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Ellagic Acid Ameliorates Ovarian Cancer via Modification of Pyroptosis and Inflammation

Yan Sun¹ Xin Hu², and Ying Wang³

¹ Department of Obstetrical, Obstetric Nursing Unit, West China Second University Hospital, Key Laboratory of Birth Defects and Related Diseases of Women and Children, Ministry of Education, Chengdu, China

² Department of Gynecology, Qingdao Municipal Hospital, Qingdao, China

³ Department of Obstetrical, Shangdong Provincial Third Hospital, Tianqiao District, Jinan, China

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ABSTRACT

Ovarian cancer is 1 of the most serious female malignancies worldwide. Despite intensive efforts to overcome ovarian cancer, there remain limited treatment options for this disease. Ellagic acid (EA), a well-known phytochemical with anti-inflammatory properties, is suggested as a therapeutical strategy as it can inhibit the growth of certain cancer cells. However, its effect on human ovarian carcinoma cells has not yet been fully elucidated. The present study aimed to explore the effect of EA on ovarian carcinoma and further expound the underlying mechanisms of EA-induced ovarian cancer cell death.

Human ovarian carcinoma cell lines, A2780 and OVCAR3, were treated with EA (0, 10, 20, 50, and 100 μ M) and assessed for viability, cell cycle (cyclin D1 and cyclin E), pyroptosis (gasdermin D [GSDMD] and gasdermin E [GSDME]), autophagy (microtubule-associated protein 1A/1B-light chain 3 [MAP1LC3] and autophagy protein 5 [ATG5]), and inflammation (interleukin [IL]-1 β and IL-6) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), real-time polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA).

The findings showed that EA could significantly inhibit cell viability, decrease cyclin D1 and E levels, downregulate GSDMD and GSDME, and suppress the levels of inflammatory markers, including IL-1 β and IL-6. However, the protein levels of autophagic markers including LC3 and ATG5 remained mostly unchanged.

The findings suggest that EA could suppress ovarian cancer cell viability and proliferation by arresting both cell lines at the G1 phase of the cell cycle through modification of cell death mediated by inflammatory-caused pyroptosis.

Keywords: Autophagy; Ellagic acid; Cell cycle; Inflammation; Pyroptosis; Ovarian cancer

Corresponding Author: Ying Wang, MM;

Department of Obstetrical, Shangdong Provincial Third Hospital, Tianqiao District, Jinan, China. Tel: (+86 136) 2541 6028, Fax: (+86 136) 2541 6028), Email: wy4848038@163.com

INTRODUCTION

Ovarian cancer (OC) is a significant health concern, ranking third in prevalence among gynecological malignancies, following cervical and uterine cancer.¹ OC is characterized by its high mortality rate and the

most unfavorable prognosis among gynecological cancers.² The primary reasons for this are its insidious and often silent progression, the delayed onset of symptoms, and the complexities in diagnosing it at advanced stages, earning it the nickname of the silent killer.^{3,4} Research has shown that a variety of factors contribute to the incidence of OC, including age, genetic predispositions, environmental and hormonal influences, lifestyle habits (e.g., diet and physical activity), reproductive events (e.g., pregnancy and lactation), and the use of oral contraceptives.^{5,6} A plethora of evidence has demonstrated that disruptions within cellular pathways, such as pyroptosis, autophagy, and inflammation, significantly contribute to the development and progression of OC.⁷⁻¹⁰ The symptoms of OC can vary greatly among individuals, often resulting in the presentation of nonspecific symptoms before a formal diagnosis is made. Studies have highlighted several key symptoms commonly experienced by those with this condition, including discomfort in the pelvic or abdominal area, an increase in abdominal size or bloating, urinary incontinence and frequency, and flatulence.^{11,12}

Individuals diagnosed with advanced-stage ovarian carcinoma undergo a standardized treatment plan that typically involves cytoreductive surgery and a combination of therapies.^{13,14} Despite the effectiveness of this approach, about 75% of patients experience a recurrence of the disease, often accompanied by a severe form of drug resistance that can be fatal. The rapid relapse and the emergence of resistance to therapy pose significant challenges in managing OC.¹⁵

Ellagic acid (EA) is a naturally occurring polyphenolic compound renowned for its potent antioxidant and anticancer properties. It is abundantly found in a wide range of fruits such as berries and pomegranates.^{16,17} The antitumor effects of EA are primarily due to its direct antiproliferative and apoptotic functions.^{18,19} Furthermore, EA has the ability to hinder tumor cell migration, invasion of the extracellular matrix, and angiogenesis, which are essential processes for tumor infiltration and metastasis.²⁰ Additionally, EA may enhance the sensitivity of tumors to chemotherapy and radiotherapy. Research indicates that EA influences the expression of various genes involved in cancer-related processes such as apoptosis, proliferation, inflammation, and oxidative stress.^{21,22}

Given the nonspecific symptoms and generally poor prognosis associated with this condition, there is a

pressing need for innovative treatment strategies tailored to OC.¹⁵ Hence, this research attempted to investigate the impact of EA on ovarian carcinoma, with a focus on elucidating the mechanisms underlying cell death in OC cells induced by EA.

MATERIALS AND METHODS

Chemicals and Reagents

EA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and a proteinase inhibitor cocktail were sourced from Sigma-Aldrich, Saint Louis, MO, USA. Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, and amphotericin B were supplied by Gibco-BRL, Grand Island, NY, USA. Cell counting kit-8 (CCK-8) was made available by Dojindo Molecular Technologies, Kumamoto Prefecture, Kyushu Island, Japan. Enzyme-linked immunosorbent assay (ELISA) kits for cyclin D1 and E, gasdermin (GSDM) D and GSDME, microtubule-associated protein 1 light-chain 3 (MAP1LC3, briefly shown LC-3), autophagy protein [ATG5], interleukin (IL)-1 β and IL-6 were obtained from MyBioSource, Inc., USA, Abbeva, Cambridge Science Park, United Kingdom, and Abcam, USA.

Cell Culture and Treatment

The human OC cell lines A2780 and OVCAR3 were procured from ATCC. Cells were then cultured in 96-well plates at a density of 1×10^4 cells/well in DMEM supplemented with 10% FBS, 100 U penicillin G, 100 $\mu\text{g}/\text{mL}$ streptomycin sulfate, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B, all under optimal conditions of 37°C and a 5% CO₂-humidified atmosphere. Following a 24-hour incubation period, the cells were subjected to various concentrations of DMSO-dissolved EA (0, 10, 20, 50, or 100 μM) for 24, 48, and 72 hours. After that, the cells were harvested through trypsinization, stained with trypan blue, and the cell count in suspension was determined using a hemocytometer in duplicate to ensure accuracy.

