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REGULATORY POTENTIAL OF RHIZOME EXTRACT FROM *PARIS POLYPHYLLA* VAR. *YUNANENSIS* ON PRO-APOPTOTIC PROTEINS IN A549 LUNG CANCER CELLS

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Summary

Regulatory Potential of Rhizome Extract from *Paris polyphylla* var. *yunanensis* on Pro-Apoptotic Proteins in A549 Lung Cancer Cells

Paris polyphylla var. *yunanensis* has been shown to affect against various types of cancer cells. The aim of this study was to investigate the effects of 80% ethanol extract from *Paris polyphylla* var. *yunanensis* rhizomes (PPRE) on cell proliferation and apoptosis in a non-small cell lung cancer cell line (A549) *in vitro via* expression of apoptosis-related proteins using western blot. Our result showed that PPRE at 2.5, 5, and 10 µg/mL concentrations significantly inhibited A549 cell proliferation in a dose-dependent manner after 24 and 48 h of treatment. Furthermore, A549 cells were treated with the PPRE at the concentrations of 4.5, 5.0, and 5.5 µg/mL for 24 h showed markedly higher Bcl-2-associated X (Bax) protein expression levels, significantly lower Bcl-2 expression levels, and obviously notably higher Bax/Bcl-2 ratio than the control. Meanwhile, the p53 protein expression levels were not significantly reduced compared to the control by the PPRE. The findings of this study implicate that *Paris polyphylla* var. *yunanensis* rhizome extract may be a potential candidate to inhibit the proliferation of A549 cells and promote apoptosis by the Bcl-2/Bax pathways. Studying the effect of PPRE on some other molecular markers involved in programmed cell death should be done to elucidate the mechanism of A549 human lung cancer cell apoptosis of the PPRE.

Keywords: *Paris polyphylla* var. *yunanensis*, *Rhizome extract, Apoptosis, Cytotoxicity, A549.*

1. Introduction

Apoptosis is the process of programmed cell death in which cells die in response to self-generated signals. This process is momentous for cellular homeostasis and plays an important role in manipulation in cancer prevention [1]. Apoptosis is a complex mechanism and involves multiple pathways including caspase activation, altered mitochondrial permeability, pro- and anti-apoptotic protein balance, and enhanced p53 protein expression. Among them, the mitochondrial-dependent intrinsic apoptosis pathway plays an important role, especially the persistence of Bcl-2 family members. The Bcl-2 family proteins are further subdivided into anti-apoptotic proteins such as Bcl-XL, Bcl-w, Mcl-1 and pro-apoptotic proteins such as Bax, Bak and Bok. The activation of Bax and Bak regulates cytochrome c to cytosol release from mitochondria through mitochondrial outer membrane permeabilization [2]. Released cytochrome c induces apoptosis by activating the caspase terminal agent. On the other hand, p53 plays an important role in the mechanism of apoptosis in cancer and activates both intrinsic pathways (Bcl-2, Puma, and Noxa protein families) and extrinsic pathways of a programmatically dead process. Thus, upregulation of p53 expression could be an effective way to treat cancer through the induction of apoptosis. Taken together, targeting the induction of cancer cell death by apoptosis may have potential in cancer therapy.

Lung cancer is increasingly common worldwide with increasing rates of poor prognosis and mortality [3]. According to World Cancer Research Fund International, there were more than 2.2 million new cases and almost 1.8

million people died from lung cancer in 2020 worldwide. Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases [4]. Despite significant advances in tumor therapy, the 5-year survival rate of patients with NSCLC remains <20%, as the majority of cases are usually diagnosed at an advanced or locally advanced stage and patients may experience serious side effects from current therapies [5]. Therefore, new potential anticancer drugs are important for NSCLC. Medicinal plants are believed to be a rich source of drugs and they have been used as novel compounds to treat many diseases, including cancer, because of their ability to inhibit the growth of cancer cells and induce programmed death of cancer cells [6].

Paris polyphylla var. *yunanensis* is a perennial herb of the genus *Paris*, family Trilliaceae. Currently, there are two species, *Paris polyphylla* var. *chinensis* (Franch.) Hara and *Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz., have been identified by official sources. In traditional medicine, it has the function of reducing fever, detoxifying, reducing swelling and relieving pain. It is also widely used to treat swelling, sore throat, snakebite, sharp pain, and convulsions [7]. Modern studies have shown that compounds isolated or extracted from *Paris polyphylla* have potential pharmacological effects such as anticancer [8], antioxidant [9], antibiotic, and anthelmintic effects [10]. The chemical constituents of *Paris polyphylla* include steroid saponins, steroid compounds C21, flavonoids, phytosterols, polysaccharides, phytoecdysons and amino acids [10]. Among these, steroid saponins act as the main active ingredients [11]. Previous studies demonstrated that *Paris polyphylla* var. *yunnanensis* had antitumor effect, of which steroid saponins are the main active components [8],[12],[14]. The 60% ethanol extract of *Paris polyphylla* var. *yunnanensis* induced prostate cancer cell apoptosis *via* endogenous and exogenous pathways (PARP1, Bcl-2, Bax, Caspase-8, and Caspase-3) as well as cell cycle arrest in G0/G1 and G2/M phases in PC3 cell while G0/G1 phase in DU145 cell [12]. Extracts and saponins of *Paris polyphylla* var. *yunnanensis* showed significant inhibitory cytotoxic activities on human liver cancer cell lines (SMMC-7721, HepG2, and SK-HEP-1) and human lung cancer cell line (A549). Saponins induced significant apoptosis and cell cycle arrest in three human cancer cell lines (A549, SMMC-7721, and HepG2), which was associated with the loss of mitochondrial membrane potential [13]. Polyphyllin VII induces apoptotic cell death *via* inhibition of the PI3K/Akt and NF-κB pathways in A549 human lung cancer cells [14].

