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Deciphering the effect of *Potentilla fulgens* root extract against healthy HUVEC cell line and cancer cell lines (A549 and SKOV-3)

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ABSTRACT

Background: Potentilla fulgens, a highly valued indigenous medicinal herb grown in high altitudes of the Himalayan region with anticancer, hypoglycaemic, antibacterial, anti-inflammatory, and antiulcerogenic properties, are used in traditional systems of medicine. This study was aimed to investigate the effect of *P. fulgens* root extract, as one of the natural alternatives to chemotherapeutic drugs used in cancer treatment, on proliferation, apoptosis, and autophagy of human non-small cell lung cancer cell line (A549), human ovarian cancer cell line (SKOV-3), and healthy human umbilical vein endothelial cell line (HUVEC).

Methods: Anti-proliferative effect was assessed by MTT assay. The expression of autophagy and apoptosis-related proteins was evaluated by western blotting. Total oxidant status (TOS) and total antioxidant capacity (TAC) test were determined using standard kit methods.

Results: Our results showed that the extract inhibited proliferation of HUVEC, A549, and SKOV-3 cells in a dose-dependent manner. MTT assay analysis revealed that the extract significantly ($P<0.05$) induced mortality in HUVEC, A549, and SKOV-3 cells. Western blot results revealed increased expression of NF-κB after the extract treatment but led to the down-regulation in Beclin-1, Bax, extracellular-signal-related kinase 1 and 2, Sequestosome-1, and cleaved Casp-3 levels. Treatment groups showed an increase in TOS and TAC values in A549 and SKOV-3 cell lines, while HUVEC cell line showed an increase in TAC and a decrease in TOS values, compared to the control group.

Conclusions: Our findings indicated that *P. fulgens* root extract inhibited the proliferation of healthy cells and cancer cells through cell cycle arrest, representing its limited application as therapeutic agent in cancer treatment.

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1. Introduction

Lung cancer is one of the most prevalent cancer types in the world. Unfortunately, less than 15 % of patients survive for few years, despite new treatment approaches. (Mir et al., 2013). Ovarian cancer is the deadliest of all gynecologic cancers and the fifth most common cancer in women. When this cancer is detected in the Phase I stage, the patient's survival rate exceeds 90 %, but when the disease reaches the Phase III or Phase IV stage, this rate drops below 20 % (Bunn Jr and Franklin, 2002).

The activation of the cellular apoptotic mechanism is a promising target for cancer treatment. In this regard, the suppression of the apoptotic pathway during cancer can occur through the over-expression of anti-apoptotic proteins or the under-expression of pro-apoptotic proteins (Pfeffer and Singh, 2018). Additionally, tumor cells can potentially avoid apoptosis by increasing the expression of caspase inhibitors and reducing the expression of Bax (Erdoğan and Uzaslan, 1998).

Autophagy prolongs cell survival in tumor cells with a damaged apoptosis mechanism (Hashemi et al., 2023). Autophagy is the cell's self-destructing process to clean up damaged or unnecessary proteins and organelles and balance energy sources in response to nutritional stress (Hashemi et al., 2023). Autophagy is a process that helps cells to survive under stress by breaking down and recycling cellular components. When autophagy is compromised, cells and tissues may be exposed to the toxic effects of excessive accumulation of autophagy substrates (Aman et al., 2021). Tumor cells, however, have high basal levels of autophagy and may depend on it for their survival (Aman et al., 2021). In hypoxic regions, autophagy is induced in tumor cells, which gives them a survival advantage (Kabakov et al., 2021). Therefore, inhibiting autophagy may be a new way to target cancer therapy by preventing tumor cells from surviving (Kartlaşmış et al., 2018, Sutton et al., 2018).

Chemotherapy and radiotherapy are accompanied by different side effects, and some tumors develop resistance to chemotherapy (Liu et al., 2021). To solve these problems, there is a push towards the use of natural substances that stimulate apoptosis in cancerous cells but do not harm normal tissues (Limtrakul et al., 1997). *Potentilla fulgens* is an annual plant belonging to the Rosaceae family which grows in temperate and high altitude (1800–4350 m) regions in the Northern and Northeastern regions of India, with a thick woody stem, long thin leaves, and yellow flowers (Laloo, 2013). The roots of *P. fulgens* and the whole plant have traditionally been used to treat gum and dental disorders (pus discharge, toothache, and tooth decay), diarrhea, stomach problems (peptic ulcers), coughs, colds, diabetes, and cancer (Kumar et al., 2013). Phytochemical analysis of *P. fulgens* root extract identified important bioactive compounds such as alkaloids, tannins, polyphenols, terpenoids, and flavonoid (Tomczyk and Latté, 2009). The extract's rich content of therapeutically important bioactive compounds has made it a suitable candidate in terms of the use of natural substance in cancer treatment (Anal et al., 2014; Ozukum et al., 2023). *In vitro* studies on various cancer cell lines showed that it had anti-proliferative effects (Anal et al., 2014; Ozukum et al., 2023). The current study was designed to investigate the effects of *P. fulgens* root extract on cell proliferation, apoptosis, and autophagy of human "non-small cell" lung cancer cell line (A549), human ovarian cancer cell line (SKOV-3), and normal human umbilical vein endothelial cell line (HUVEC).