Cell Viability Assays

To assess cell viability, MTT and cell counting kit-8 [CCK-8] assays were employed. Following a 24-hour treatment period, 10 μL of MTT solution were introduced into each well of the plate. The solution was then incubated at 37°C for 2 hours, after which 100 μL

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of solubilization buffer was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate spectrophotometer (ELX800, Biotek Instruments Inc, USA). Additionally, the CCK-8 reagent was administered, followed by a 1 hour incubation at 37°C in a 5% CO₂-humidified atmosphere. The absorbance was subsequently determined at 450 nm using the same microplate spectrophotometer (ELX800, Biotek Instruments Inc, USA). The viability of control cells was considered 100% for comparison purposes.

Cytotoxicity Assay

The assessment of cytotoxicity prior to and following treatment was conducted by evaluating lactate dehydrogenase (LDH) levels. The LDH measurement process involves transferring 10 µL of culture medium into a fresh plate, followed by the addition of the LDH reaction mix. This mixture was then incubated at room temperature for 30 minutes. The optical density (OD) at 450 nm was subsequently analyzed using a microplate reader.

RNA Extraction and Real-time PCR

The extraction of total RNA was accomplished using the PureLink RNA Mini Kit (Catalog #: 12183018A, Thermo Fisher, USA), adhering strictly to the manufacturer's instructions. The RNA's purity and concentration were assessed with the Biotek Nanodrop system. Subsequent to cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Catalog #: 4368814, Thermo Fisher, USA), the cDNA was quantified through polymerase chain reaction (PCR) utilizing the StepOne Real-Time PCR System (Applied Biosystems, USA) and Maxima SYBR Green qPCR Master Mix (Catalog #: K0253, Thermo Fisher, USA). Expression data was normalized in relation to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The results were presented as fold change, employing the 2^{-ΔΔCT} method. The primer sequences employed in this study are outlined in Supplementary Table 1. A 2-step reaction protocol was employed, commencing with a 95°C denaturation phase for 5 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 15 seconds.

Enzyme-linked Immunosorbent Assay

The evaluation of cell cycle markers, including cyclin D1 (sensitivity=33 pg/mL, Catalog#: ab214571, Abcam, USA) and cyclin E (sensitivity=1.2 ng/mL,

Catalog#:ab231929, Abcam, USA), pyroptosis indicators, GSDMD (sensitivity = 0.154 ng/mL, Catalog#:ab272463, Abcam, USA) and GSDME (sensitivity = 0.06 ng/mL, Catalog#:abx386859, Abbexa, Cambridge Science Park, United Kingdom), autophagy markers, microtubule associated protein 1 light-chain 3 (LC3) (sensitivity = 2.1 pg/mL ng/mL, Catalog#:ab239432, Abcam, USA) and ATG5 [autophagy related 5, sensitivity = 0.28 ng/ml, Catalog #: abx251031, Abbexa, Cambridge Science Park, UK], and inflammatory markers, IL-1β (sensitivity = 5.64 pg/mL, Catalog#:ab214025, Abcam, USA) and IL-6 (sensitivity=1.6 pg/mL, Catalog#: ab178013, Abcam, USA) was conducted using ELISA, adhering to the instructions provided by the manufacturer.

Statistical Analysis

The experiments were replicated at least 3 times, and the obtained results are presented as mean ± standard deviation (SD). The Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, version 24) and GraphPad Prism (version 8.0.2.263) were used to perform statistical analyses and provide graphs. The statistical significance was obtained using one-way ANOVA and two-way ANOVA analysis. A *p* value < 0.05 was considered statistically significant.

RESULTS

Cytotoxic Effects of Ellagic Acid on A2780 and OVCAR3 Cell Lines

EA inhibited cancer cell viability in a dose- and time-dependent manner (*p* value < 0.001) (Figure 1). The cells that were treated for 24 hours revealed the highest cell viability, and the cell viability decreased gradually after 48- and 72-hour treatments. In this regard, the viability of A2780 and OVCAR3 cells began to decrease significantly after treatment with 10 µM EA for 24 hours and intensified in higher doses and durations of treatment (*p* value < 0.001). The IC₅₀ of EA in A2780 cells was 340.8, 33.97, and 28.27 µM after 24, 48, and 72 hours of treatment, respectively. Moreover, >20000, 378.4, and 210.6 µM were determined as IC₅₀ of EA in OVCAR3 cells after 24, 48, and 72 hours of treatment, respectively. Based on the results of the present study as well as a similar recent study,²³ 48-hour treatments were considered for further investigations.

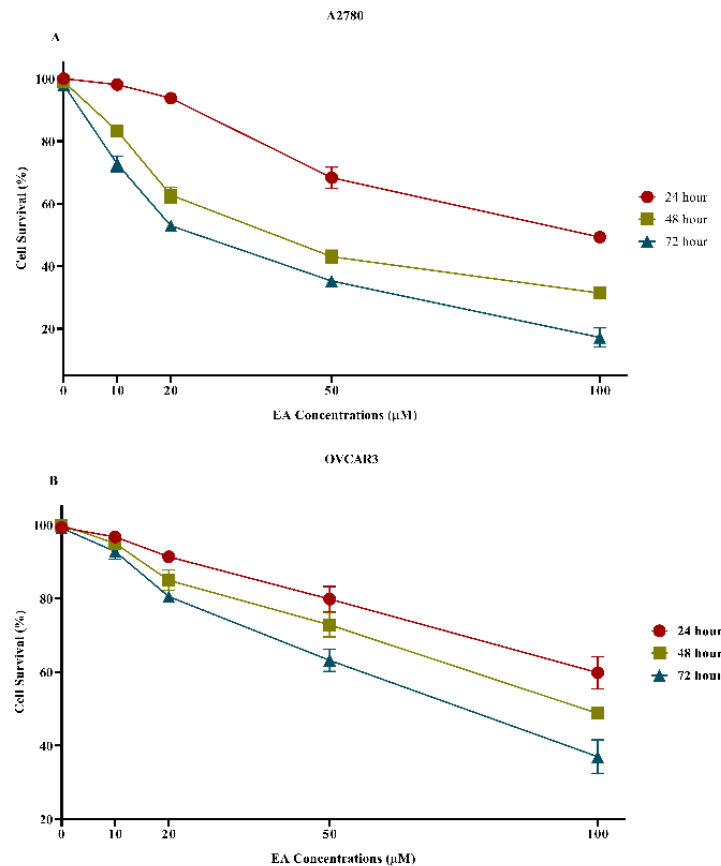


Figure 1. Cell viability after treatment with ellagic acid (EA). The survival percentage of A2780 (A) and OVCAR3 (B) cells after treatment with 0, 10, 20, 50, and 100 µM of EA for 24, 48, and 72 hours are presented.

