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# Anticancer Activity of Pterostilbene in Human Ovarian Cancer Cell Lines

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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**Background:** Epithelial ovarian cancer is a major cause of mortality in women and one of the most common gynecologic disorders. Pterostilbene (PTS), a trans-3,5-dimethoxy-4'-hydroxystilbene, was chosen for this work due to its reported effectiveness as a chemotherapeutic agent in cancer studies. In this work, we studied underlying molecular mechanisms of PTS treatment in various ovarian cancer cell lines such as OVCAR8, OV1063, IGROV-1, and SKOV3.

**Material/Methods:** We used the cytometric bead array (CBA) method and real-time PCR analysis to analyze the secretion level of tumor necrosis factor alpha (TNF-α) and to measure the TNF-α mRNA expression. NF-kappa B (NF-κB) promoter analysis, Western blot analysis, electrophoresis mobility shift assay (EMSA), and immunostaining analyses were performed to measure the NF-κB activity and other relative proteins levels.

**Results:** The PTS treatment decreased the release of TNF-α in IGROV-1 ovarian cancer cells. It also showed significant inhibitory effect on nuclear NF-κB p50, and NF-κB p65 protein levels.

**Conclusions:** From the results obtained, we suggest that PTS has the potential to treat ovarian cancer by reducing the level of TNF-α cytokine and to have a limited effect on NF-κB, AKT, and ERK signaling pathways.

**MeSH Keywords:** **Cytokines • Ovarian Neoplasms • Tumor Necrosis Factor-alpha**

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## Background

In recent years, epithelial ovarian cancer (EOC) has been considered one of the most common gynecologic malignancies in developed countries and the most common cause of cancer mortality in women worldwide [1,2]. Detection at an earlier stage remains the most favorable approach for long-term survival of ovarian cancer patients. Although progress has been made in early detection and treatment, the mortality rate for ovarian cancer has remained virtually unchanged over the past few decades [3,4]. Stage I ovarian cancer has a 90% survival rate for cancer patients at five years, and surgery is the only method known to cure most patients from this disease [5–8]. Ovarian adenocarcinomas occur as four main histologic subtypes: endometrioid, mucinous, clear cell, and serous; with serous the most common subtype. The systematic examination of the molecular mechanisms supporting ovarian cancer is needed to better understand each of these subtypes, and there is an urgent need for new technology for detection of early stage ovarian cancer.

Blueberries and grapes comprise a class of natural polyphenolic compounds called stilbenes [9]. Stilbenes contain resveratrol, a naturally occurring compound which can exhibit potential antioxidant properties. Resveratrol tends to induce apoptosis and has been used in carcinopreventive activity studies [10,11]. Resveratrol has also been shown to possess significant inhibitory activities against different kinds of malignant primary tumors, including breast cancer, colon cancer, and prostate cancer [9]. Pterostilbene is the dimethyl analogue of resveratrol or trans-3,5-dimethoxy-4'hydroxystilbene (Figure 1), and has also garnered increased attention as an effective chemopreventive agent for malignant tumors [12,13]. Pterostilbene is considered a natural dimethylated analog of resveratrol and has been proposed to have similar properties as that of resveratrol, including antioxidant, anticancer, anti-inflammation, antiproliferation, apoptosis, and analgesic ability. In addition, the plasma level of pterostilbene and its corresponding pterostilbene-sulfate are greater than plasma levels of resveratrol and its corresponding sulfates [14]. Previous reports have shown that pterostilbene can inhibit the growth of tumors both *in vitro* as well as *in vivo*. The existence of significant bioavailability of pterostilbene indicates that it could have potential clinical application. The resveratrol analogue of pterostilbene that is present in blueberries has been shown to exhibited tumor growth arrest in cases of colon cancer, breast cancer, gastric cancer, melanoma, and leukemia in different animal models and *in vitro* studies [15–19]. In addition, the molecular skeleton of pterostilbene makes it more bioavailable compared to that of resveratrol upon oral ingestion. However the exact pharmacological mechanisms of pterostilbene and its effects in humans are still under investigation. Based on the aforementioned points, we hypothesized that pterostilbene could act against tumor growth in ovarian carcinoma cell lines *in vitro*.

