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Pyrogallol acts as a novel anticancer factor to enhance the sensitivity to cisplatin in ovarian cancer cells through inducing miR-15a upregulation

Azin Shirani¹, Razieh Mirabbasi², Leila Shabani³ and Somayeh Reisi^{1*}

Abstract

Introduction Pyrogallol is a catechin compound, as an active ingredient extracted from *Embllica officinalis*, has anti-proliferative and antiapoptotic effects on some of the human cancer cells. Cisplatin is known as an effective chemotherapeutic agent against cancer by inducing DNA damage and apoptosis. However, the effect of pyrogallol alone and in combination with cisplatin on proliferation and apoptosis of ovarian cancer has not yet been reported.

Methods In the present study, ovarian carcinoma cell line A2780 was cultivated, and the anticancer properties of pyrogallol and cisplatin determined by cell treatment of the two drugs separately and combination. Cell viability and apoptosis rate was investigated by MTT assay and flow cytometry. The effects of the compounds in inhibiting colony formation and migration were measured by colony formation and scratch methods.

Results The results of the present study revealed that pyrogallol significantly inhibited the proliferation of A2780 cells in a dose-dependent manner. The combination treatment of pyrogallol and cisplatin exhibited a prominent inhibitory effect on the growth, colony formation and migration, and increased the induction of apoptosis up to 2.5 times compared to single treatments. Also, the qPCR results showed a significant increase in the expression of miR-15a and a decrease in the expression of miRNA target genes under treatment with the drugs both separately and in their combination.

Conclusion This study suggests that the combination treatment of pyrogallol and cisplatin significantly increases the death of ovarian cancer cells by inducing apoptosis. Therefore, it can boost the efficacy of chemotherapy and reduce its side effects. It is hoped that the findings of the present study can provide a new perspective for pyrogallol as a therapeutic agent in the treatment of ovarian cancer.

Keywords Pyrogallol, Cisplatin, Ovarian cancer, Cell proliferation, miR-15a

Introduction

Ovarian cancer is the most lethal malignancy of the female reproductive system and has the fifth highest mortality rate among women's cancers [22]. The risk of developing ovarian cancer during a woman's lifetime is 1 in 75, and the probability of her dying from this disease is 1 in 100. The disease is rarely diagnosed in the early stages due to the absence of specific symptoms; And since most patients are diagnosed with an advanced stage

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of the disease, it causes an increase in mortality among all women's malignancies [31]. In fact, more than 70% of ovarian cancers are not diagnosed until the disease has progressed to stage III or IV [35]. Pathologically, over 90% of ovarian cancers originate from the epithelial surface of the ovary. And the others are related to germ or stromal cells [37]. The standard treatment for advanced ovarian cancer is primary cytoreductive surgery followed by platinum-based chemotherapy. Cytoreductive surgery is performed to confirm the diagnosis, remove poorly perfused tissue, which may be the site of disease, and reduce tumor volume to enhance chemotherapy [27]. However, despite promising clinical results, many studies have reported noticeable side effects associated with cisplatin monotherapy, while others have shown significant drug resistance in some cancer patients [5]. Both drug resistance and safety issues in cancer monotherapy with cisplatin have led to the development and implementation of new treatment strategies that include combination therapies with other anticancer drugs such as doxorubicin, gemcitabine, paclitaxel, or herbal drugs such as berberine, curcumin, and numerous polyphenols and flavonoids [36, 41]. Evidence from numerous in vitro and in vivo studies shows that many medicinal plants contain bioactive compounds that are promising candidates for cancer treatment and medicinal uses. It has been shown in preclinical studies that the combination of natural products with cisplatin not only increases its therapeutic activity but also reduces the toxicity due to chemotherapy [7]. Therefore, the combination of anticancer drugs increases efficacy compared to a monotherapy approach, as it targets key pathways synergistically or additively [9]. This approach potentially reduces drug resistance, while simultaneously providing anticancer therapeutic benefits such as reducing tumor growth and metastatic potential, arresting mitotically active cells, reducing the cancer stem cell population, and inducing apoptosis [2].

Pyrogallol is a simple phenolic compound which is also known as pyrogallol acid or 1,2,3-trihydroxybenzene. Pyrogallol is a reductant capable of producing free radicals, especially superoxide anions ($O_2^{\bullet-}$) [15]. This compound is found in various plants, including *Emblica officinalis*, *Terminalia chebula* (black nightshade), *Jatropha neopauciflora*, *Jatropha karx*, *Entada abyssinica*; tea and coffee; *Cassia siamea*, *Labisia pumila*, *Rumex obtusifolius* [34]. Pyrogallol has various biological properties such as antibacterial, antioxidant, antifungal, antiviral, disinfectant, anti-dermatitis, cardioprotective, and anti-mutagenic. In recent studies, its anticancer properties have also been demonstrated [29]. Based on available evidence, pyrogallol produces antiproliferative effects on human tumor cell lines such as K562 (chronic myelogenous leukemia), Jurkat (lymphoma T), Raji (lymphoma

B) and HEL (erythroleukemia) and prevents the growth of tumor cell lines [20]. Given the above-mentioned findings and arguments, in this study, the effect of the combination of cisplatin and pyrogallol in inhibiting the proliferation and migration of ovarian cancer cells was investigated with the aim of increasing the sensitivity of cancer cells to cisplatin.

Material and methods

Cell line and reagents

The A2780 human ovarian cancer cell line was obtained from the cell bank of Pasture Institute of Iran (NCBI). Pyrogallol was procured from Sigma-Aldrich Chemical Co. (St. Louis, MO) and was dissolved in distilled water at 100mM as a stock solution. As well as, 2,2-diphenyl-1-picrylhydrazil (DPPH), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma. All solution obtained in molecular grade. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [38].

Cell cytotoxicity

The A2780 human ovarian cancer cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium complemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Sigma, USA). Cells were sustained at 37 °C under 5% of CO₂ and 95% humidity. After every 4 days, old media was changed with fresh media, to stimulate growth until the cells reach a confluence of 75–80%. The MTT assay was performed to investigate the cytotoxicity and viability percentage of A2780 cells in the treatment with cisplatin and pyrogallol individually and in combination with each other. The cells were cultured in 96-well plates at the number of 1×10^4 cells/well and were incubated for 24 h at 37 °C until the cells were attached to the cell culture plate. Then, the medium of the cells was replaced with the drug treatment medium. Cells were treated with cisplatin (at 0–6 µg/ml) and pyrogallol (at 0–20 µg/ml) separately for 48 h. To conduct the combination treatment, concentrations of 0.5, 1, 3 and 6 of pyrogallol were combined with concentrations of 0–6 µg/ml of cisplatin. After 48 h of cell treatment, to investigate cell viability, 20 µl of MTT solution (5 mg/ml) was added to each well of the plate and the cells were incubated for 3 h at 37 °C in the dark. Then, 100 µl of DMSO was added to the wells to dissolve the formed formazan crystals, and optical absorbance was measured by plate reader at 490-nm wavelength. The percentage of cell viability in the treatment with the studied drugs was calculated using the formula below:

$$\% \text{Cell viability} = \frac{\text{Absorbance of treated cells} - \text{Absorbance of blank}}{\text{Absorbance of untreated cells} - \text{Absorbance of blank}} \times 100$$