Effects of Ellagic Acid on Cell Cycle Arrest in A2780 and OVCAR3 Cells

Expression of coding genes and levels of cyclin D (Figure 2) and cyclin E (Figure 3) were evaluated in OC cells treated with different doses of EA. The findings showed that in A2780 and OVCAR3 cells, doses of 50 (35.10% in A2780 and 34.95% in OVCAR3) and 100 (55.70% in A2780 and 54.13% in OVCAR3) µM EA significantly decreased the expression of cyclin D encoding gene (p value < 0.001). Similarly, the expression of cyclin E after treatment with doses of 50 (29.38% in A2780 and 34.40% in OVCAR3) and 100 (51.81% in A2780 and 46.99% in OVCAR3) µM EA was significantly decreased (p value < 0.001), whereas, the doses of 10 and 20 µM EA did not cause significant changes in the expression of cyclin D and cyclin E (p value > 0.05).

In addition, measuring the level of cyclin D by ELISA showed that doses of 50 and 100 µM of EA in A2780 (18.3% and 40.8%, respectively) and OVCAR3 cells (20.3% and 49.4%, respectively) had decreased this cell cycle marker significantly (p value < 0.01). Moreover, both 50 and 100 µM doses of EA significantly reduced the level of cyclin E in A2780 cells (p value < 0.001), although no significant difference was found in terms of the level of cyclin E in OVCAR3 cells after treatment with different doses of EA (p value > 0.05).

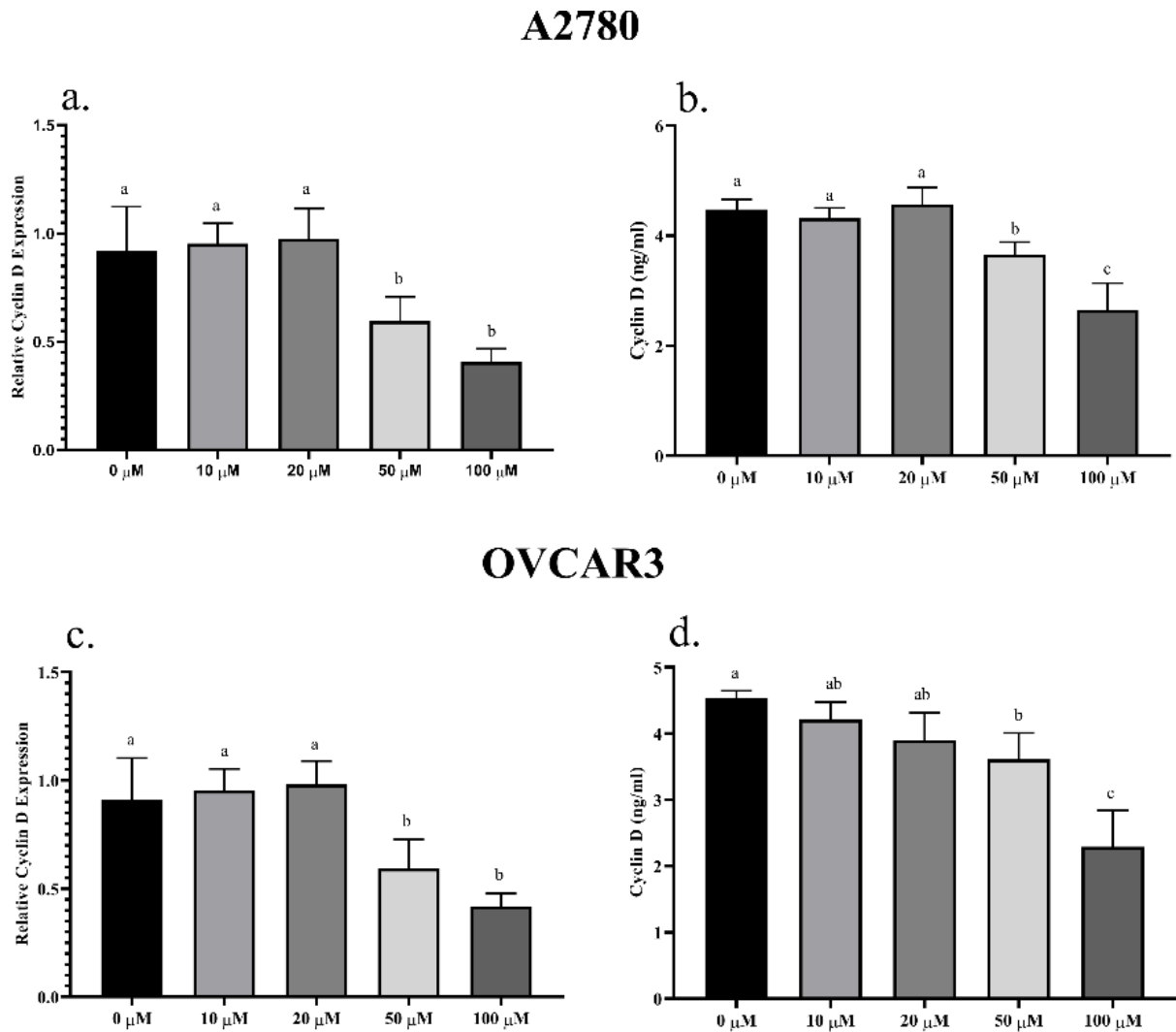
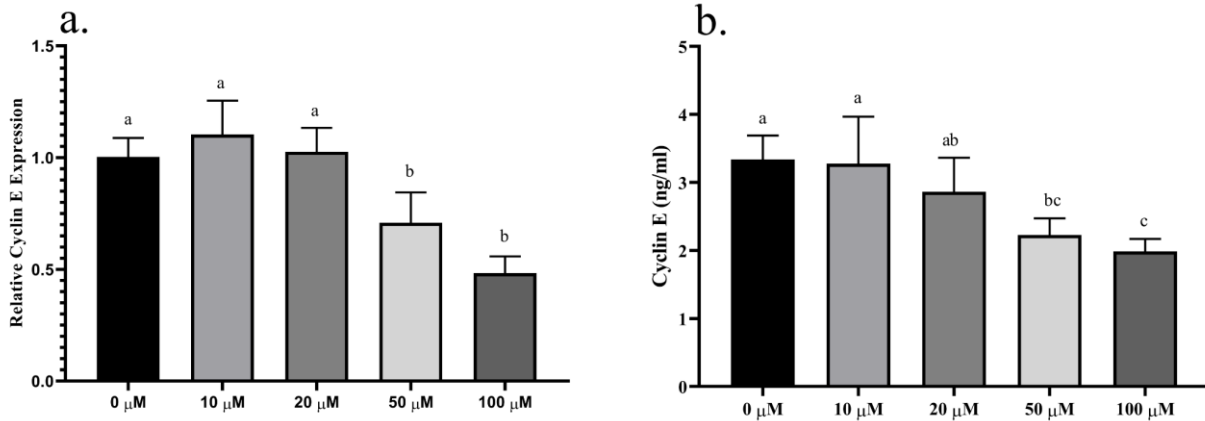


