrations. A slight increase in the G2/M population was also observed in treated cultures. These changes indicate accumulation of HEC-1-B cells in the S phase of the cell cycle following exposure to *C. pyrenoidosa* extract.

In contrast, in KLE cells treated with the extract, there was practically no change in the cell cycle distribution. The percentage of cells in the G0/G1 phase remained comparable to that in the control cultures, and no changes were observed in the S-phase population either. At the same time, no differences were observed in the cultures treated with the extract in the G2/M phase.

DISCUSSION

Natural compounds have garnered significant attention as potential anticancer agents because of their capacity to modulate various cellular processes implicated in tumour progression [10, 15, 16]. Dysregulation of proliferation and cell cycle control is a key factor in cancer development [4, 17, 18]. This study evaluated the effects of *Chlorella pyrenoidosa* extract on proliferation markers, epithelial phenotype, and cell cycle distribution in human endometrial cancer cells. The results offer insights into the mechanisms underlying the antiproliferative activity of chlorella-derived compounds. Given the limited number of studies examining chlorella in the context of endometrial cancer, these findings contribute valuable knowledge. Nevertheless, the scarcity of existing literature restricts the ability to conduct direct comparative analyses with previous research. In our previous studies evaluating the anticancer activity of *C. pyrenoidosa,* we assessed the cellular safety of its water extract in noncancerous human cell models. Under the experimental conditions applied, the extract did not significantly affect cell viability, proliferative activity, or morphology, as demonstrated in normal human colon epithelial cells (CCD 841 CoN) [19] and human skin fibroblasts [11]. These results support a favourable cellular safety profile of the investigated extract and provide an important context for the interpretation of its biological activity in cancer cells. The primary objective of the present study was therefore to further characterise the antiproliferative effects of the extract specifically in endometrial cancer cells.

Microscopic evaluation using May-Grünwald-Giemsa staining demonstrated that treatment with *C. pyrenoidosa* extract induced visible morphological alterations in the analysed cancer cell lines. A reduction in cell density, accompanied by pronounced changes in cellular morphology, was observed, indicating impaired cell viability. Similar morphological features have been reported in cancer cells treated with Chlorella-derived compounds and were typically associated with the induction of apoptosis [12,20]. In particular, Sawasdee et al. demonstrated that treatment with Chlorella extract leads to characteristic apoptotic morphological changes alongside reduced cell viability [20], while Hamouda et al. observed structural alterations and a decreased cell number following exposure to *Chlorella vulgaris* in cancer models [12]. Collectively, these observations indicate that the morphological changes identified in the present study are consistent with previously described cytotoxic effects of chlorella, and further support its ability to impair endometrial cancer cell integrity and growth.

A reduction in the expression of the proliferation marker Ki-67 was observed following treatment with chlorella extract. Ki-67 is widely recognized as a reliable indicator of proliferative activity, and its elevated expression is associated with increased tumour aggressiveness and poor prognosis [21, 22]. In the present study, Ki-67 expression decreased in all analyzed cell lines, although the magnitude of this effect varied between models. The strongest inhibition was observed in HEC-1-B cells, where a reduction in proliferative activity was already evident at lower extract concentrations. In contrast, a more pronounced effect in EDC cells was detected only at higher concentrations, while KLE cells exhibited the weakest response. The observed reduction in Ki-67 expression is consistent with previous findings reported by Lemieszek et al., who demonstrated that chlorella-based extracts significantly inhibit colon cancer cell proliferation, as evidenced by decreased DNA synthesis and reduced metabolic activity in in vitro models. In that study, the antiproliferative effect was confirmed using BrdU incorporation assays [19]. Given that Ki-67 is closely associated with actively proliferating cells, the reduction in its expression observed in the present study likely reflects a similar inhibition of proliferative activity. Moreover, in our earlier studies on endometrial cancer cells, we demonstrated that *Chlorella pyrenoidosa* extract reduced metabolic activity, DNA synthesis, and migratory potential, while simultaneously activating cell death pathways [14]. A cell line-dependent response was identified, with varying sensitivity among HEC-1-B, KLE, and EDC models. This variability aligns with the differences in Ki-67 reduction observed in the present study and supports the hypothesis that chlorella-derived compounds may modulate proliferative activity in accordance with the intrinsic biological characteristics of cancer cells.