Synergistic effect of pyrogallol and cisplatin

Compusyn software was used to study the synergistic or antagonistic effect of cisplatin and pyrogallol. Basically, one of the important factors in pharmacology and combination drug therapy is an index called Combination Index (CI). This index is a measure of the effect of the combination of drugs and shows that the combination drug has a synergistic or antagonistic effect. The software uses the following formula to calculate the index:

$$CI = (D)A/(Dx)A + (D)B/(Dx)B$$

where, (D)A and (D)B represent the doses of drugs A and B, respectively, which must be combined to obtain effective Fa. As well, (DX)A and (DX)B indicate the doses of drugs A and B, respectively, that are used individually to obtain effective Fa. $CI < 1$ represents the synergistic effect of medicinal compounds, $CI = 1$ additive effect and $CI > 1$ the effect of the combination drug as antagonism [6].

Colony formation

Clonogenic assay or colony formation assay is an in vitro cell viability assay based on the ability of a single cell to grow and form a cell colony. A cell colony is defined as at least 50 cells. This assay essentially tests each cell in the population for its ability to undergo unlimited division [4].

This method can be used to determine the effect of drugs and agents with cytotoxic effects. In the present study, to perform the colony formation test, first, 500 cells were cultured in each well of a 12-well plate. After 24 h, the treatment with the IC10s of studied drugs including pyrogallol, cisplatin and pyrogallol/cisplatin combination was done. After 48 h, the drug medium was replaced with fresh medium and the cells were incubated at 37 °C for 14 days. Then, to stain the colonies, the supernatant of the cells was removed and 1 ml of the mixture of ethanol and acetic acid was added to each well. After the cells were fixed, crystal violet stain was added to the wells and then the wells were incubated for 1 h at room temperature. In the next step, washing was done with distilled water and after the plate dried, the colonies were counted. The ratio of the number of colonies to the number of cultured cells was calculated.

Migration assay

In the scratch test method, the attached cells on the bottom of the cell culture plate are scratched and a

cell-free area is created. The migration of cells from around the scratched area toward the center is analyzed by imaging. In this study, cells were cultured at a density of 95% in 12-well plates to examine the effect of drug treatment on the inhibition of cell migration. A vertical scratch was made in the middle of the well by the tip and the isolated cells were washed with PBS and the medium containing pyrogallol, cisplatin and the combination of the two agents at a concentration of IC10 was added to each well. Imaging of the cells at the scratch area was done at 0, 24, 48 and 72-h intervals. The extent of the scratch area at different intervals was calculated using ImageJ software.

Flow cytometry

The amount of cell apoptosis was determined by annexin V-fluorescein isothiocyanate (FITC) staining and PI labeling according to the protocol of kit's manufacturer. Annexin V can be used to identify the externalization of phosphatidylserine throughout the progression of apoptosis, and therefore can identify cells in the early stages of apoptosis. For the investigation of apoptosis rate under the treatment of studied drugs, 1×10^6 cells/well were cultured. After 24 h, the cells were treated with the IC30 of pyrogallol and cisplatin alone and their combination. After 48 h of cell treatment, the cells were separated by trypsin and then the cell mass was separated by centrifugation. The collected cells were washed twice with cold PBS and then, 90 µl of annexin binding buffer (10mM HEPES/NaOH, pH 7.4, 140mM NaCl, 2.5mM CaCl₂) was added to the cells and the cells were completely suspended in the buffer. Then 5 µl of annexin V-FITC and PI were added to the cells and the cells were incubated for 20 min in the dark, and then they were examined using a flow cytometry device. Viable cells are negative for both annexin V-FITC and PI stain. Early apoptotic cells are positive for annexin V-FITC and negative for PI, and late apoptotic cells are strongly positive for both staining. Necrotic cells are displayed as PI positive and annexin V-FITC negative.

Investigation of the antioxidant capacity of pyrogallol

DPPH is a stable chromogenic radical with dark purple color and its inhibition test is done based on electron donation of antioxidants to neutralize DPPH radical. This reaction is accompanied by the color change of DPPH, and the color change serves as an indicator of antioxidant effectiveness. The result obtained for a compound is generally compared with a conventional antioxidant compound such as ascorbic acid. To perform the test, a 100 µM alcoholic solution of DPPH was prepared and to investigate the antioxidant activity, it was mixed with an equal amount of pyrogallol (at 0–20 µg/ml). The samples

were incubated for 30 min in the dark at room temperature and then absorbance was read by a spectrophotometer at 517 nm wavelength. Prepared DPPH substance is used as a blank. Next, the scavenging rate of free radicals in DPPH solution is calculated with the following formula:

$$\begin{aligned} &\text{DPPH scavenging effect (\%)} \\ &= (A \text{ Blank} - A \text{ Sample}) / (A \text{ Blank}) \end{aligned}$$

Measurement of cellular superoxide dismutase activity

Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion into hydrogen peroxide and molecular oxygen. In this assay, SOD activity is measured using tetrazolium salt, which produces a water-soluble formazan dye after reduction with superoxide anion. The rate of formation of formazan is limited by the presence of SOD in the environment and it can be measured photometrically, in such a way that an increase in the activity of SOD causes a decrease in the amount of color and lower absorption. In this study, in order to check the level of SOD activity, cells were cultured at the rate of 1×10^6 cells/well in a 6-well plate. After 24 h, the cells were treated with concentrations of 0, 1, 3, 6, 10, 12 and 20 µg/ml of pyrogallol and incubated for 48 h at 37 °C in a CO₂ incubator. Then, the cells were separated without using trypsin and with the help of scraping and were lysed by repeated freezing and thawing in cold PBS. After centrifugation, the cell supernatant was collected and reagents were added for SOD assay according to the kit protocol. After 20 min of incubation at 37 °C, absorbance reading was done by a plate reader at 450-nm. The amount of SOD inhibitory activity was calculated by the following formula:

$$\begin{aligned} &\text{SOD Activity(\%)} \\ &= \frac{(A \text{ blank1} - A \text{ blank3}) - (A \text{ sample} - A \text{ blank2})}{(A \text{ blank1} - A \text{ blank3})} \times 100 \end{aligned}$$

qPCR analysis

qPCR was used to determine the effect of the studied compounds on the apoptotic pathway (CASP-3, -8, -9, BCL2) as well as the expression of mir-15a and two of the target genes involved in cell proliferation pathway and metastasis (CDC25A, KRAS). First, 1×10^6 cells/well were cultured in a 6-well plate. Then the cells are treated with IC₃₀ concentration of pyrogallol, cisplatin and pyrogallol-cisplatin combination. After 48 h, RNA was extracted using RNX-Plus solution. The cDNA synthesis of miR-15a was completed by specific miRNA primers in the form of stem-loop and MMLV enzyme. Oligo-dt and random-hexamer primers were used for total cell

cDNA synthesis. The real-time PCR was completed by specific primers for miR-15a, miRNA target genes as well as genes of the apoptotic pathway using the SYBR Green method. The U6 gene was used as miRNA internal control and GAPDH as internal control for target genes. Amplification was conducted with an initial denaturation for 5 min at 94 °C, followed by 35 cycles of amplification (94 °C for 15s, annealing temperature for 10s, and 72 °C for 10s) using Mic qPCR cycycler (Bio-Molecular Systems). All amplification reactions were performed in triplicate. Relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method.