Figure 2. Ellagic Acid (EA) decreased the level of cell cycle-related markers in ovarian cancer cell lines. As shown, doses of 50 and 100 μM of EA significantly reduced the levels of cyclin D. (a) gene expression of cyclin D in A2780 cells; (b) cyclin D levels in A2780 cells; (c) gene expression of cyclin D in OVCAR3 cells; (d) cyclin D levels in OVCAR3 cells; Similar lowercase letters on the bars represent no significant difference. *p* value <0.05 was considered significant.

A2780



OVCAR3

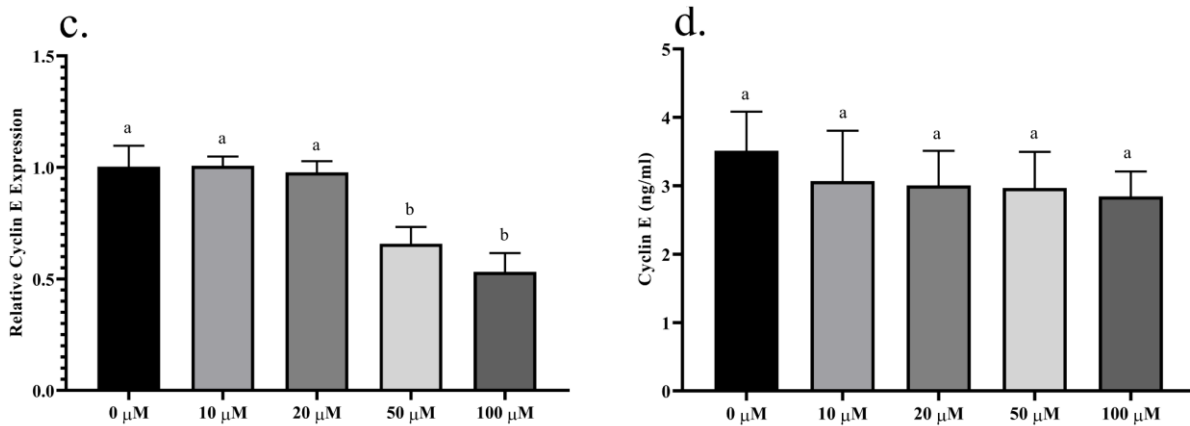


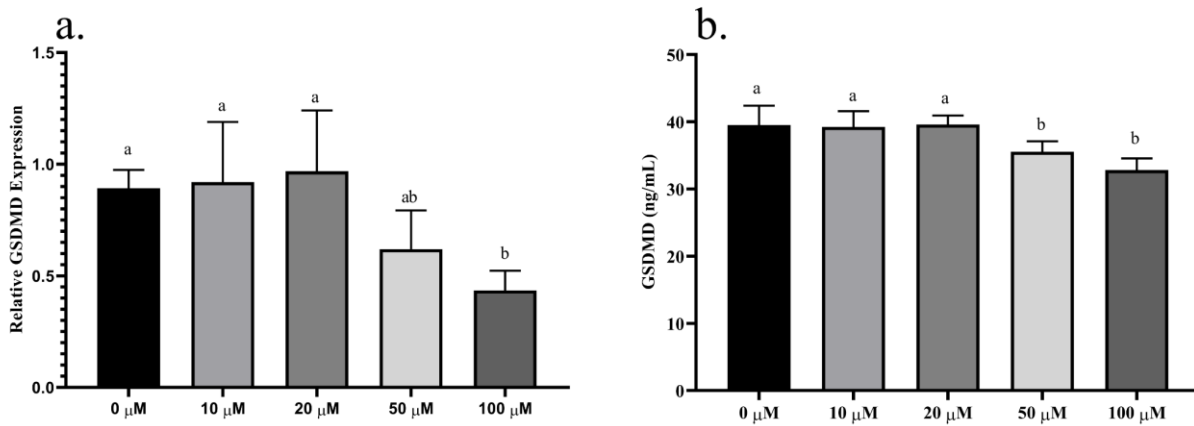
Figure 3. Ellagic Acid (EA) decreased the level of cell cycle-related markers in ovarian cancer cell lines. As shown, doses of 50 and 100 μM of EA significantly reduced the levels of cyclin E. (a) gene expression of cyclin E in A2780 cells; (b) cyclin E levels in A2780 cell; (c) gene expression of cyclin E in OVCAR3 cells; (d) cyclin E levels in OVCAR3 cells. Similar lowercase letters on the bars represent no significant difference. p value < 0.05 was considered significant.

Effects of EA on Pyroptosis in A2780 and OVCAR3 Cells

Treatment of OS cells with different doses of EA caused a significant difference in the expression of genes related to pyroptosis (Figure 4, Figure 5). In this regard, the expression of *GSDMD* in cells A2780 (51.23%) and OVCAR3 (42.54%) was significantly reduced after treatment with 100 μM of EA (p value < 0.001). Moreover, the dose of 50 μM of EA in OVCAR3 cells caused a significant decrease of 46.15%

in the expression of *GSDMD* (p value < 0.05). On the contrary, in both studied cells, increasing the dose of EA was accompanied by a significant increase in *GSDME* gene expression (p value < 0.001).