2. Materials and methods

2.1. Preparation of P. fulgens root extract

The aqueous extract of *P. fulgens* roots used in the study was supplied in dry powder form from Xi'an Yuensun Biological Technology Company (China Spe:20:1 Batch No:YS-EM-131030A). It was dissolved in 2 % ethanol and heated in a boiling water bath for 10 min before being cooled. The solution was centrifuged at 2000 rpm for 10 min in glass tubes, and the resulting supernatant was stored at 4 °C for further use (İpek et al., 2019).

2.2. Cytotoxic activities of extract

Cell viability tests were performed against cancer cell lines (A5459 and SKOV-3) and the healthy HUVEC cell line using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). The cell lines were obtained from the American Type Culture Collection (ATCC). A549, SKOV-3, and healthy HUVEC cell lines were grown in a T75 flask using RPMI-1640 medium (Sigma-Aldrich R8758, USA) with 10 % FBS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin. The flask was incubated at 37 °C with 5 % CO_2. Cells were counted using the hemocytometric method after reaching 80–90 % confluency. At regular time interval (24, 48, and 72 h), cells (5×10^3 cells) were seeded into 96-well plates in three replications. The cells were left to adhere before further experimentation. Then, the cells were washed with phosphate buffered saline and the extract was added at different concentrations (100, 80, 60, 40, 20, 10, and 5 μg/mL). Ethanol (2 %) was used as negative control. At 24, 48, and 72 h, the medium was aspirated and 25 μL of MTT solution was added into each well. After 4 h of incubation at room temperature, 100 μL of dimethyl sulphoxide was added to each well and left undisturbed for 1 h. The absorbance was read at 570 nm using micro plate reader. The control wells' average absorbance value was considered 100 % viable cell. The absorbance values obtained from the wells that were treated with the extract were compared to the control absorbance value and represented as % viability.

2.3. Total oxidant status (TOS) and total antioxidant capacity (TAC) level

The $\log IC_{50}$ values obtained from the MTT results were used to determine the TOS and TAC. After 24 h, the cell suspensions were removed using Trypsin-EDTA, and then centrifuged at 1000 × g (2–8 °C) for 20 min to collect supernatant. The commercially available TOS Assay Kit and TAC Assay Kit (Rel Assay Diagnostic-Turkey) were used for analyzing TOS and TAC, respectively using the collected supernatant (Öztoprak et al., 2022). Further, oxidative stress index (OSI) was calculated as:

$$OSI = (TOS/TAC) \times 100$$

2.4. Western blot

Cells were cultured and exposed to the extract at effective doses determined by MTT, with 2 % ethanol control group. Cell lysates were made with RIPA lysis buffer and a protease-phosphatase inhibitor. Total cellular protein concentration was measured with a BCA protein assay kit (Kandemir and Ipek, 2023).

Protein samples were adjusted to 20 μg and mixed with 2X Laemmli sample buffer with 5 % p-mercaptoethanol. After heating at 95 °C for 5 min, samples were separated on an SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane and blocked in PBS-T using 5 % skim milk for 1 h. Samples were incubated with primary antibodies overnight at 4 °C, followed by the addition of secondary antibodies for 1 h at room temperature. After washing, the signal was visualized and analyzed using software. Antibodies used included Beclin, cleaved caspase, Bax, ERK ½, p-ERK, NF-κB, p-NF-κB, SQSTM1, and β-actin as the internal control (Kandemir and Ipek, 2023).

2.5. Statistical analysis

The GraphPad Prism 8 program calculates the value of extract's inhibitory concentration (IC_{50}). The data were analyzed using Statistical Package for the Social Sciences (SPSS) Inc Chicago, IL, USA, software

version 21. Data were compared between groups using unpaired *t*-test and among multiple groups by one-way ANOVA, followed by Tukey's post hoc tests. $P<0.05$ was considered statistically significant (Cui et al., 2023).

3. Results and discussion

3.1. Cytotoxicity assay

Figs. 1 and 2 represent the cytotoxic activities of extract against A549, SKOV-3, and healthy HUVEC cells. The logIC_{50} values were estimated as 1.740, 1.436, and 1.177 µg/mL for A549 cells; 2.268, 1.970, and 1.487 µg/mL for SKOV-3 cells; and 1.561, 1.214, and 1.051 µg/mL for HUVEC cells at 24, 48, and 72 h, respectively. No significant ($P>0.05$) difference at 48 and 72 h was observed for A549 and HUVEC cells, while significant ($P<0.05$) differences were observed for SKOV-3 at 24–72 h.