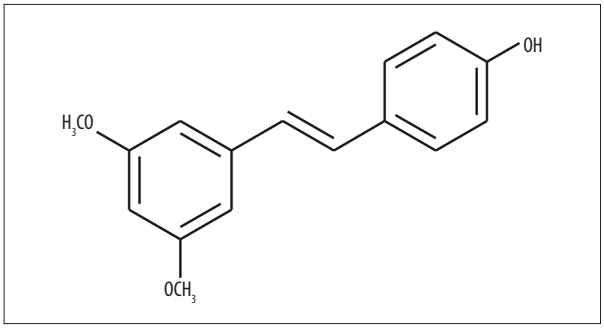


**Figure 1.** Structure of pterostilbene.

## Material and Methods

### Chemicals

Pterostilbene, β-actin antibodies, dimethyl sulfoxide (DMSO), and other reagents and chemicals used in our experiments were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). All the monoclonal primary antibodies, including NF-kappa (NF-κ) Bp50, NF-κ Bp65, phospho-IκB, phospho-IKK(Ser176), AKT, phospho-AKT, ERK(p44/42), phosphor-ERK(p44/42), Egr-1, secondary antibody (FITC conjugated goat anti-mouse IgG (1: 100 dilution), α-tubulin, and PCNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primers for control, actin, and TNF-α, and 2X SYBR Green PCR Master Mix were obtained from Life Technologies. Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Chemicon International (Temecula, CA, USA).

### Cell cultures

The human ovarian cancer cell lines OVCAR8, OV1063, IGROV-1, and SKOV3 were purchased from American Type Culture Collection (ATCC). The medium used varied with the cell line as follows: OV1063 was grown in RPMI-1640; OVCAR8, IGROV-1, and SKOV3 were maintained in DMEM. All cells were grown in 10% fetal calf serum (FCS) (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with penicillin and streptomycin and L-glutamine at 37°C, and 5% $CO_2$ and 95% air.

### Cytometric bead array (CBA) method and flow cytometry

IGROV-1 cells at a density of about $3\times10^5$ cells/well were cultured using 24-well culture plates. These cultured cells were then treated with different PTS concentrations: 0, 100, 200, and 500 μg/mL in DMEM/RPMI 1640 medium, respectively for about 48 hours. After the set period, media were collected and centrifuged. Collected media were used to measure the TNF-α level using CBA assay based on the reported literature [20]. Human Th1/Th2 CBA kit (BD Biosciences, San Diego, CA, USA) and flow cytometry [21] were used for the quantification. The experiments were performed in triplicate.





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### Real-time PCR analysis

IGROV-1 cells at a density of $6\times10^5$ cells/well were treated with 500 µg/mL of PTS for about 6, 12, and 24 hours and then total RNA was extracted using ReliaPrep™ RNA Miniprep Systems (Promega, MA, USA) as per previously reported methods [22]. These samples were then subjected to reverse transcription to produce cDNA at a temperature of 42°C for 30 minutes. This was performed using high capacity cDNA reverse transcription kit as per the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). The following measurement conditions were used for the quantitative PCR analysis: 2 minutes at 50°C; 10 minutes at 95°C; 15 seconds at 95°C, and 1 minute at 60°C using 200 nm of forward (F) and reverse (R) primers. All the assays were analyzed using MiniOpticon Real-Time PCR system (Bio-Rad, USA). The experiments were repeated in triplicate and the fold change expressions were determined using CT as mentioned in the reported method [23].

### Western blot analysis

A density of $3\times10^6$ cells/well of IGROV-1 cells were cultured in 24-well culture plates, then treated with 500 µg/mL PTS for different time periods of about 0, 2, 4, and 6 hours. At 24 hours, parallel experiments were also carried out using PTS treatment at different concentrations of 0, 100, 200, and 500 µg/mL to identify changes in protein levels. Then cells were harvested and the proteins were detected according to the literature [24]. Proteins were resolved over 12% SDS-PAGE; Western blots were then incubated using monoclonal primary antibodies individually. The blots were stained using horseradish peroxidase conjugated secondary antibody as per Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences, New Jersey, USA) and autoradiography using x-ray film [24]. Phosphorimage system was used to quantify the intensity of the bands.