A2780



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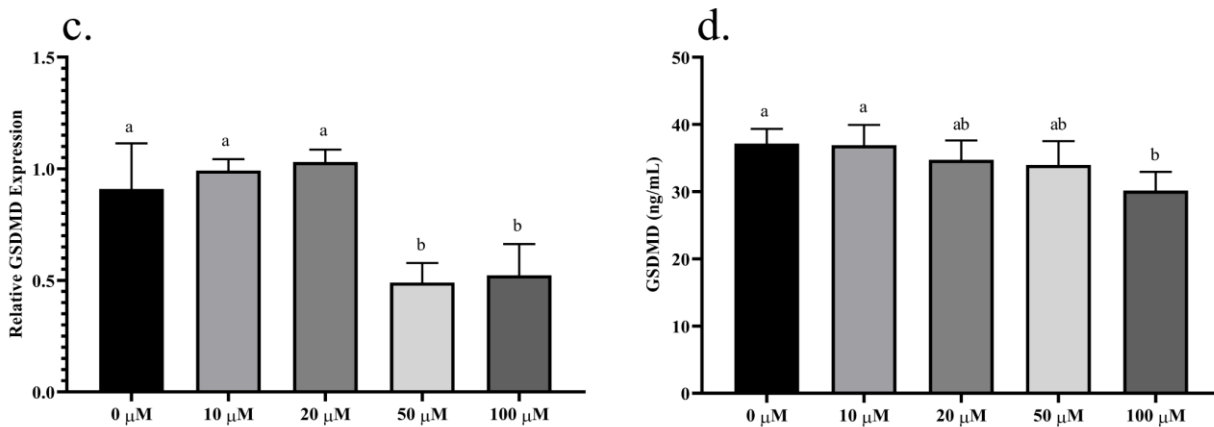


Figure 4. The levels of pyroptotic-related markers. EA altered the levels of GSDMD in human ovarian carcinoma cells. (a) gene expression of GSDMD in A2780 cells; (b) GSDMD levels in A2780 cell; (c) gene expression of GSDMD in OVCAR3 cells; (d) GSDMD levels in OVCAR3 cells; Similar lowercase letters on the bars represent no significant difference. p value<0.05 was considered significant. GSDMD: gasdermin D.

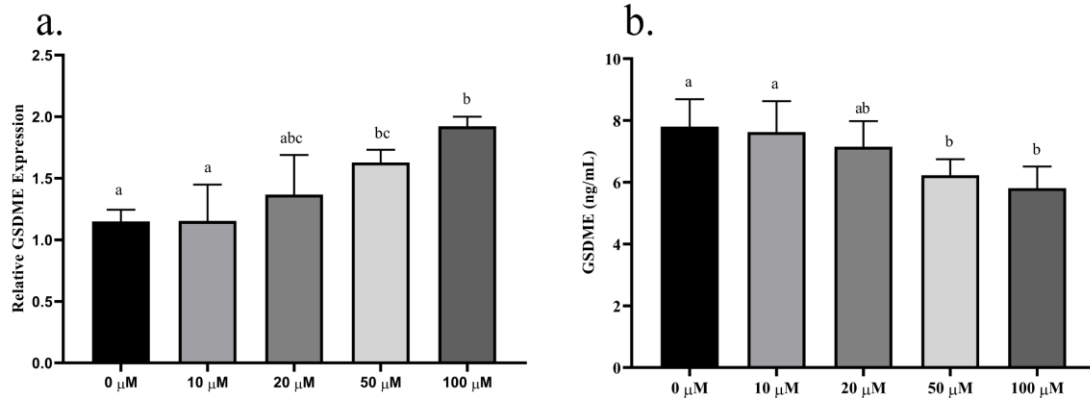
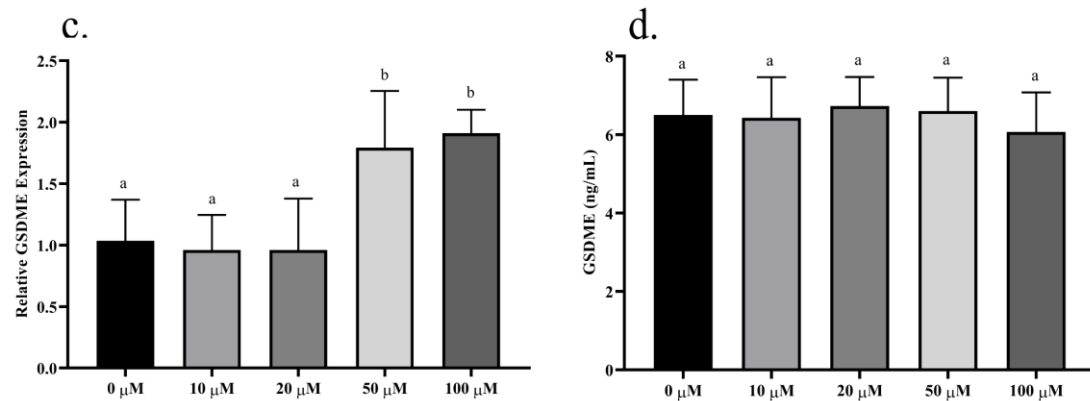
A2780**OVCAR3**

Figure 5. The levels of pyroptotic-related markers. EA altered the levels of GSDME in human ovarian carcinoma cells. (a) gene expression of GSDME in A2780 cells; (b) GSDME levels in A2780 cell; (c) gene expression of GSDME in OVCAR3 cells; (d) GSDME levels in OVCAR3 cells. Similar lowercase letters on the bars represent no significant difference. p value<0.05 was considered significant. GSDME: gasdermin E.

In A2780 cells, doses of 50 and 100 μ M of EA caused a significant decrease (p value<0.01) in the levels of GSDMD (10.1% and 17.0%, respectively) and GSDME (20.2% and 25.6%, respectively). Moreover, the dose of 100 μ M of EA significantly reduced the level of GSDMD in OVCAR3 cells, although no significant difference was obtained in terms of levels of GSDME between different doses of the substance (p value>0.05).