P. fulgens root extract (methanolic, butanolic, and dichloromethane) have been found to be cytotoxic against glioblastoma cancer cell lines (U87, U118, and T98G) (Kandemir and Ipek, 2023). In another study, the methanolic extract also increased the survival rate of mice with Ehrlich ascites cells and inhibited the growth of MCF-7 cells in a dosage-dependent manner (Radhika et al., 2012). In the present study, *P. fulgens* root extract inhibited the growth of A549 and SKOV-3 cells, which was consistent with the findings of previous studies. However, it has been determined that the growth inhibitory effect in healthy cell line is similar to the cancer cell lines (Kandemir and Ipek, 2023).

3.2. TOS and TAC analyses

Treatment groups showed an increase in TOS and TAC values in A549 and SKOV-3 cell lines, while HUVEC cell line showed an increase in TAC and a decrease in TOS values compared to the control group (Fig. 3). However, OSI values were significantly decreased in all three cell lines.

Polyphenols in *P. fulgens* root extract has potent antioxidant capacity and protective effects on human health, including neoplastic diseases, as they contribute to radical scavenging and antioxidant activity. Other antioxidant compounds such as carotenoids and vitamins also contribute to radical scavenging activities (Kumar et al., 2013). Our study demonstrated the antioxidant properties of *P. fulgens* root extract by causing an increase in the total antioxidant status of the extract in cancer cells and healthy cells *in vitro*. However, despite the decrease in total oxidant saturation in healthy cells, the increase in TOS levels in cancer cells suggests that different mechanisms of the extract come into play in these cells. The decrease in OSI values in all cell lines suggests that the extract may cause a decrease in the formation of oxidant molecules or the release of reactive oxygen species in the cell.

3.3. Bax and Beclin-1 expression

In A549, SKOV-3, and HUVEC cells, the level of Bax protein was significantly ($P<0.05$) lower than the control groups. Fig. 4 displays the relative amounts of Bax protein expression. Bax can be found either in the cytoplasm or weakly bound to the membrane when inactive. However, exposure to a death signal can activate Bax in the mitochondria. Higher levels of Bax may suggest that apoptosis is being induced (Perego et al., 1996). Our findings showed that *P. fulgens* root extract exerted anti-proliferative effects and caused apoptosis in all tested cell lines, thereby indicating cell death by the extract through a Bax-independent pathway.

Fig. 4 shows the relative amounts of Beclin-1 protein expression. Our results showed that the extract substantially plummeted Beclin-1 expression. Autophagy is a crucial process that helps maintain the balance within cells by breaking down and recycling damaged cytoplasmic components, such as organelles and macromolecules. Without autophagy, survival against stress is compromised, and cells and tissues are exposed to the toxic effects of excessive accumulation of autophagy substrates. Cancer cells can use autophagy to recover from the stresses of adverse conditions, and this protective mechanism can also make them resistant to anticancer therapy. Inhibiting autophagy can enhance the therapeutic response and increase the effectiveness of cancer treatment.

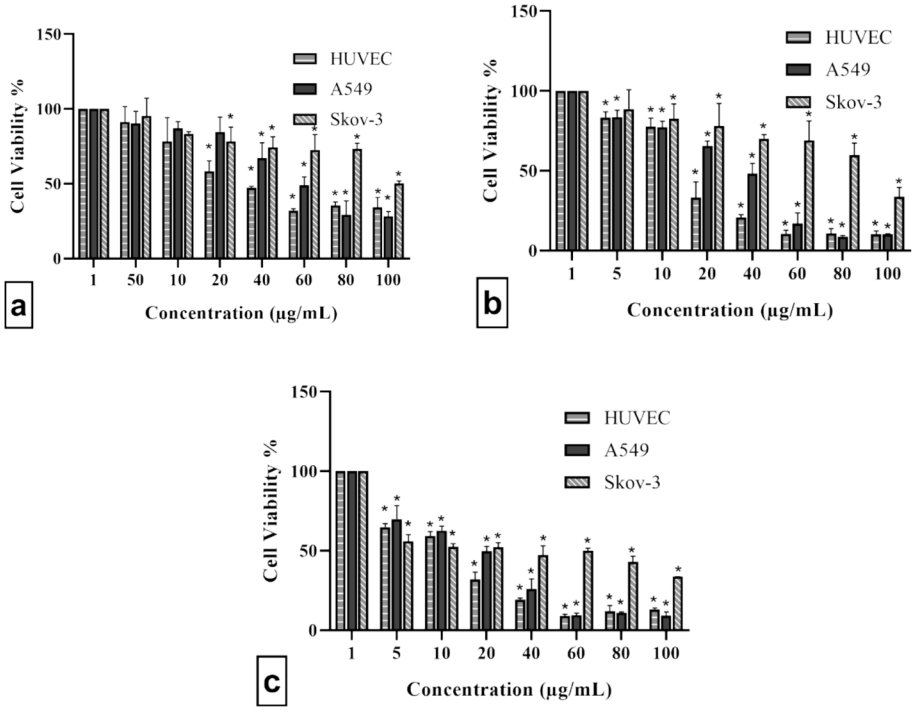

Fig. 1. Treatment of HUVEC, A549, and SKOV-3 cells with different concentrations of extract for (a) 24, (b) 48, and (c) 72 h. The extract suppressed HUVEC, A549, and SKOV-3 cells viability in a dose–dependent manner. Data were presented as mean ± standard deviation with significance level of $*P<0.05$.