### Immunostaining

A density of $2\times10^6$ cells/wall of IGROV-1 cells were cultured first using 24-well culture plates. Then the cells were treated with 500 µg/mL PTS for about 24 hours and fixed using 4% formaldehyde for 10 minutes. Then 0.1% of Triton-X 100 in PBS was used for cell permeabilization and 2% BSA was used for blocking. Anti-human NF-κB p65 antibody with 1: 100 dilution was added and incubated overnight; then exposed to the FITC-conjugated goat anti-mouse IgG secondary antibody followed by PI staining. Cells were then imaged using Zeiss fluorescence microscope [25].

### NF-κappaB promoter assay

This assay was performed to determine NF-κB activity. Before transfection with lipofectamine 2000, a density of $5\times10^6$ IGROV-1 cells were co-transfection with NF-κB promoter (0.5 µg) and β-galactosidase (0.5 µg) expression vectors and grown in 24-well cultured plates. The cells were then incubated at room temperature for 30 minutes, then, lipofectamine 2000 was added into every well and kept for 24 hours for transfection. After which, completed PTS were added and incubated again. Lysates were prepared by adding 100 µL of Reporter Lysis Buffer to each group, followed by scraping of cells from the dishes, and centrifuging lysates for one minute at 13,000 rpm to collect the supernatants. An equal amount of protein was added into opaque black wells followed by the addition of an equal amount of luciferase substrate. Then the luminescence was determined using a GloMax® 96 Microplate Luminometer (Promega) [26].

### Mobility shift electrophoresis (EMSA)

CelLytic™ NuCLEAR™ Extraction Kit obtained from Sigma-Aldrich (St. Louis, USA) was used to measure the nuclear extracts from the PTS-treated IGROV-1 cells (density of $5\times10^6$ cells/well). The protein concentrations were detected followed by the development of NF-κB related biotin end labeled oligonucleotide sequences. The extracted nuclear proteins were used for EMSA determination using chemiluminescent EMSA kit from Beyotime (Shanghai, China) as per the supplier's instructions. After preparing the biotin end labeled duplex DNA, it was treated with nuclear extracts and incubated, and then electrophoresis on 6% polyacrylamide gel followed by the addition of 100 fold of unlabeled double stranded oligonucleotide to carry out the competitive experiment, then the DNA was quickly transferred into the nylon membrane. Then it was cross linked with UV and probed with the biotin-horseradish peroxidase and then treated with substrate and incubated according to the instructions given by suppliers of Amersham ECL Western Blotting Detection Kit [27].

### Data statistics analyses

One-way ANOVA test was used to measure the significance of changes occurred between the control and the test compound variables. Bonferroni test was used for multiple comparisons. All the experiments were performed in triplicate and the data were expressed as mean ±S.D. finalized at $p<0.05$.

## Results

### PTS effects on the release of cytokines

Figure 2 shows the CBA analysis and results, which found that a decrease in the release of TNF-α was observed in IGROV-1 cells upon PTS treatment. Meanwhile, there was not any significant effect of PTS treatment on other ovarian cell lines on





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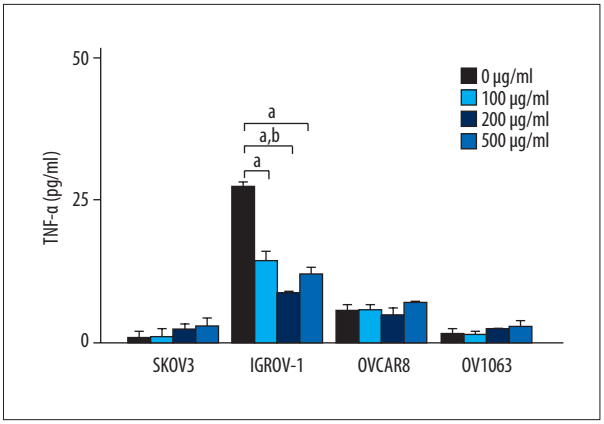


**Figure 2.** TNF-α release identification in ovarian cancer cell lines SKOV3, IGROV-1, OVCAR8, and OV1063 upon PTS treatment of 0, 100, 200, and 500 µg/mL for 48 hours. Each experiment was done in triplicate and represented as mean ±SD: 'a' represents $p<0.05$, significant comparison with control of 0 µg/mL and 'b' represents $p<0.05$, significant comparison with 200 µg/mL treatment of PTS.