. Statistical analysis

Prism software was used to investigate the results of data analysis related to migration test, colony formation, antioxidant activity, flow cytometry and also to calculate the expression level in the treated and control samples and compare them. To make comparisons two group, t-test was used and ANOVA test were used for comparison of more than two groups. *P*-value < 0.05 was considered as a significance level.

Results

Effects of pyrogallol and cisplatin alone or in combination on A2780 cell proliferation

In the present study, ovarian cancer cells A2780 were treated with different concentrations of pyrogallol and cisplatin separately. Cell viability significantly decreased in the treatment with pyrogallol and cisplatin with increasing dose. Also, the cell morphology changed significantly due to the increase in the concentration of the drugs, so that these changes were clearly visible at concentrations above 6 µg. As Fig. 1A illustrates, the treatment of A2780 cells with pyrogallol caused a significant decrease in cell viability at concentrations of 3 to 20 µg after 48 h. This reduction was observed from 20% of viability at 3 µg/ml to 60% of viability at 6 µg/ml. In addition, treatment with cisplatin at 48-h interval showed a significant decrease in all studied concentrations (Fig. 1B). IC₅₀ for pyrogallol treatment was calculated at 5.32 µg and for cisplatin at 1.39 µg. For the combination treatment, four concentrations of pyrogallol were selected to combine with different concentrations of cisplatin. Significantly, the treatment at all concentrations caused a greater decrease in viability compared to the single treatment. The decrease in viability at concentrations of 0.5 µg/ml of both compounds was also clearly visible (*p* < 0.01) (Fig. 1C). The IC₅₀s of the combination concentrations are shown in Table 1. The results obtained from the combination of pyrogallol and cisplatin caused an increase in this effect compared to single treatment, so that at high concentrations of pyrogallol, the consumption of

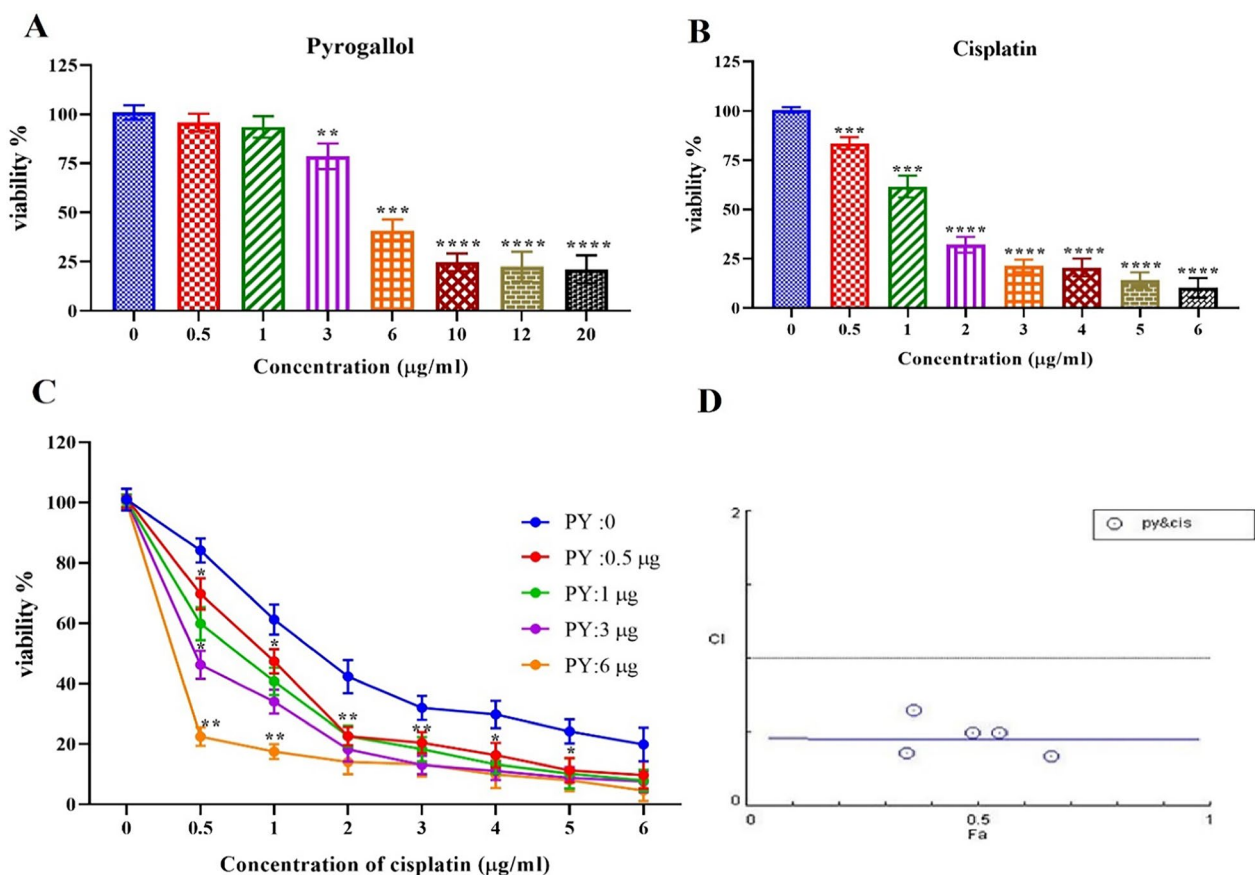


Fig. 1 Combination of cisplatin and pyrogallol repressed ovarian cancer cell growth. A2780 cells were treated with the different concentrations of pyrogallol (A), cisplatin (B), and their combination (C). Combination treatment showed a more cytotoxicity effect on A2780 cells compared to each agent alone. (D) CI Value-Fa (Fa: fraction affected level) curve for pyrogallol and cisplatin generated from CompuSyn. It demonstrated that synergistic effect is starting from 35% effective level (Fa=0.35) and this synergistic effect continues to other drug levels in pyrogallol and cisplatin. Data displays the mean and the standard deviation from three independent experiment (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

cisplatin decreased. Compusyn software was used to confirm the increase in the effect of drug combination and synergistic mode. In the Fa-CI plot, drug-related doses had a CI less than 1, which indicates drug synergism (Fig. 1D). In the combination of pyrogallol and cisplatin at constant and variable concentrations, CI was less than 1 in all cases. The CI values for constant concentrations are shown in Table 2.