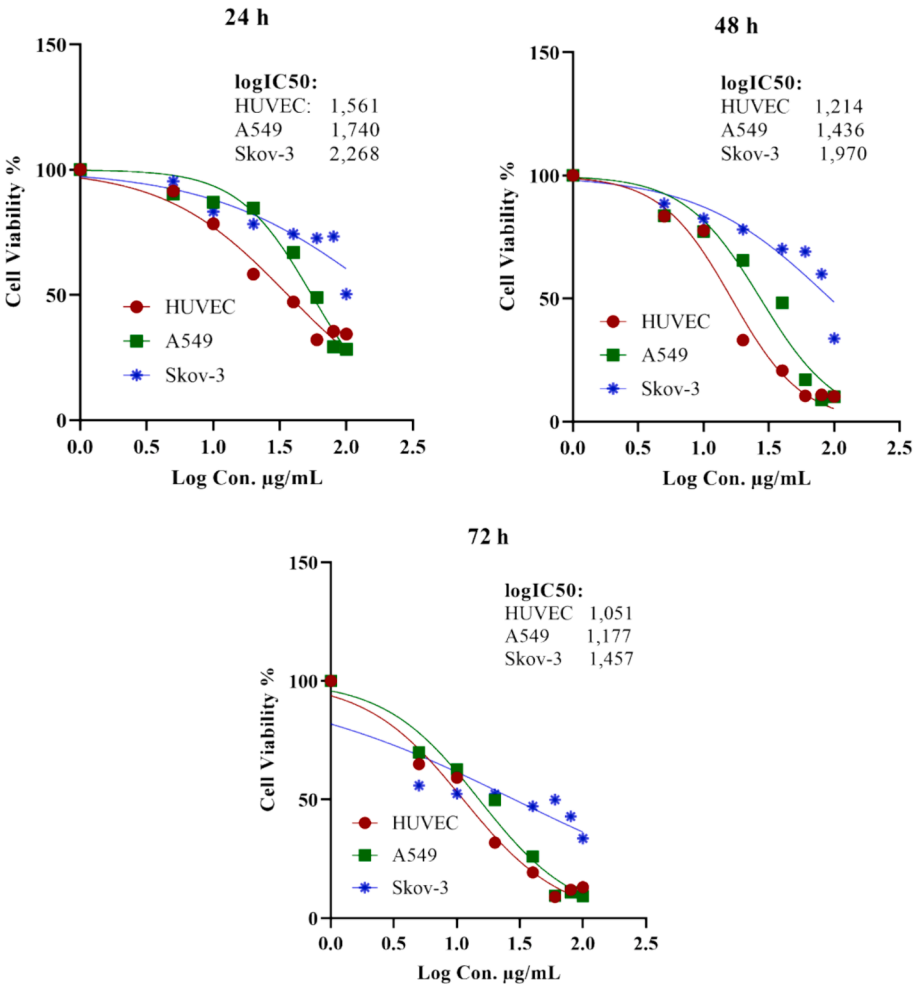

Fig. 2. $LogIC_{50}$ values for HUVEC, A549, and SKOV-3 cell lines after extract exposure for 24, 48, and 72 h.

Beclin-1 is one of the first autophagy genes reported in association with cancer. This protein plays a central role in autophagy. In association with Beclin-1, autophagy can occur in both tumor and normal tissue. Autophagy in tumor cells increases tumor cell viability (Sahni et al., 2014, Liu et al., 2017). Inhibition of autophagy may be a new target for cancer therapy, as it could prevent the survival of tumor cells. (Schweichel and Merker, 1973). In this study, the expression of Beclin-1 was plunged in treatment groups, which has been associated with the tumor-suppressive property of autophagy (Yue et al., 2003).

3.4. Nuclear factor-kappa B (NF-kB) expression

Western blotting was used to evaluate the phosphorylation level and expression of NF-kB protein. The results showed that the phosphorylation level decreased (p-NF-kB) by inducing NF-kB protein expression compared to the control groups. Statistical analyses showed that this decrement was significant ($P<0.05$) for HUVEC and A549 cell lines. The relative amounts of NF-kB protein expression are shown in Fig. 5.