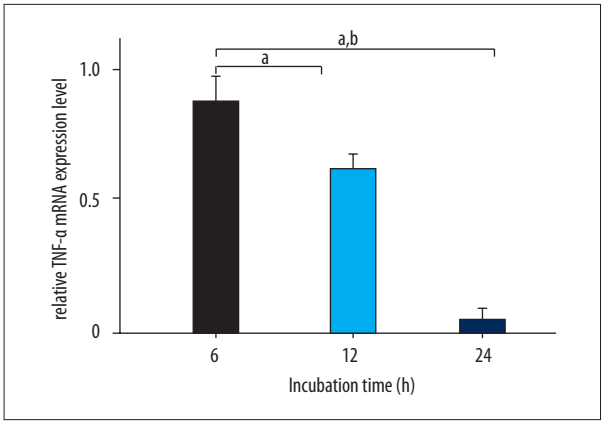


**Figure 3.** Inhibitory effect of PTS on TNF-α gene expression in IGROV-1 cells using RNA extracts obtained from IGROV-1 upon 500 µg/mL PTS treatment of about 0, 12, and 24 hours. Each experiment was done in triplicate and represented as mean ±SD: 'a' represents $p<0.05$, significant comparison with a control at 0 hour and 'b' represents $p<0.05$, significant comparison with 12 hour PTS treatment.

TNF-α release. Therefore, IGROV-1 cells should be considered for further studies. Real-time PCR analysis was used to study the PTS effects on TNF-α mRNA gene transcription and the results are shown in Figure 3. Upon treatment with 500 µg/mL of PTS with IGROV-1 cells resulted in a suppression of TNF-α mRNA expression in a time dependent response. This suggested that the production of TNF-α cytokine can be regulated by PTS at a transcription level.

### Changes associated with protein levels of NF-κB, NF-κB translocation, and NF-κB activity

To determine the NF-κB signaling pathway involved in the secretion of TNF-α cytokine, we studied the PTS effects on NF-κB. Figure 4 shows the time and dose dependent response of PTS on NF-κB. The results shown in Figure 4A indicate that PTS showed great inhibitory effect on nuclear NF-κB p50 and NF-κ p65 protein levels. Similar results were also observed using Western blot analysis as shown in Figure 4B. Figure 4C and 4D show the inhibitory effect of PTS on the p-IKK and p-IκB protein levels and Western blots in a time- and concentration-dependent response, respectively. PTS attenuation of NF-κB p65 translocation to nuclear by immuno-staining analysis is shown in Figure 5A. The promoter activity assay of NF-κB indicated that PTS inhibited the NF-κB expression in a time-dependent response at the transcription stage and is shown in Figure 5B. EMSA results are shown in Figure 5C, which indicates that PTS also decreased NF-κB binding level with the DNA in a time-dependent manner. All these results suggested that PTS inhibited the cytokine secretion of TNF-α via inhibiting the NF-κB mediated pathway in IGROV-1 cancer cells.

### AKT and ERK related protein expressions upon PTS treatment

To prove the role of AKT and ERK mediated pathways in cytokine secretion of TNF-α, we studied the effects of PTS on it. The inhibitory effects of PTS on p-AKT and p-ERK protein levels are shown in Figure 6A as time- and dose-dependent response. Similar results were observed using Western blot analysis as shown in Figure 6B. Similarly, PTS inhibitory effects were identified with Egr-1 nuclear protein levels for ERK transcriptional factor as a time- and dose dependent response and are shown in Figure 6C. The corresponding Western blot results are shown in Figure 6D. However, it was observed that PTS showed negative response towards JNK, p-JNK, p38, and p-p38 protein levels of IGROV-1 cells (data not given). The aforementioned results further confirmed the PTS inhibitory effects towards cytokine production of TNF-α via inhibition of AKT and ERK mediated pathways in IGROV-1 cells.