The antiproliferative effect of chlorella extract was further evidenced by cell cycle analysis results. Different patterns of cell-cycle changes were observed across cell lines. In EDC cells, an accumulation of cells in the G0/G1 phase with a decrease in the S phase was observed, indicating inhibition of cell cycle progression at the G1 checkpoint. In HEC-1-B cells, a higher proportion of cells were in S phase, suggesting disruption of DNA synthesis and replication. In contrast, no changes in the cell cycle distribution were observed in KLE cells, which is consistent with this cell line's lower sensitivity to the extract under investigation.

These findings are consistent with previous reports showing that chlorella-derived compounds and related nanocomposites can induce cell cycle arrest at different phases, depending on the cellular context. For instance, studies on colon cancer cells have shown accumulation of cells in the G0/G1 phase, accompanied by an increase in the subG1 fraction, reflecting both cell cycle arrest and apoptosis induction [23]. In contrast, breast cancer models have been associated with S-phase arrest, indicative of replication stress and impaired DNA synthesis [24].

It should be emphasized that chlorella extract reduced cytokeratin 7 expression in the analyzed cell lines. Cytokeratins are essential structural proteins responsible for maintaining epithelial cell integrity and are widely used as markers of the epithelial phenotype [8]. However, accumulating evidence indicates that their modulation may reflect biologically relevant responses to anticancer agents rather than non-specific cellular damage. For example, in studies investigating natural compounds such as resveratrol, antiproliferative and proapoptotic effects are frequently accompanied by cytoskeletal reorganisation and alterations in the expression of structural proteins, including cytokeratins [25, 26]. In the present study, the most pronounced decrease in cytokeratin expression was observed in EDC cells, whereas HEC-1-B and KLE models exhibited a less marked response. These findings may indicate differential susceptibility of the analyzed cell lines to chlorella-induced alterations in cellular architecture, potentially associated with cytoskeletal remodelling and cellular stress responses. The variability in response between the analyzed cell lines represents an important observation. Differences in sensitivity to chlorella extract may be related to variations in tumour characteristics, including genetic background, differentiation status or intrinsic proliferative potential.

In our study we revealed that the extract consisted mainly of sugars (42.32%), proteins (26.86%) and nucleic acids (24.43%). While the precise composition of the analyzed extract remains incompletely characterized, the use of aqueous extraction and the identification of key bioactive constituents indicate that the observed biological activity is likely attributable to the presence of Chlorella Growth Factor (CGF). CGF is generally described as a complex fraction of water-soluble bioactive compounds derived from chlorella cells. The components most frequently associated with this fraction include nucleic acids, peptides, free amino acids, polysaccharides, glycoproteins, vitamins, and minerals [27]. The extract examined in this study exhibits the primary characteristics associated with CGF, including a heterogeneous mixture of proteins, polysaccharides, nucleic acids, and low-molecular-weight water-soluble compounds derived from chlorella biomass. Currently, there is no universally recognized compositional standard for CGF. The observed heterogeneity primarily results from differences in Chlorella species, culture conditions, and extraction methods, each of which can significantly affect both the qualitative and quantitative composition of the resulting fraction [28].

Limitations of the study. Several limitations of this study should be acknowledged. The experiments were performed *in vitro,* which may not fully reflect the complexity of

tumour behaviour *in vivo*. Biological effects were observed at relatively high concentrations of the whole extract. However, these values must be interpreted in light of the extract's complex and heterogeneous composition, as they reflect the total concentration of multiple constituents rather than that of a single, well-defined bioactive compound. Future studies should examine key regulators of cell cycle progression, including cyclins, cyclin-dependent kinases, and their inhibitors. Studies should characterize all bioactive substances present in the extract and check the bioavailability of the extract after oral administration. Furthermore, in vivo models are required to evaluate the therapeutic potential of chlorella extract.

CONCLUSIONS

Chlorella pyrenoidosa extract shows significant antiproliferative effects in human endometrial cancer cells, demonstrated by reduced Ki-67 expression, altered cytokeratin 7, and cell line specific changes in cell cycle progression. These findings suggest the extract affects both proliferation and cellular phenotype, supporting its potential as a multitarget chemo-preventive agent. However, further molecular and *in vivo* studies are needed to confirm this clinical relevance. In summary, *C. pyrenoidosa* might be a promising natural compound for this purpose in endometrial cancer prevention and as a supportive therapy.

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