NF-kB prevents cell death by activating genes that produce proteins inhibiting apoptosis, such as Bcl-2 family members, c-Flip, and IAPs. It also increases the production of antioxidant proteins that prevent reactive oxygen species-induced apoptosis and necrosis. However, the effect of NF-kB on apoptosis depends on the type of death stimulus present, and in unfavorable conditions, it can activate proteins that resist apoptosis (Karin, 2006). NF-kB probably exerts both anti- and pro-apoptotic functions, determined by the state of death stimulus rather than tissue origin. In inappropriate physiological conditions, NF-kB causes resistance to the apoptotic stimulus through the activation of a host of complex proteins. However, NF-kB activation in response to certain stimuli can lead to the induction of apoptosis. Activating some pro-apoptotic proteins such as caspases, interferon-regulated factor-1, c-myc, and p53 can explain this issue (Ghobrial et al., 2005). The function of NF-kB in apoptosis is bidirectional. Previous findings reported that NF-kB mediated the apoptosis, while few studies reported the anti-apoptotic effect of NF-kB. Continuous suppression of NF-kB in tumors prevents cell proliferation, inhibits the cell cycle, and causes apoptosis. The NF-kB pathway may have different effects in various types of cancer (Shishodia and Aggarwal, 2002). In our study, it is thought that the anti-proliferative effect of the extract may be due to the suppression of the NF-kB pathway.

3.5. ERK ½ expression

Fig. 6 displays the relative amounts of ERK ½ protein expression. Our findings indicated that the extract decreased ERK ½ expression and caused a significant reduction in phosphorylation levels of A549 cells. The ERK ½ pathway is responsible for regulating various cellular processes, including cell proliferation. It has been reported that interfering with the ERK signal cascade can interrupt DNA repair processes (Mi et al., 2023). Additionally, ERK ½ plays a crucial role in signal transduction, stimulating cell proliferation, growth, and differentiation. Studies have revealed that the activation of the ERK pathway is associated with the pathology, progression, and oncogenic behavior of various types of human cancers, including non-small lung cell cancer,

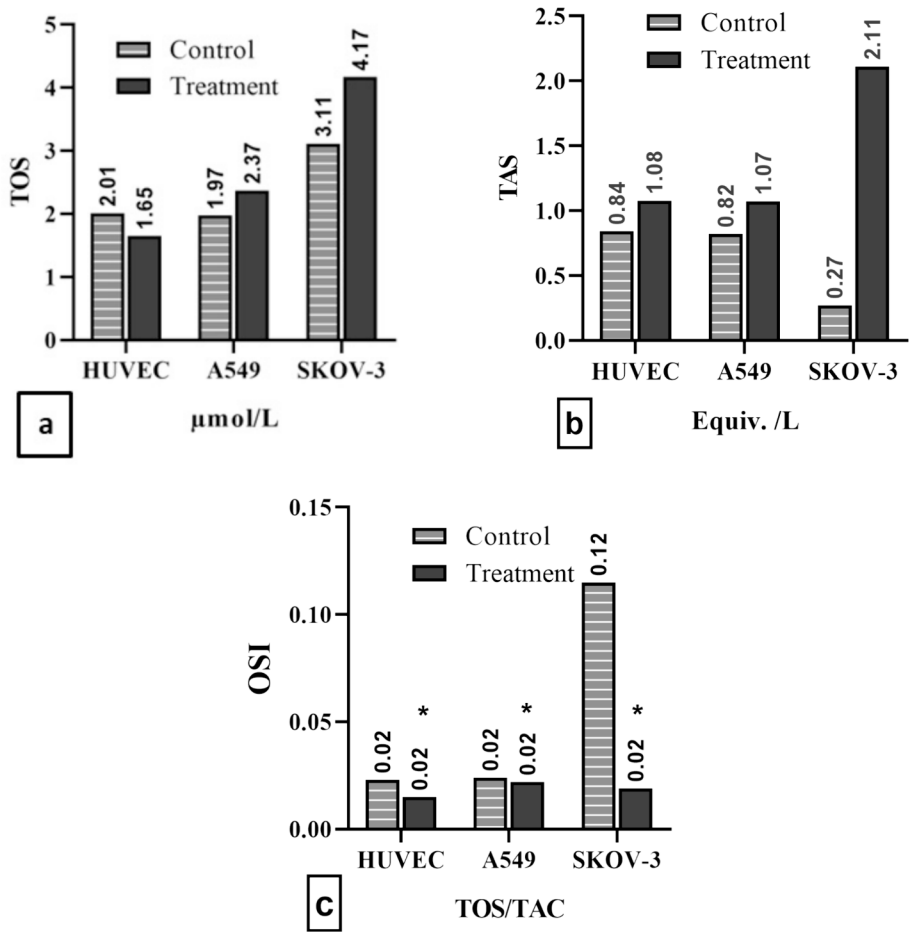

Fig. 3. (a) TOS, (b) TAC, and (c) OSI in cell lines. Data were presented as mean ± standard deviation with significance level of $^{*}P<0.05$.

breast cancer, head and neck squamous cell cancer, and colorectal cancer (De Luca et al., 2012). ERK ½ activates p90RSK, which, in turn, activates CREB, inducing the expression of Bcl-xL and Bcl-2. These Bcl-2 family members play a critical role in preserving the integrity of mitochondria by preventing cytochrome *c* release and subsequent caspase-9 activation (Liu et al., 2015).