Inhibition of colony formation

In the present study, the colony formation test was used to investigate the effect of pyrogallol, cisplatin and their combination on the ability of colony formation in A2780 ovarian cancer cells. Figure 2A illustrates the colonies formed in this treatment compared to the untreated control sample. It was clearly observed that the number of colonies in the cells treated with pyrogallol and cisplatin decreased compared to the controls, and the combination of these two drugs led to a further reduction in the

number and size of colonies, which can be a confirmation of the synergistic effect of pyrogallol and cisplatin. Also, according to the diagram in Fig. 2B, the ratio of the number of colonies to the number of cultivated cells in the combination of two drugs has decreased significantly compared to the alone treatment, so that in the control samples, the ratio of colony formation was calculated at 50%, in the pyrogallol- and cisplatin-treated samples, 30% and 25%, respectively, and in the combination of two drugs, at 15%.

Reduced in migration rate

In order to determine the effect of the studied drugs on the cell migration inhibition rate, a scratch test was used. To this end, the cells were scratched on the bottom of the plate and treated with IC10s of pyrogallol, cisplatin and their combination. Then, images of the scratch area were taken in the control and treatment

Table 1 Primer sequence used in this study for gene expression

Gene name	Sequence (5' -> 3')	Product size
CASP3	F: TTCTTCACCATGGCTCAGAAGCACAC R: AAGCACTGGAATGACATCTCGGTCTG	202 bp
CASP8	F: ACAAGGGCATCATCTATGGCA R: TCTCGTTTGAGGTGATGATAAATC	218 bp
CASP9	F: GTGGATTGCACGTGGCCTCTT R: TTGGCACCACCTCAGGAAGACG	216 bp
BCL2	F: ACTTTGCAGAGATGTCCAGT R: CGGTTCAGGTACTCAGCAT	217 bp
CDC25A	F: ACTGCGAGTTTTCTTCTGAGAG R: AGGACATACAGCTCAGGGTAG	109 bp
KRAS	F: AGTGCTTGACGATACAGCTA R: CCTGCTGTGTCGAGAATATCC	131 bp
GAPDH	F: ACAACTTTGGTATCGTGAAGG R: GCCATCACGCCACAGTTTC	101 bp
miR-15a	Stem: GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGAC CACAAAC F: GCGGCTAGCAGCACATAATGG R: CTGTGTGCAGGGTCCGAGGT	–
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT	–

Table 2 IC50 for cisplatin in combination with pyrogallol and combination index (CI) for constant treatment

Pyrogallol concentration (µg/ml)	IC50 of cisplatin (µg/ml)	CI
0	1.39 ± 0.73	–
0.5	0.96 ± 0.28	0.28779
1	0.77 ± 0.41	0.51702
3	0.47 ± 0.89	0.52200
6	0.32 ± 0.69	0.21658

groups at intervals of 0, 24, 48 and 72 h. As Fig. 3A illustrates, the scratched area in the cells treated with pyrogallol and cisplatin alone, compared to the cells in the control group at 24 and 48 h, included less migration of cells. In addition, pyrogallol and cisplatin combination treatment had a much greater effect on preventing the migration of ovarian cancer cells than either of these drugs alone, and the scratched area was observed almost free of cell migration, which indicates the synergistic effect of these two compounds. Figure 3B illustrates the percentage of open wound area in different treatments calculated by ImageJ software. According to this figure, the extent of the scratch area in the combination treatment does not differ much between 0 and

72 h, and the filling of the scratch occurred to a very small extent in this treatment.

Apoptosis induction

The amount of apoptosis induced by pyrogallol and cisplatin individually and by their combination was determined by Annexin V/PI staining. As illustrated in Fig. 4A, treatment with pyrogallol or cisplatin significantly increased the number of apoptotic cells. In the combination treatment, the increase in apoptosis was significantly observed compared to single treatments. In calculating the percentage of these cells according to the division of different parts of the diagram based on the percentage of positive and negative cells for each color, the percentage of cells at early apoptosis in the treatment with cisplatin and pyrogallol was 9.62% and 9.37%, respectively. The final apoptosis rate was calculated at 15.7% and 6.03%, respectively. The early and final apoptosis rates in the combination treatment were 23.3% and 26.3%, respectively (Fig. 4B). Therefore, the co-treatment with the two drugs increased the rate of apoptosis in ovarian cancer cells.

Radical scavenging potential

Free radicals are molecules that do not have a complete electron shell, which causes the chemical reaction to boost. Free radicals play a significant role in causing cancer, and to remove these active molecules, antioxidants serve as a broom and remove them from cells. DPPH measurement is a quick and easy way to determine the antioxidant activity of pyrogallol using a spectrophotometer. For this purpose, concentrations of 0 to 20 µg/ml of pyrogallol were prepared with 96% alcohol, and after reading the absorbance according to the formula, the percentage of free radical scavenging effect was obtained for each of the concentrations and the respective graph was drawn (Fig. 5A). The antioxidant activity of pyrogallol was measured in comparison with the standard compound ascorbic acid. Based on the obtained results, the percentage of DPPH scavenging activity and consequently the antioxidant activity of pyrogallol increased with increasing concentration, so that the highest level of free radical scavenging activity was observed at 3 µg/ml. Also, compared to ascorbic acid, pyrogallol exhibited a higher antioxidant activity.

SOD activity

To measure the activity of superoxide dismutase (SOD), treatment with different concentrations of pyrogallol (0 to 20 µg/ml) was done. According to the results obtained from the assay, with the increase in pyrogallol concentration, the enzyme activity also increased (Fig. 5B). At zero concentration of pyrogallol, the activity of SOD was