Effects of Ellagic Acid on Autophagy in A2780 and OVCAR3 Cells

Gene expression and protein levels of LC3 (Figure 6) and ATG5 (Figure 7) in OC cells were evaluated to

investigate autophagic flux. The findings showed that in A2780 cells, only the dose of 100 μ M of EA caused a 4.23 times significant increase in the expression of LC3 (p value<0.0001), while in OVCAR3 cells, no significant difference was found in terms of the level of LC3 after treatment with different doses of EA (p value>0.05). Additionally, the doses of 50 and 100 μ M of EA caused a 3.98 times significant increase in the expression of ATG5 in A2780 cells, although the studied doses of EA did not cause a significant difference in the expression of this gene in OVCAR3 cells (p value>0.05).

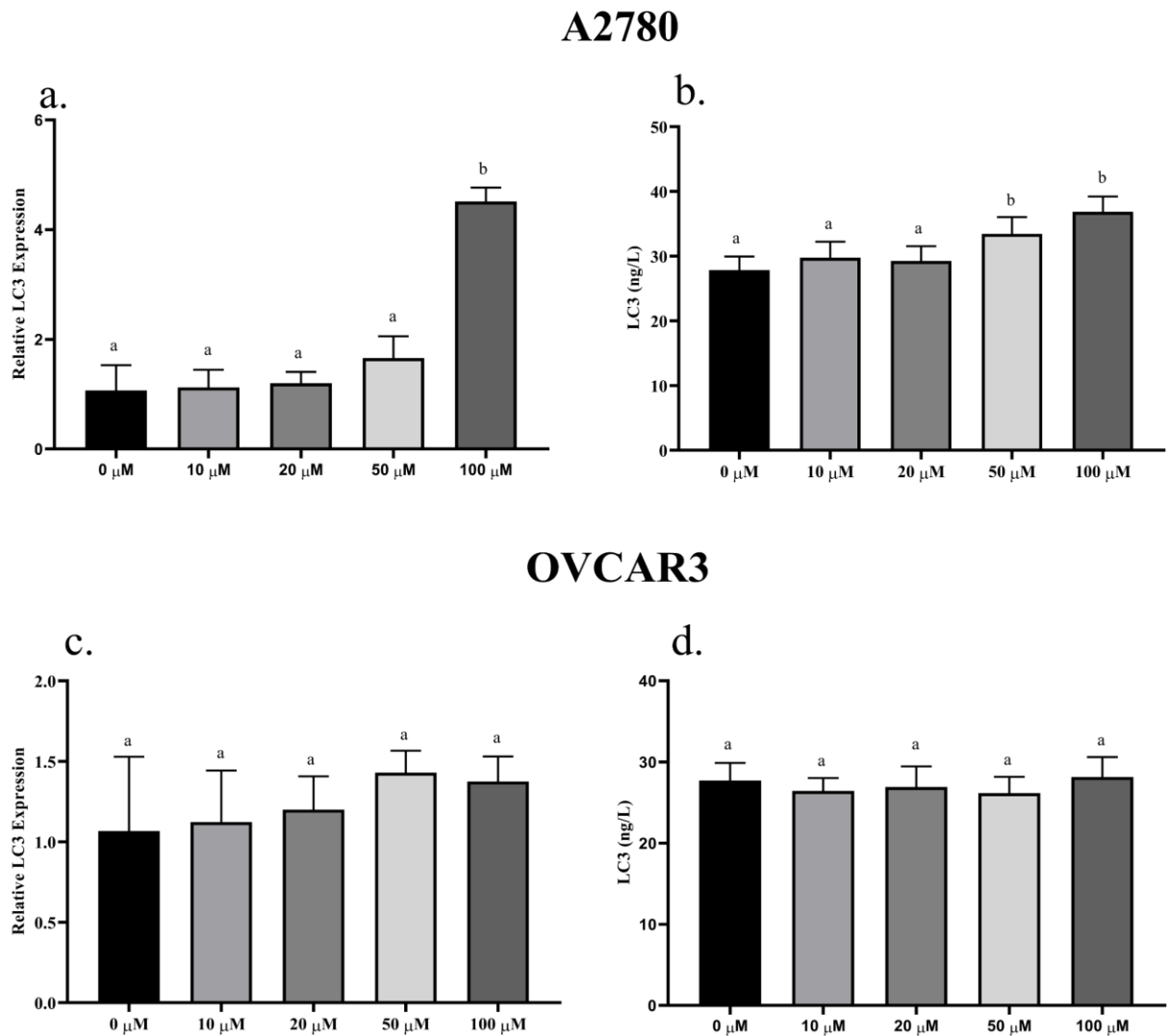


Figure 6. The levels of autophagic markers. (a) gene expression of LC3 in A2780 cells; (b) LC3 levels in A2780 cell; (c) gene expression of LC3 in OVCAR3 cells; (d) LC3 levels in OVCAR3 cells; Similar lowercase letters on the bars represent no significant difference. p value < 0.05 was considered significant. LC3: microtubule-associated proteins 1A/1B light chain 3.