Ras mutations are common oncogenic mutations in many human tumors. Oncogenic Ras continuously activates the ERK-1 and ERK-2 pathways, which allow tumor cells to proliferate. Therefore, inhibitors of the ERK ½ pathway are considered potential anticancer agents. In our current study, *P. fulgens* root extract might have inhibited the proliferation of lung cancer cells by inhibiting DNA damage repair via ERK ½.

3.6. Cleaved caspase-3 and SQSTM1 expression

The analysis of the cleaved caspase-3 protein expression was done through the western blot method, with β-actin protein serving as a loading control. The results of the western blot analysis indicated a significant reduction in the expression of the cleaved caspase-3 protein (Fig. 7). Caspases are considered central regulators of apoptosis, and their expression is a marker of the process of various types of cancer (Coutinho-Camillo et al., 2011). Caspases involved in apoptosis have two types, initiator caspases (caspase-8 and −9) and executioner caspases (caspase-3, −6, and −7), which was classified according to their mechanism of action. For apoptosis to occur, caspase-3 must be fully activated, which involves cleaving it to an aspartate residue to form cleaved caspase-3. This activated form of caspase-3 breaks down multiple cellular proteins and causes morphological changes and DNA fragmentation in cells (Liu et al., 2017; D'arcy, 2019; Hu et al., 2020).

In this investigation, the expression of cleaved caspase-3 decreased. This reduction indicates that the caspase-dependent apoptosis pathway did not activate, and therefore, the extract did not cause cell death through this pathway. It is possible that the extract suppressed cell viability through alternative pathways, such as a caspase-independent apoptosis pathway or other cell death pathways.

Fig. 7 illustrates the relative amounts of Sequestosome 1 protein (p62/SQSTM1) expression. There was a significant ($P<0.05$) reduction in the expression of p62/SQSTM1. Autophagy is a process of programmed release of toxic components through the lysosomal system, and p62/SQSTM1 is one of the receptors involved in selective autophagy (Kumar et al., 2022). According to Li et al. (2021), the excessive production of p62 leads to the spread of cancer to the bone by stimulating migration rather than the growth of lung adenocarcinoma cells. Autophagy can prevent the accumulation of p62 and suppress tumor formation. Additionally, specific proteins that regulate autophagy, including p62 (SQSTM1), have been found to influence the activity of NF-kB (Li et al., 2021). Our findings revealed that the groups that received the extract treatment had weaker expression of SQSTM1 expression, compared to the control groups. Lower levels of SQSTM1 expression have been associated with the tumor suppressor function of autophagy.

4. Conclusions

Our findings demonstrated that *P. fulgens* root extract had the ability to inhibit the ERK½, NF-kB, p62/SQSTM1, and Beclin-1 signaling pathway, leading to a decrease in the cell viability in lung (A549) and ovarian (SKOV-3) cancer cells. This anti-proliferative activity of the