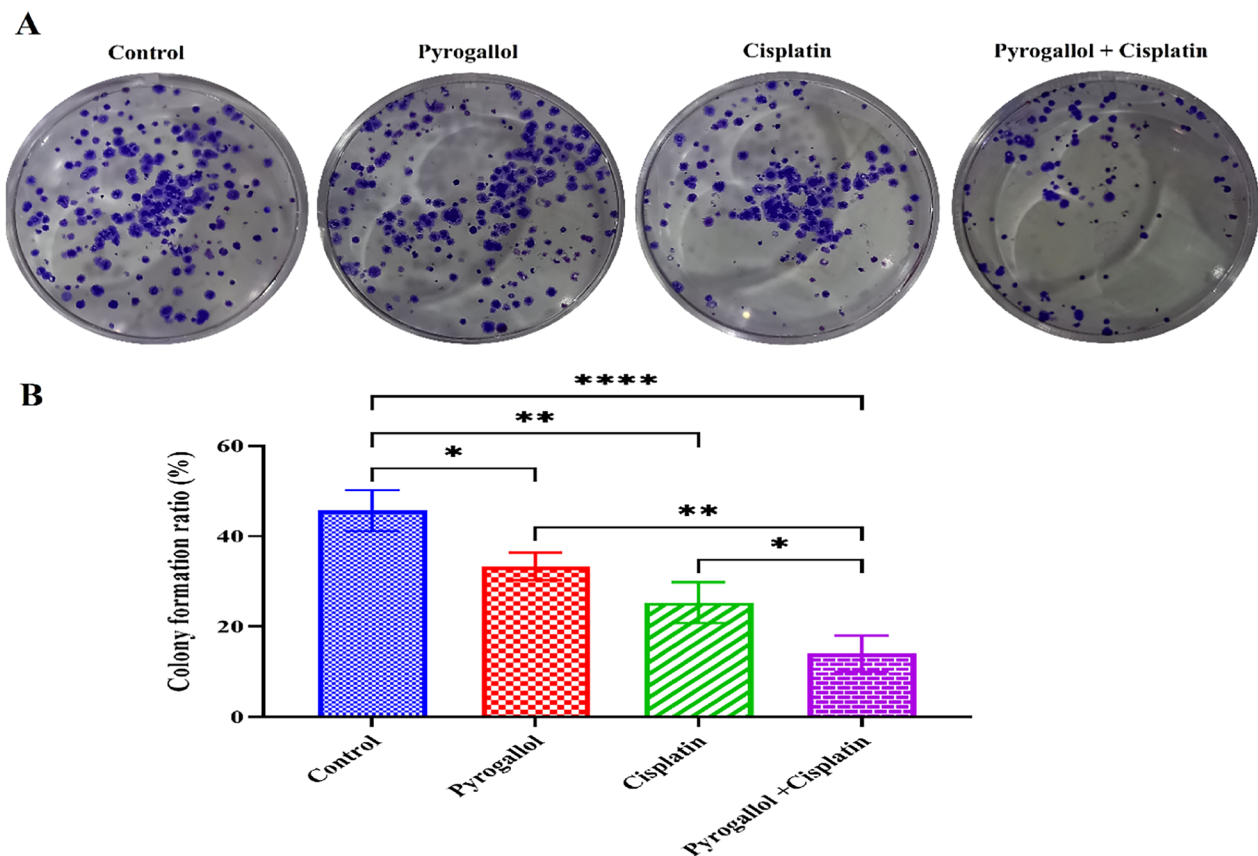


Fig. 2 Assessment of colony inhibition of pyrogallol and cisplatin alone or in combination on A2780 cell line. **A** Clonogenic assay was evaluated after 2 weeks of treatment, and wells were stained with crystal violet at the end of the experiment. **B** the colony ration estimation. Results are presented as the median of three independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

estimated to be about 20%, and at the highest concentration of pyrogallol, the enzyme activity reached about 60%. Therefore, by increasing the pyrogallol concentration, the activity of SOD and consequently the conversion of superoxide anion increases.

Gene expression

For examining the expression of apoptotic pathway genes in cells treated with cisplatin and pyrogallol, the expression of CASP3 gene showed a 1.8 and 1.5-fold increase compared to control cells, respectively, and this rate was measured as 2.2 in cells treated with a combination of two drugs. This expression of CASP8 and CASP9 genes also increased significantly ($p < 0.05$). However, regarding the BCL2 gene, the treatment with the studied compounds caused a significant decrease in its expression, so that in the combination of cisplatin and pyrogallol, an approximately fivefold decrease was observed for this gene (Fig. 6A). In addition to apoptotic genes, the expression of miR-15a was also measured to determine the effect of the compounds on the miRNA expression level. As Fig. 6B illustrates, the expression of miR-15a

increased significantly in the studied treatments. Also, the expression of two target genes of miR-15a, which are involved in the pathways of cell proliferation and metastasis, decreased significantly (Fig. 6C). The CDC25A gene showed a significant decrease by 4.5 times and KRAS by 3.3 times compared to the control.

Discussion

Platinum-based chemotherapy is used as the main treatment for advanced and recurrent ovarian cancer. However, the observations of the last three decades have shown that the overall viability rate in ovarian cancer has not significantly improved in this treatment [30]. Clinically, in a large number of patients after receiving first-line chemotherapy, the disease relapses and they suffer many complications from chemotherapy, which is associated with a poor prognosis in patients. In addition, the development of resistance to chemotherapy is clearly observed over time [23]. Therefore, many studies have been conducted to resolve these challenges. One of the proposed strategies is the use of combination treatments, especially combination with agents that are less