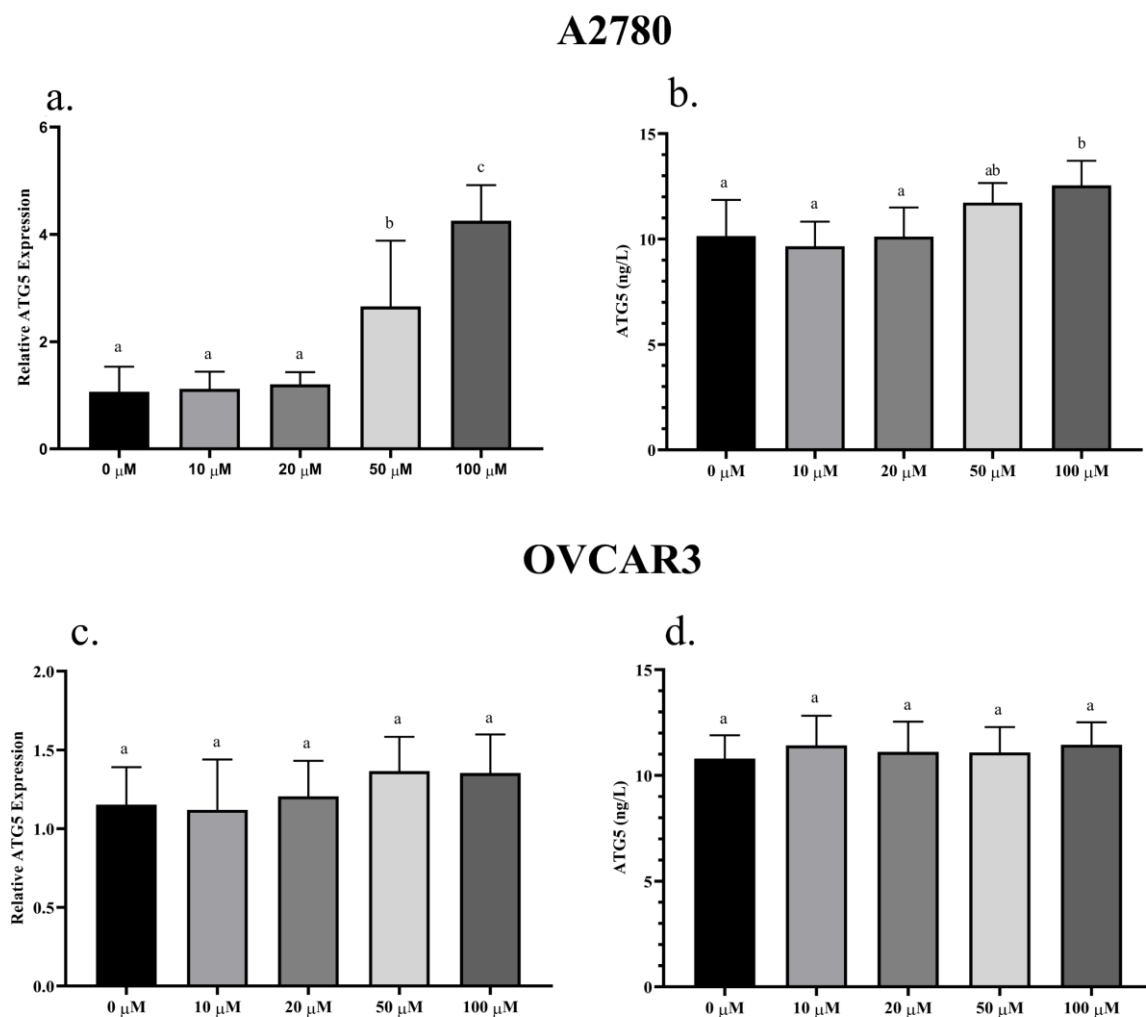


Figure 7. The levels of autophagic markers. (a) gene expression of ATG5 in A2780 cells; (b) ATG5 levels in A2780 cell; (c) gene expression of ATG5 in OVCAR3 cells; (d) ATG5 levels in OVCAR3 cells. Similar lowercase letters on the bars represent no significant difference. p value <0.05 was considered significant. ATG5: autophagy protein 5.

In A2780 cells, the doses of 50 and 100 μ M of EA caused a significant increase in the level of LC3 (1.2 times and 1.3 times, respectively, p value <0.001). In addition, EA at a dose of 100 μ M caused a significant increase of 23.8% in ATG5 level in A2780 cells (p value <0.001). On the contrary, the studied doses of EA could cause a significant change neither in the level of LC3 nor in ATG5 in OVCAR3 cells (p value >0.05).

Effects of Ellagic Acid on Inflammation in A2780 and OVCAR3 Cells

The obtained results indicated that the dose of 100 μ M of EA caused the level of IL-1 β in A2780 and OVCAR3 cells to experience a significant decrease of

21.1% and 18.2%, respectively (p value <0.001). However, the doses of 10, 20, and 50 μ M of EA did not cause a significant difference in the level of IL-1 β either in A2780 cells or in OVCAR3 cells (p value >0.05). Meanwhile, the level of IL-6 in A2780 cells showed a significant dose-dependent decrease after treatment with the studied concentrations of EA (Figure 8). Meanwhile, in OVCAR3 cells, only doses of 50 and 100 μ M of EA caused a significant decrease in the level of IL-6 (p value <0.05).

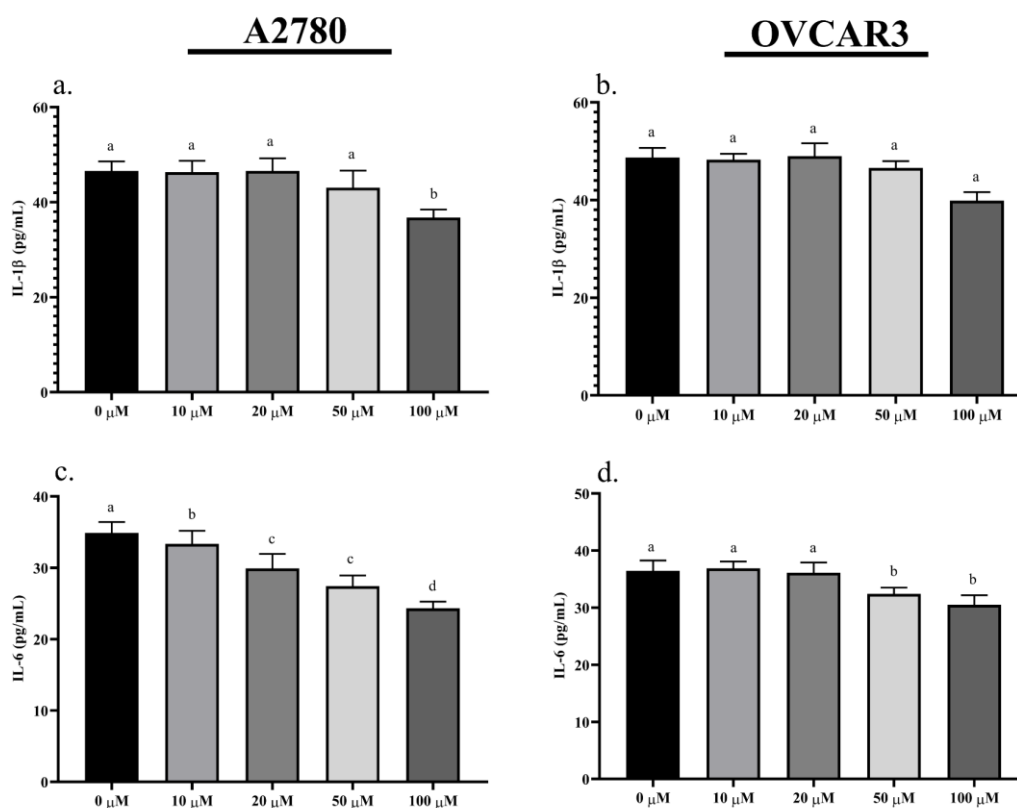


Figure 8. Ellagic acid (EA) suppressed inflammation in ovarian cancer cell lines. The levels of interleukin (IL)-1 β and IL-6 were measured in A2780 and OVCAR3 cells. (a) Levels of IL-1 β in A2780 cells; (b) Levels of IL-1 β in OVCAR3 cells; (c) Levels of IL-6 in A2780 cells; (d) Levels of IL-6 in OVCAR3 cells. Similar lowercase letters on the bars represent no significant difference. p value <0.05 was considered significant.