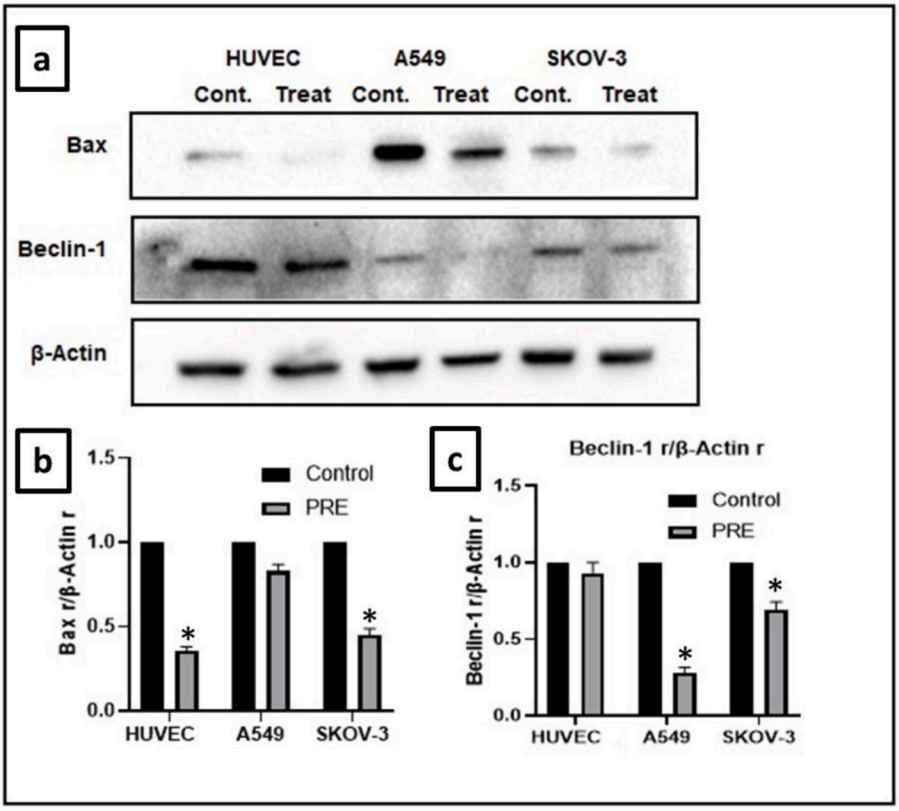

Fig. 4. Effect of extract on expression of Bax and Beclin-1 protein in cell lines. **(a)** In A549, SKOV-3, and HUVEC cell lines, the Bax levels and the autophagy marker Beclin-1 levels were decreased compared to the control groups. The reduction in Bax was significant ($P<0.05$) in both HUVEC and SKOV-3 cell lines, while the reduction in Beclin-1 was significant ($P<0.05$) in both A549 and SKOV-3 cell lines. (β-actin is the loading control). **(b)** The intensity of Bax was analyzed through densitometry and presented as a ratio to the total level of β-actin. **(c)** The intensity of Beclin-1 was analyzed through densitometry and presented as a ratio to the total level of β-actin. (Control, arbitrarily set to 100 %; $*P<0.05$). PRE – *P. fulgens* root extract.

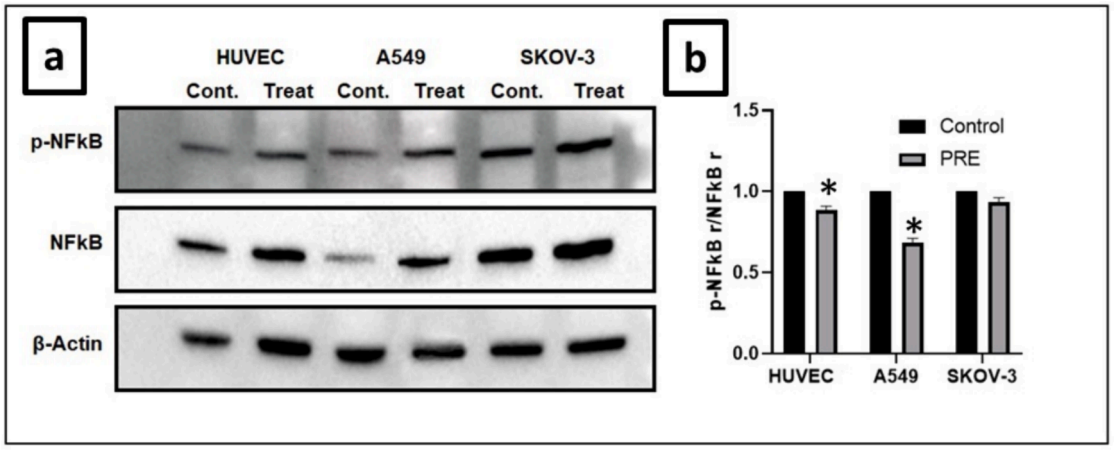

Fig. 5. Effect of extract on NF-kB expression in cell lines. **(a)** In A549, SKOV-3, and HUVEC cells, the extract caused an increase in phosphorylation by promoting the expression of NF-kB protein. This increase was significant in the HUVEC and A549 cell lines. **(b)** The intensity of p-NF-kB was analyzed through densitometry and presented as a ratio to the total level of NF-kB. (Control, arbitrarily set to 100 %). ($*P<0.05$). PRE – *P. fulgens* root extract.

extract indicated its potential to be used as a new chemotherapeutic agent for the treatment of lung and ovarian cancers. However, the toxicity of extract against healthy HUVEC cell line confines its application as anticancer agent. In addition, the extract, which has high antioxidant properties, reduced the formation of oxidant molecules or the release of reactive oxygen species in healthy cells, suggesting that *P. fulgens* root may offer a new approach for cancer treatment but its effect on healthy cells should not be ignored.

CRediT authorship contribution statement

Polat İpek: Conceptualization, Investigation, Methodology, Writing – original draft. **Ayşe Baran:** Conceptualization, Investigation, Methodology, Writing – original draft. **Mehmet Fırat Baran:** Conceptualization, Investigation, Data curation. **Aziz Eftekhari:** Project administration, Supervision, Visualization, Writing – review & editing. **Ameer Khusro:** Writing – review & editing, Methodology,