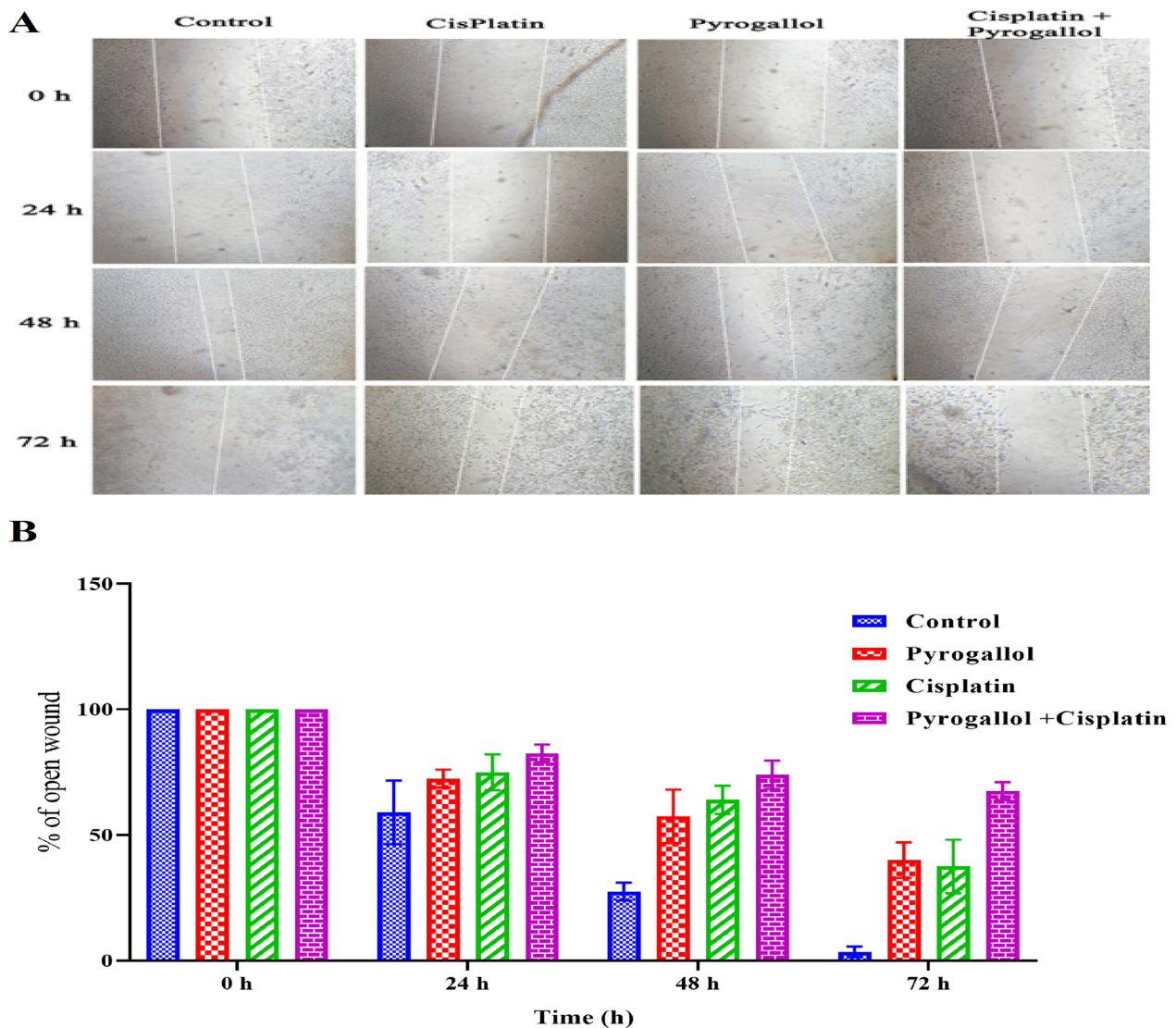


Fig. 3 **A** A2780 cell morphology after alone and combinational treatment with IC10 of cisplatin, pyrogallol and combination of cisplatin/pyrogallol for 72 h. **B** The wound healing scratch width ratio of control and treated cells was calculated using the ImageJ software

toxic, such as natural products [7]. Therefore, the present study is also focused on the combination of cisplatin and pyrogallol to increase sensitivity to cisplatin and reduce its dosage. The results of the present study showed that pyrogallol effectively inhibited cell proliferation, colony formation and migration in A2780 ovarian cancer cells. Also, its combination with cisplatin significantly increased the lethal effect of cisplatin in ovarian cancer cells, in such a way that the amount of IC₅₀ in cisplatin treatment only decreased from 1.39 to 0.32 µg/ml in combination with 6 µg/ml of pyrogallol. The reduction in the number and size of the colonies as well as the inhibition of cell migration also confirms the effect of pyrogallol on increasing the sensitivity of cells to cisplatin. To confirm

the synergistic effect of the combination of cisplatin and pyrogallol, the combination index and dose reduction index (DRI) were calculated using Compusyn software. In all the doses used, the CI was less than 1 and the DRI greater than 1. The CI less than 1 indicated the synergism of the compounds and DRI greater than 1 at constant and variable concentrations of the compounds indicated the reduction of the appropriate dose for the combination of drugs. Various studies have investigated the effect of pyrogallol on cancer cells, but in a very limited number of them, the combination of pyrogallol with other compounds has been investigated. In a study, the combination of pyrogallol and doxorubicin was investigated with respect to oral squamous cell carcinoma (OSCC) cells. In

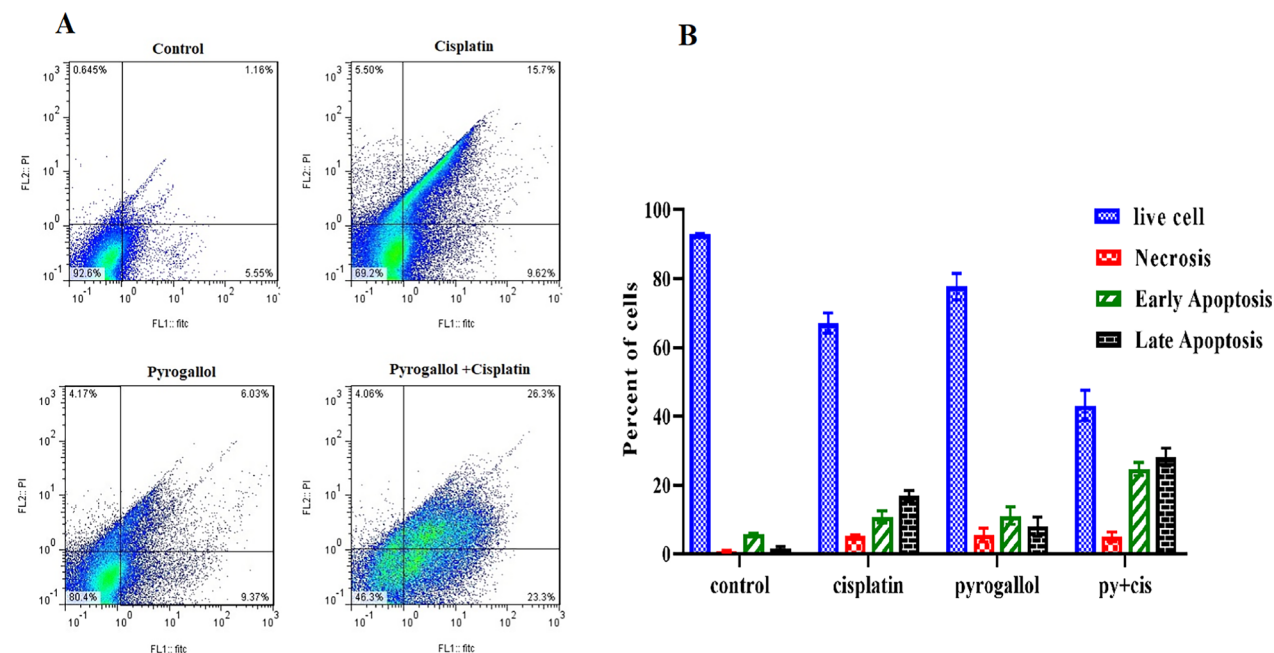


Fig. 4 pyrogallol promoted cisplatin-mediated apoptosis in ovarian cancer cells. A2780 cells were treated with cisplatin and pyrogallol in alone and combination with IC30 concentration; apoptosis rate was measured by Annexin V/PI double staining. **A** AnnexinV+/PI– cells (the lower right) and Annexin V+/PI+ cells (the upper right) were considered to be in the early and late of apoptosis, respectively. Annexin V–/PI+ cells (the upper left) were necrotic. **B** The total percentage of apoptotic cells in the combination treatment was 52.80% for A2780 cells, which was much greater than cisplatin (27.66%) or pyrogallol (19.20%) treatment alone