## Discussion

We determined the effect of PTS on cytokines among various ovarian cancer cell lines OVCAR8, OV1063, IGROV-1, and SKOV3 and their possible signaling pathways. A previous study showed that there was a relationship between ovarian cancer and inflammation [28]. To check whether the decrease in the release of TNF-α was not due to the cytotoxic effect of PTS treatment, we performed the cytotoxic test with various concentrations of PTS from 0 to 800 µg/mL. The results showed that there was not any cytotoxic effect (up to 800 µg/mL) on





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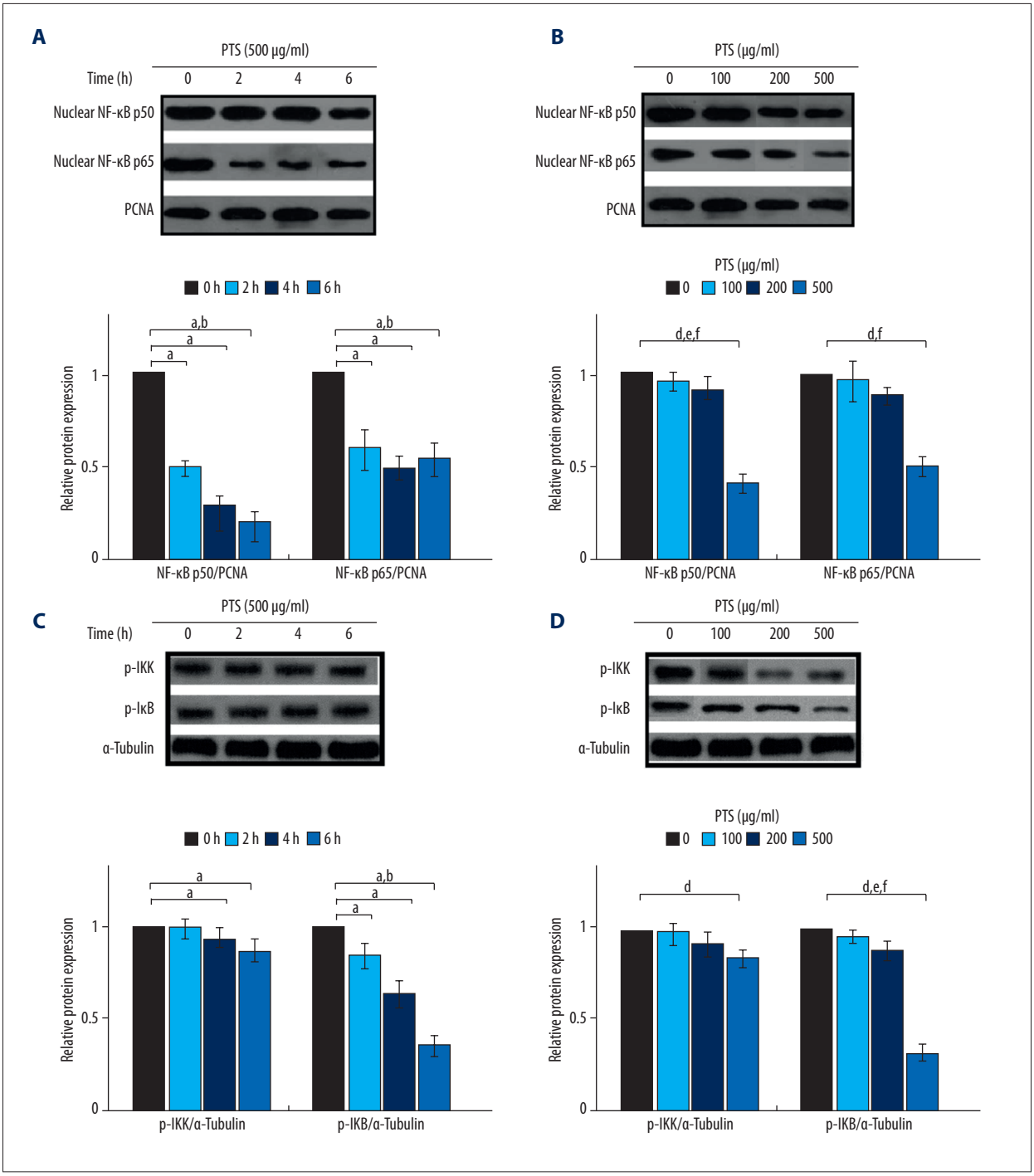