DISCUSSION

OC is recognized as the leading cause of gynecological cancer-related death.²⁴ Although new diagnostic and treatment strategies have increased the survival rate in recent decades, increasing prevalence at a young age, resistance to chemotherapy, and side effects caused by treatment approaches are considered the most important challenges of disease management.^{24,25} Therefore, ongoing studies are looking for novel treatment options. The present study aimed to evaluate the effect of EA on the inhibition of 2 OC cell lines by evaluating cell cycle, pyroptotic, autophagic, and inflammatory pathways.

The findings of the present study showed that EA was able to inhibit the growth of cancer cells in a dose-

dependent manner, although the IC₅₀ values in A2780 OC cells were lower compared to OVCAR3 cells. The lower IC₅₀ values show that EA has been able to inhibit the growth of cancer cells in lower doses. It has already been proven that the OVCAR3 cell line is chemoresistant, contrary to A2780 cells,^{26,27} hence it can be assumed that the higher values of IC₅₀ in OVCAR3 cells are due to its chemoresistant feature. It has been previously shown that EA inhibits OC cell lines including ES-2 and PA-1 cells through the induction of programmed cell death pathways such as apoptosis.²⁸ To further elucidate the mechanisms underlying the inhibition of A280 and OVCAR3 human OC cells by EA, the present study measured cell cycle arrest and inflammation as well as pyroptotic and autophagic pathways.

The results showed that doses of 50 and 100 μM of EA significantly reduced the gene expression and protein levels of cyclin D and cyclin E in both studied OC cell lines. It has been found that increased levels of cyclin D are major characteristics of a variety of cancers, including OC, which is associated with poor prognosis.²⁹⁻³¹ Concordantly, cyclin E is assumed as a novel target to treat OC.^{32,33} Indeed, the upregulation of cyclin D and cyclin E in cancer cells is assumed to be an indicator of elevated rates of cell proliferation and therefore disease progression.^{34,35} Therefore, the decrease in the level of cyclin D and E by EA can be attributed to its ability to inhibit the proliferation of OC cells. Similarly, phytochemicals such as quercetin and resveratrol have been proposed as a new option for the treatment of OC due to their ability to inhibit the proliferation of cancer cells by downregulating the levels of cyclins.^{36,37}

Immortality is considered a main feature of cancer cells, thereby the viability of OC cells crucially requires compromising with different mechanisms related to cell death.³⁸ Nowadays, the mechanisms involved in programmed cell death fall into 2 main subgroups: apoptotic (including apoptosis) and non-apoptotic (including pyroptosis, autophagy, and ferroptosis) cell death programs.³⁸ Pyroptosis is an inflammasome-induced type of programmed cell death that is mediated by GSDMs. GSDMs are a group of pore-forming proteins that are responsible for membrane permeabilization leading to pyroptosis.³⁹ The findings of the present study showed that EA in doses of 50 and 100 μM caused a significant downregulation of GSDMD in both studied OC cells, although downregulation of GSDME occurred only in A2780 cells after treatment with EA. It was previously shown that the increase in GSDMD level is an indicator of OC progression.⁴⁰ Interestingly, the induction of pyroptosis was assumed to be one of the mechanisms involved in OC management.^{41,42} In addition, it is demonstrated by Berkel et al that in ovarian tumors GSDMD is significantly upregulated and GSDME is downregulated in comparison with surrounding nontumoral tissue.⁴³ Contrary to the findings of the present study, the level of GSDME in OC was reported to be lower than that of healthy ovarian samples.⁴⁰ Genetic variation in genes encoding GSDMs may be one of the main reasons for the inconsistencies in the findings related to GSDME levels, although further studies are pivotally required to clarify this issue.

Along with pyroptosis, autophagy is another type of programmed cell death, which with a dual approach may lead to both cell survival and regulated cell death. Indeed, autophagy may inhibit carcinogenesis by inhibiting the transition of normal to tumor cells, although it may promote cell proliferation by providing energy to cancer cells. In OC, autophagy promotion has been shown to induce resistance to chemotherapy.⁴⁴ Induction of autophagy has also been demonstrated to increase the survival of OC cells.⁴⁵ On the contrary, some chemicals are suggested to inhibit ovarian tumors by inducing autophagy and apoptosis.^{46,47} The findings of the present study revealed that the administration of EA significantly induced autophagy in A2780 cells, although there was no significant change in OVCAR3 cells. As mentioned earlier, OVCAR3 cells are considered chemoresistant OC cell lines, hence in these cells, it seems that pyroptosis may contribute as the main mechanism of cell death.

A plethora of evidence has shown that the growth and proliferation of cancer cells are accompanied by increased production of inflammatory cytokines.^{48,49} In fact, increased levels of inflammatory mediators such as IL-1 β and IL-6 are vitally present in the tumor microenvironment and provide the necessary conditions for tumor progression.^{50,51} The results of the present study showed that EA in high doses has significantly reduced the level of IL-1 β and IL-6. Anti-inflammatory activity has been assumed to be one of the main features of phytochemicals such as EA, quercetin, and resveratrol, which has made these compounds suggested as favorable options for cancer management.⁵²⁻⁵⁴

The findings of the present study revealed that EA is able to inhibit the growth of 2 human ovarian tumor cell lines, including A2780 and OVCAR3, via downregulation of cyclin D1 and cyclin E, suppress inflammation, and prevent pyroptosis. Thereby, it appears that EA arrests the cell cycle in ovarian cancer cell lines via the inhibition of inflammation-mediated pyroptosis, hence it may be considered a therapeutical option to confront ovarian cancer. Nevertheless, further *in vitro* and *in vivo* investigations along with complementary clinical trials are required to elucidate the possible role of ellagic acid in ovarian cancer treatment.

STATEMENT OF ETHICS

No human or animal samples were used in the current study.

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

Not applicable.

Data Availability

The data are available upon reasonable request from the corresponding author.

AI Assistance Disclosure

No artificial intelligence (AI) tools were used in preparing this manuscript.

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