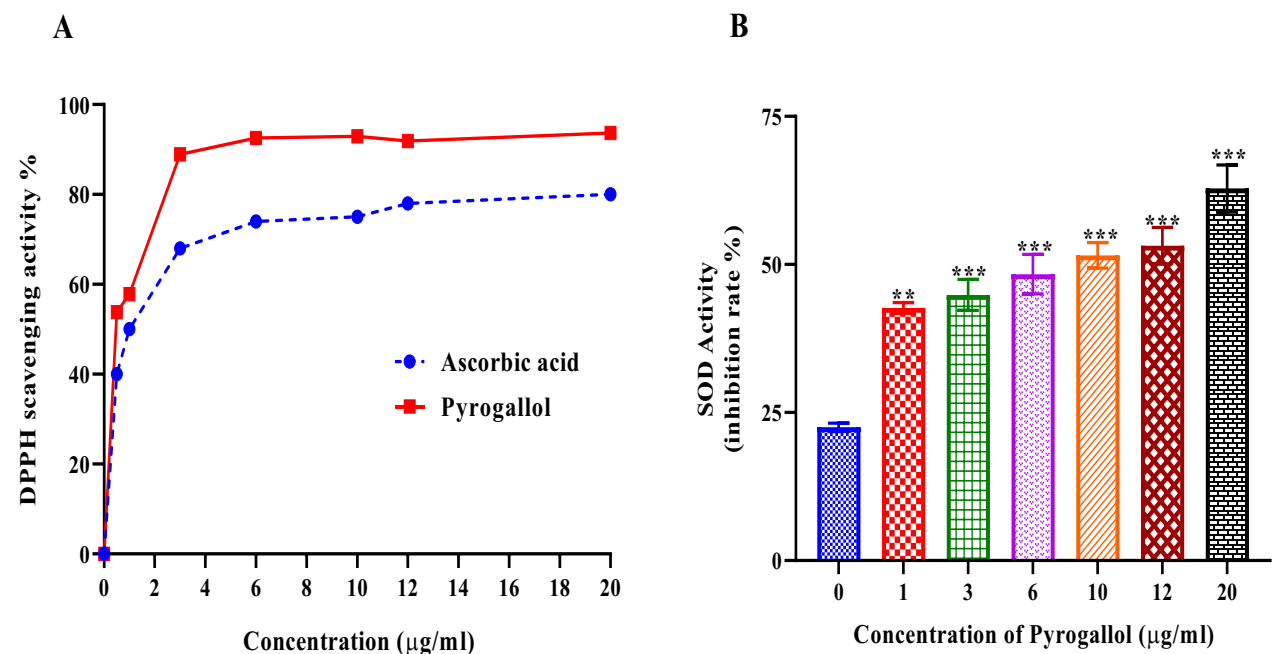


Fig. 5 **A** Detection of antioxidant properties of pyrogallol in free radical DPPH scavenging. Red line related to pyrogallol and ascorbic acid (blue line) used as a positive control. **B** Superoxide dismutase enzyme activity as a result of pyrogallol treatment in A2780 cells

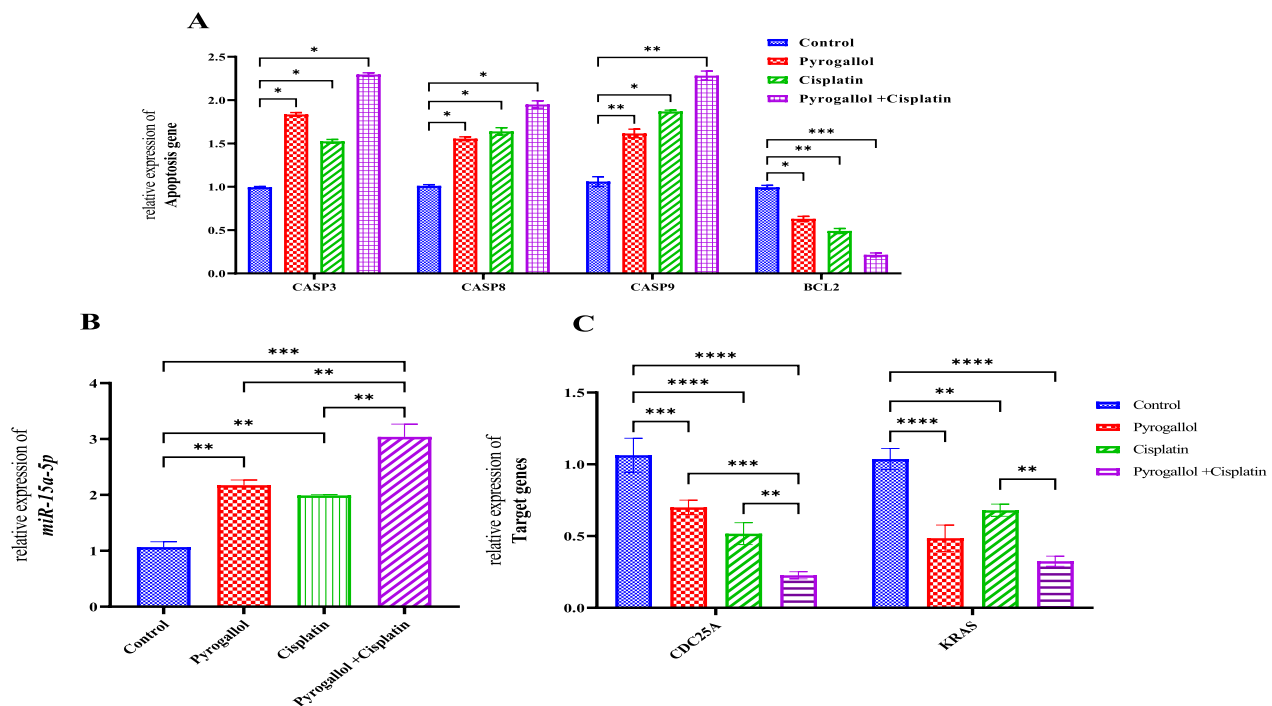


Fig. 6 **A** CASP3, -8, -9 and BCL2 expression test using qPCR analysis; A2780 cells were treated with IC30 concentrations of cisplatin, pyrogallol in alone and in combination for 48 h. The effects of cisplatin and pyrogallol in single and co-treatment on the expression level of miR-15a **B** and CDC25A and KRAS target genes. Relative gene expression was normalized to the U6 for miRNA and GAPDH housekeeping gene for other genes. *, ** and *** Symbol denotes significance at $P < 0.05$, 0.01 and 0.001 levels

this study, it was found that the combination of pyrogallol and doxorubicin increased caspase 3 and cell death [24]. However, this effect has been demonstrated regarding other plant secondary metabolites with low toxicity, such as polyphenols so that it has reduced the dose required for toxic therapeutic factors in cancer treatment [32]. Pyrogallol, as a simple phenolic compound, produces significant apoptotic effects in various cancer cells such as gastric cancer cells (SNU-484) [26], human histiocytic cells (U937) [33] and pulmonary adenocarcinoma cells (Calu-6) [16]. One of the action mechanisms of pyrogallol is to induce apoptosis through the increase of Bax and the simultaneous decrease of the Bcl-2 gene, and it also arrests the cells in the G2/M phase by affecting cyclin B1, Cdc25C and increasing the phosphorylation of Cdc2 (Thr14). In addition, pyrogallol can inhibit tumor growth in an animal model of lung cancer, and after 5 weeks of treatment with this component, tumor regression was visible. The interesting thing to note in this study was that pyrogallol did not show a toxic effect on the healthy cell line and only lung cancer cells died in the treatment with it [40]. In addition, cisplatin also interacts with cellular macromolecules after being absorbed into the cancer cell and exerts its cytotoxic effects through biochemical mechanisms through binding to DNA and forming

intercalating agent within the DNA strand, which leads to the inhibition of DNA synthesis and cell growth [12]. The primary molecular mechanism of cisplatin action is realized by inducing intrinsic and extrinsic pathways of apoptosis caused by the production of reactive oxygen species, activation of various signal transduction pathways, induction of p53 signaling and cell cycle arrest. Up-regulation of apoptosis-inducing genes/proteins and down-regulation of proto-oncogenes and antiapoptotic genes/proteins are associated (Tchounwou et al. 2021). Therefore, co-treatment with pyrogallol and cisplatin can strengthen these molecular pathways and inhibit proliferation and induce apoptosis in cancer cells. Various tests in the present study have also confirmed this observation.