**Figure 4.** PTS effects on NF-κB p-50 and NF-κB p-65 protein levels (**A, B**); p-IKK and p-IκB protein levels (**C, D**) in IGROV-1 cells. (**A, C**): IGROV-1 cells with 500 μg/mL PTS treatment for 0, 2, 4, and 6 hours. (**B, D**): Western blot assay with IGROV-1 cells treated with 0, 100, 200, and 500 μg/mL PTS for about 24 hours. Each experiment was done in triplicate and represented as mean ±SD: 'a' represents $p<0.05$, significant comparison with a control at 0 hour; 'b' represents $p<0.05$, significant comparison with 2 hour PTS treatment; 'd' represents $p<0.05$, significant comparison with 0 μg/mL PTS treatment; 'e' represents $p<0.05$, significant comparison with 100 μg/mL PTS treatment; and 'f' represents $p<0.05$, significant comparison with 500 μg/mL PTS treatment.





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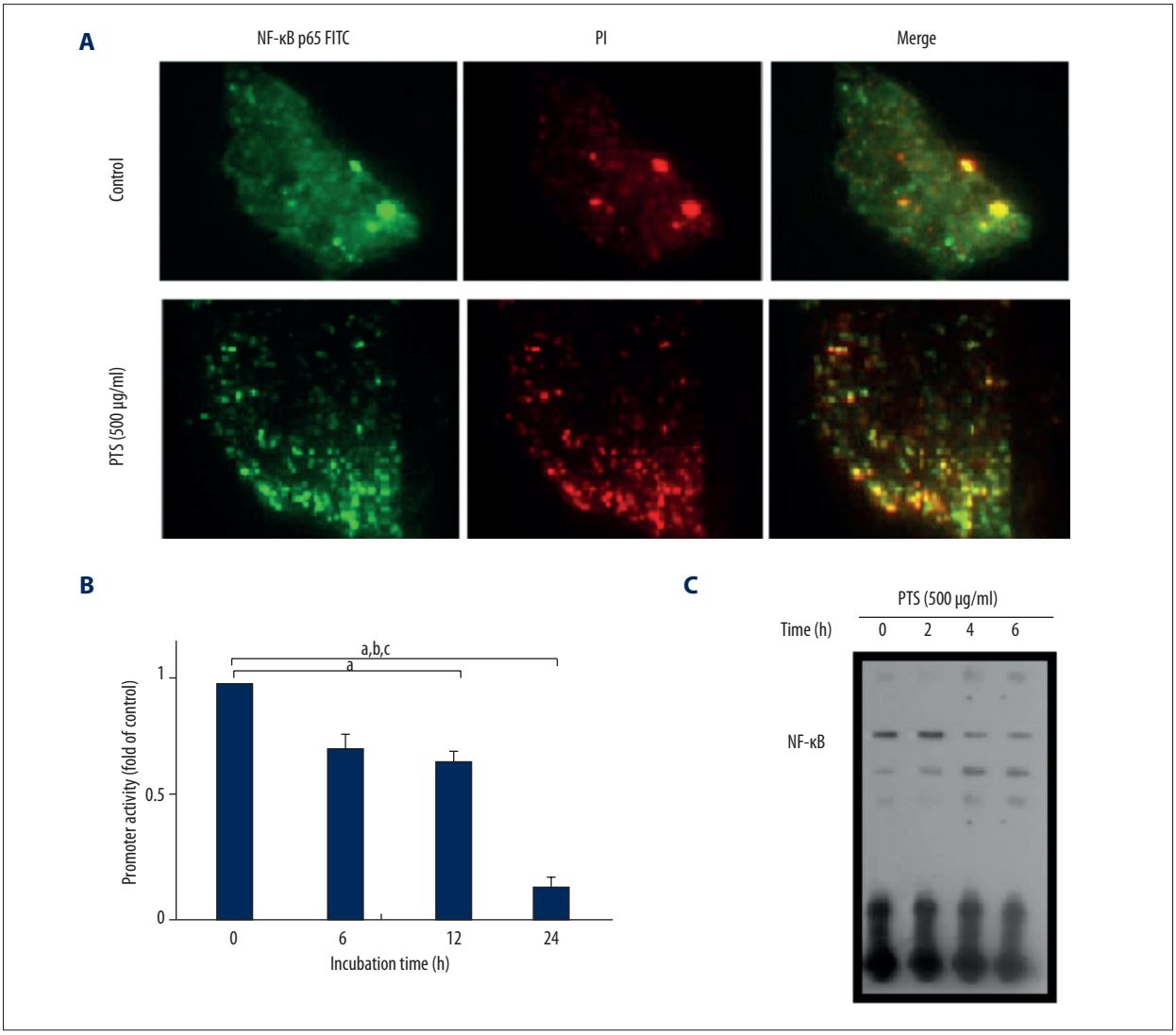