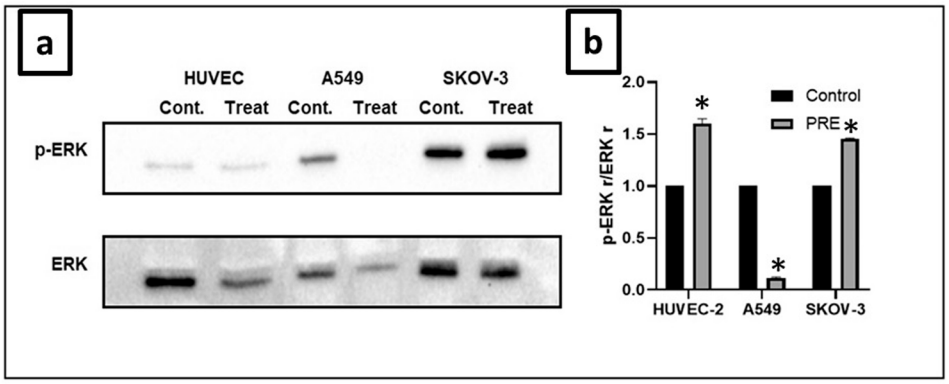

Fig. 6. Effect of extract on ERK ½ protein expression in cell lines. **(**a) The extract inhibited ERK ½ protein expression in A549, SKOV-3, and HUVEC cells, reducing phosphorylation. (b) The intensity of p-ERK was analyzed through densitometry and presented as a ratio to the total level of ERK ½ (Control, arbitrarily set to 100 %). (*P<0.05). PRE – *P. fulgens* root extract.

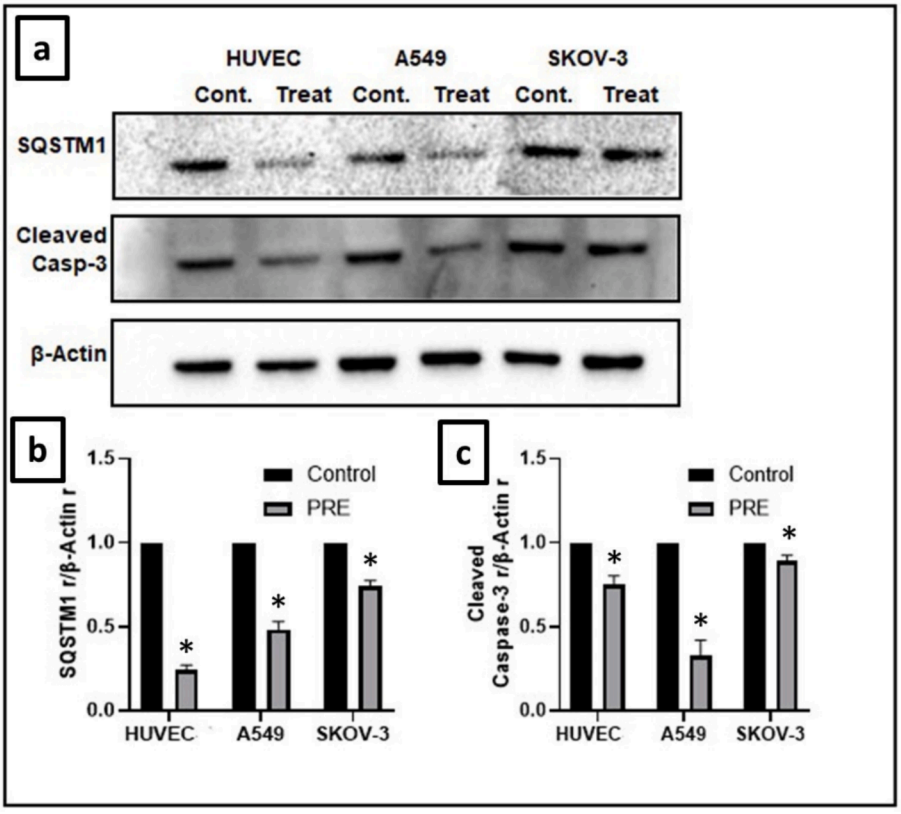

Fig. 7. Effect of extract on cleaved caspase-3 and SQSTM1 expression in cell lines. **(a)** Levels of cleaved caspase-3 and the autophagy marker SQSTM1 were notably lowered in HUVEC, A549, and SKOV-3 cell lines as compared to the control groups. The loading control was β-actin. **(b)** The intensity of cleaved caspase-3 was measured by densitometry and presented as a ratio to the total level of β-actin. **(c)** The intensity of SQSTM1 was analyzed through densitometry and presented as a ratio to the total level of β-actin. (Control, arbitrarily set to 100 %). (*P<0.05). PRE – *P. fulgens* root extract.

Visualization, Conceptualization, Validation. **Mohammad Mehdi Ommati:** Resources, Visualization, Writing – review & editing. **Elvin Aliyev:** Data curation, Visualization. **Rovshan Khalilov:** Visualization, Supervision, Formal analysis. **D. Esther Lydia:** Conceptualization, Validation. **Mohamed Farouk Elsadek:** Formal analysis, Funding acquisition. **Saeedah Musaed Almutairi:** Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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