In the study of apoptosis under the combination treatment of cisplatin and pyrogallol, the percentage of apoptotic cells was calculated to be around 50%, which is about 2.5 to 3 times higher compared to single treatments. As well, pyrogallol has antioxidant and prooxidant properties, that is, it has the ability to produce superoxide anion (O_2^-), which is known as reactive oxygen species (ROS). The regulation of intracellular ROS is very important for cellular homeostasis, so that different ROS levels can produce different biological responses. Low and moderate levels of ROS act as signaling molecules

that maintain cell proliferation and differentiation and cycle progression, and also activate viability pathways in response to stressful activities [18]. However, excess ROS levels trigger severe cell damage and apoptotic pathways [10]. Cell damage caused by ROS depends not only on its intracellular concentration but also on the balance between ROS and various intracellular antioxidants. When the prooxidant/antioxidant balance is lost, oxidative stress occurs and changes many intracellular molecules [39]. In addition, various laboratory assays such as the DPPH inhibition method, ABTS inhibition, DMPD inhibition, and H₂O₂ inhibition activities have confirmed the antioxidant properties of pyrogallol. In these investigations, compared to standard antioxidant compounds such as BHA, BHT, and α -tocopherol, as a natural antioxidant, pyrogallol was found as being a comparably stronger antioxidant [25]. The presence of hydroxyl groups in the ortho position of ring B in pyrogallol plays the main role in contributing to the antioxidant properties of pyrogallol. On the other hand, pyrogallol was observed to induce O₂-mediated cell death in cancer cells such as human lymphoma cells, human glioma cells and Calu-6 lung cancer cells. In a study, pyrogallol was found to strongly produce ROS, especially O₂⁻, in As4.1 JG cells [14].

Therefore, according to the dual role of pyrogallol in creating ROS and also activating SOD, it can be emphasized its high capability in two modes of inhibiting cancer cells and providing protection to other cells against ROS. According to various studies, regulation of antioxidant defense systems allows cells to overcome cell death caused by excessive levels of ROS. However, excessive production of ROS can affect cancer cells, resulting in cell cycle arrest and apoptosis [17].

In this study, the effect of pyrogallol, cisplatin and their combination on miR-15a expression was also investigated. In ovarian cancer, the expression level of miR-15a decreases significantly. miR-15a acts as a tumor suppressor in various cancers, including ovarian cancer, and can inhibit growth and metastasis in cancer cells by targeting various oncogenes. In addition, miR-15a has pro-apoptotic properties by inhibiting the BCL2 gene [8]. The noticeable point is the significant decrease in the expression of miR-15a in resistance to cisplatin. In this condition, BCL2 expression is increased and this gene, by stopping the cisplatin action pathway, causes a decrease in autophagy and apoptosis in cancer cells [3]. Therefore, increased expression of this miRNA can be an appropriate therapeutic target for various cancers, including ovarian cancer. A number of polyphenolic compounds can be effective on the expression of different miRNAs, including miR-15a. For example, treatment with curcumin increases

the expression of miR-15a in leukemia cell lines (K562 and HL-60) [11]. As well, in the study of Gordon et al. [13], treatment of MM1 multiple myeloma cells with benzo[a]pyrene increased the expression of miR-15a. In the treatment of acute lymphoblastic leukemia (ALL) cell line (CCRF-CEM) with resveratrol, as a phenolic compound, increased expression of miR-15a induced apoptosis in the cancer cells [1]. In the present study, an increase in the expression of miR-15a was observed under the treatment with pyrogallol, and to confirm the effect of miRNA on targeting important genes in cell proliferation and metastasis, the expression levels of CDC25A and KRAS genes were measured. The CDC25 family expresses protein phosphatase, a cell cycle regulatory protein. This family has three isoforms, A, B and C, which are responsible for cell transition from the G1/S phase and the G2/M phase. Substantially increased expression of CDC25A has been reported in a number of human malignancies, including liver, ovarian, colorectal, esophageal, and non-Hodgkin's lymphoma. Overexpression of CDC25A may lead to cell cycle dysregulation and decreased response to DNA damage, thus causing abnormal cell proliferation and increased carcinogenesis [42]. In addition, the G1 phase is considered to be the most sensitive phase to cisplatin [28]. CDC25A knockdown may cause cell cycle arrest in G1 or G2 phases, allowing cells to repair DNA damage or abnormalities. The KRAS gene, as a proto-oncogene, plays an important role in cell proliferation and differentiation in the MAPK signaling pathway. Increased expression in this gene has been reported in many cancers, including epithelial ovarian cancer [19]. Due to this change in expression, the investigation of the effect of different miRNAs in targeting this oncogene has been the main purpose of various studies. Increasing the expression of miRNAs through the inhibitory role of tumor can reduce the expression of KRAS and play a part in reducing cell proliferation and inhibiting carcinogenesis [21].

To the best of our knowledge, this is the first study to investigate the effect of pyrogallol alone and in combination with cisplatin on cell proliferation, apoptosis and migration in ovarian cancer. The results of the present study highlight the functional role of pyrogallol in increasing the sensitivity of ovarian cancer cells to cisplatin and also reducing its effective dose. Also, these results show that pyrogallol may play its role in inducing apoptosis and inhibiting carcinogenesis by increasing the expression of miR-15a and decreasing its important target genes such as CDC25A and KRAS. Therefore, pyrogallol can be introduced as an effective agent for the greater effect of chemotherapy and to inhibit possible resistance to chemical drugs.

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Author contributions

A. Shirani performed most of the experiments as part of his master's degree in Genetics. R. Mirabbasi performed some cellular assay and miRNA expression. L. Shabani was thesis advisor and participated in intellectual discussions of the data. S.Reisi coordinated the study, designed the experiments, and revised the manuscript.

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Availability of data and material

The authors declare that all generated and analyzed data are included.

Declarations

Ethical approval and consent to participate

This article does not contain any studies on human or animals. Also, this article does not contain any individual person's data in any form.

Consent for publication

This article does not contain any individual person's data in any form.

Competing interests

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