**Figure 5.** Changes in NF-κB p65 translocation levels of IGROV-1 cells upon PTS treatment. (**A**) Promoter assay; (**B**) DNA binding effect; (**C**) NF-κB activity. Each experiment was done in triplicate and represented as mean ±SD: 'a' represents $p<0.05$, significant comparison with a control at 0 hour; 'b' represents $p<0.05$, significant comparison with 6 hours PTS treatment; 'c' represents $p<0.05$, significant comparison with 12 hours PTS treatment.

ovarian cells due to PTS treatment and the inhibition was only due to the action of PTS. The cytotoxic data was excluded as it did not have any significance. This work proved the inhibitory effects of PTS towards TNF-α secretion of cytokine in IGROV-1 ovarian cells.

The CBA study analysis showed that PTS increased interleukin of IL-4 and IFN-γ levels in all the tested ovarian cancer cells, however, we observed a minor change with the following concentration of 1 pg/mL of IL-4 as control and 123.2 pg/mL of PTS as test compound, and 122.7 pg/mL of IFN-γ as control and 224 pg/mL of PTS (data not shown). Furthermore, CBA analyses also revealed that PTS decreased the TNF-α level investigated particularly in IGROV-1 cells (control: 28 pg/mL; PTS: 12.6 pg/mL) (Figure 2). The aforementioned analyses indicated that PTS helps to regulate the TNF-α level and further inhibits the cytokine secretion of TNF-α via NF-κB, AKT, and ERK mediated pathways in ovarian cancer cells IGROV-1. In a previous study, it was reported that the expression of TNF-α was correlated with the tumor grade in epithelial ovarian cancer cells [27]. In another study, immunohistochemical analysis of tissue specimens identified the positive localization of TNF-α in tumor tissues whereas a negative response was shown on the normal ovarian tissue with TNF-α staining [28]. These reports support our hypothesis that a decrease in TNF-α in IGROV-1 ovarian cells is due to the PTS treatment.





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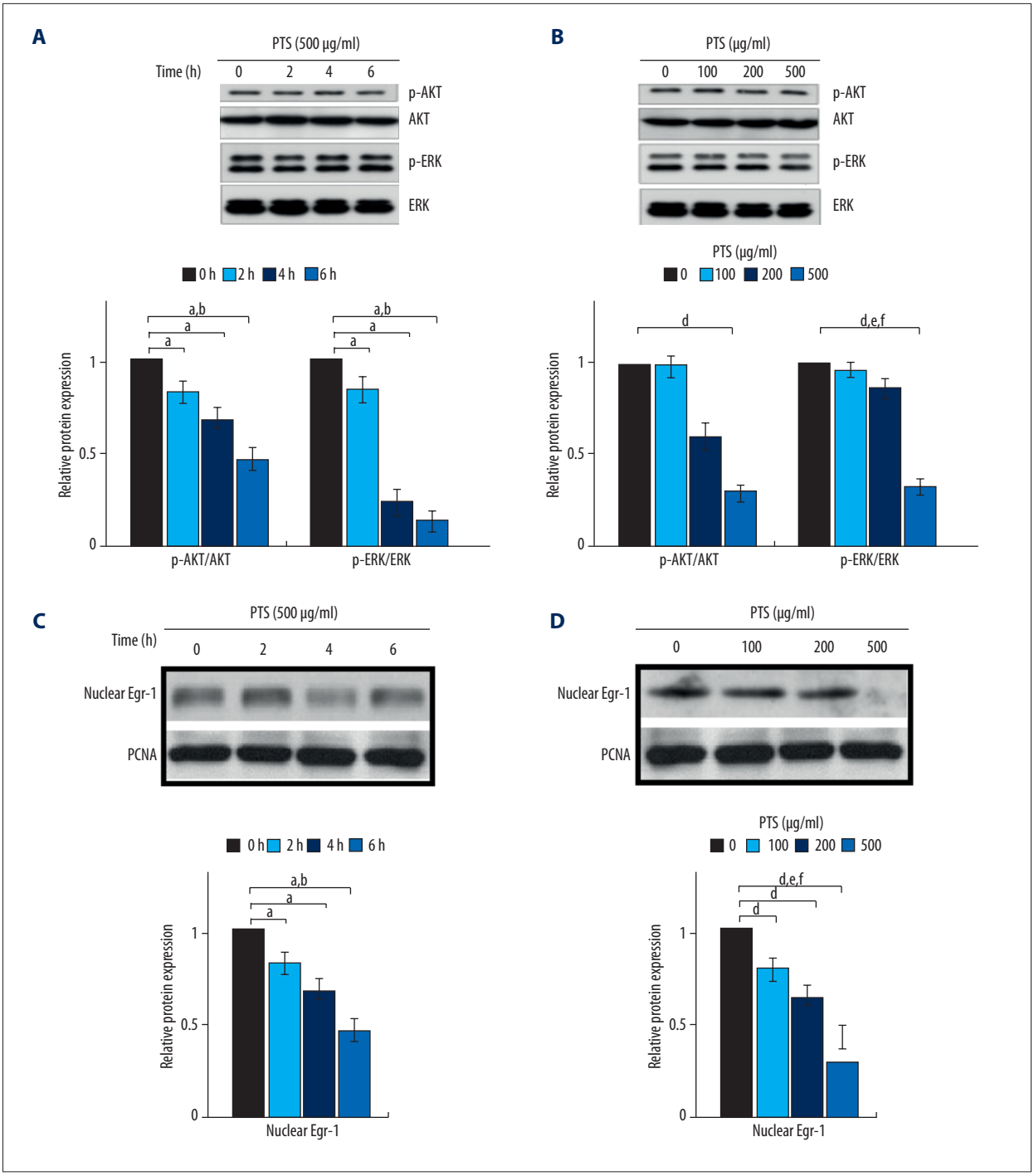


**Figure 6.** (**A, B**) Changes in AKT, p-AKT, ERK, p-ERK (**A, B**) protein levels upon PTS treatment; (**C, D**): Changes in Egr-1 protein level upon PTS treatment. Each experiment was done in triplicate and represented as mean ±SD: 'a' represents $p<0.05$, significant comparison with a control at 0 hour; 'b' represents $p<0.05$, significant comparison with 2 hours PTS treatment; 'c' represents $p<0.05$, significant comparison with 4 hours PTS treatment; 'd' represents $p<0.05$, significant comparison with 0 µg/mL PTS treatment; 'e' represents $p<0.05$, significant comparison with 100 µg/mL PTS treatment; and 'f' represents $p<0.05$, significant comparison with 500 µg/mL PTS treatment.





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The CBA analysis determined that PTS significantly decreased the TNF-α in IGROV-1 ovarian cancer cells. The PTS treatment showed suppressed mRNA expression of TNF-α in a time-dependent response in IGROV-1 cells which further indicated that TNF-α was a main target in PTS-treated IGROV-1 ovarian cells. Figures 3 and 4 show the time- and concentration-dependent Western blot analysis that demonstrated the PTS inhibitory effects on NF-κB, p-IKK, p-IκB, p-AKT, and p-ERK protein levels. Figure 4A–4C revealed that PTS suppressed the NF-κB translocation, NF-κappaB activity using promoter assay and ESMA. Our study showed that PTS inhibited the release of TNF-α cytokines in IGROV-1 cells through the inhibition of NF-κB, AKT, and ERK pathways.

## Conclusions

This study revealed that PTS possessed the ability to alter the cytokine production and significantly decreased the mRNA levels of TNF-α. PTS further decreased the protein expression in NF-κB, AKT, and ERK signaling pathways, and helped to reduce the effect of NF-κB activity and translocation in IGROV-1 ovarian cells. Further studies on PTS can aid in the development of a potential drug for ovarian cancer treatment.

### Conflict of interest

The authors declare that they have no competing interests.

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