In this study, the effects of *Paris polyphylla* var. *yunanensis* rhizome extract on the proliferation and regulation of pro-apoptotic proteins in A549 human lung cancer cells were examined by MTT assay and western blot technique, respectively.

2. Materials and methods

2.1. Plant materials

Paris polyphylla var. *yunanensis* rhizomes were collected in March 2018 from Mang But Ward, Kon Plong District, Kon Tum Province, Vietnam. The plant sample were identified and authenticated by MSc. Le Duc Thanh (Research Center of Ginseng and Medicinal Materials Ho Chi Minh City-CGMM) and a voucher specimen (TNDL-BLMH-2018) was kept at the Department of Natural Resources and Medicinal Materials Development (CGMM). Rhizomes were washed and dried (Loss of drying (LOD) ≤ 13%). These dried rhizomes were ground to a fine powder and kept individually in airtight PVE bag at the CGMM (Sample code: TTS-BLMHKT-2018).

2.2. Extract preparation

Rhizome powder was extracted with 80% ethanol (OPC Pharmaceutical Company, Vietnam) at room temperature by percolartion, the liquid extract was continuously dripped after 24 hours of immersion at a rate of 2 mL/min and 80% ethanol was also added in parallel to achieve a ratio of 1/6 (*w/v*). The liquid extract was concentrated using a rotary evaporator at 70°C under reduced pressure to get *Paris polyphylla* var. *yunanensis* rhizome extract (PPRE). The extraction yield of the extract was 18.82%. The extract had the presence of saponin components and gracillin compound by chemical reaction and thin layer chromatography. The total saponin content in the extract was 32.73% determined by photometric method using standard gracillin [15]. The crude extract were stored at 2 - 8°C and dissolved in DMSO (Dimethyl sulfoxide, Sigma) to yield a stock solution.

2.3. Cell culture

Human lung cancer cells A549 were cultured in Roswell Park Memorial Institute 1640 (RPMI, Sigma-Aldrich, UK) with 10% fetal bovine serum (Cytiva-HyClone, US), 1% penicillin-streptomycin (Sigma-Aldrich, Israel), in 5% CO_2 and air in an incubator (Esco Lifesciences, Singapore) at 37°C. Cells are removed from the monolayer using 0.25% trypsin (Cytiva-HyClone, US) in phosphate buffered saline, resuspended in medium and seeded at a suitable density in plate.

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cytotoxicity

The viable cells were seeded at a density of 1×10^4 (100 µL/well) in a 96-well plate (Corning) and incubated in a condition of 5% CO_2 at 37°C for 24 h to form a cell monolayer. After 24 h, 100 µL of medium containing the extract at concentrations of 1.25, 2.5, 5, 10, and 25 µg/mL were added and incubated for 48 h. After that, 100 µL MTT agent (Sigma-Aldrich, US) was added into each well and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Supernatants were removed and 100 µL of DMSO (Sigma-Aldrich), was added and the plates were incubated for 15 min to solubilize the formazan crystals and absorbance was measured at 570 and 630 nm using Multifunctional ELISA System (Biotek, USA). Doxorubicin (Sigma) was used as a positive control. The viability of A549 cells treated with and without PPRE was expressed as cell viability percentage (%) by comparing the mean absorbance of cells from different groups as Cell viability (%) = (Mean absorbance treated cells - Mean absorbance blank)/(Mean absorbance untreated cells - Mean absorbace blank) ×100 [16].

2.5. Morphological assessment of A549 cells post-PPRE treatment

The morphological assessment of PPRE treated and untreated cells were additionally undertaken. Briefly, 1×10^4 cells were seeded, treated with 2.5, 5, and 10 µg/mL PPRE and incubated for 24 and 48 h. After incubation, these treated cells were visualized to observe any changes in their appearance compared to the untreated control cells using a microscope Eclipse Ts2R-FL (Nikon, China).

2.6. Protein isolation and western blot analysis

A549 cells were seeded at a density of $5x10^4$ cells/mL on 6-well plates (Corning) and cultured for 24 h at 37 °C in 5% CO_2. The cells were then treated with and without the extract at the concentration of 4.5, 5.0, and 5.5 µg/mL and cultured in a medium supplemented with FBS 10% for 24 h. The cells were collected and washed with Dulbecco's phosphate-buffered saline (Sigma-Aldrich, US). The harvested cells were then lysed on ice for 1 h in 70 µL cell lysis buffer containing 10 mM Tris-HCl (pH 7.6), 1% Triton X100, 100 mM sodium chloride, 10% glycerol, 1 mM EDTA (pH 8.0), 1 M sodium orthovanadate, 25 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 1M sodium fluoride, 0.5 M glycerol-2-phosphate, protease, and phosphatase inhibitor II, III (Sigma-Aldrich). The cell lysate was centrifuged at 16,000 rcf for 15 min to remove cell debris using Centrifuge 5430R (Eppendorf, US). Supernatants were collected from the lysates and protein concentrations were determined using the Pierce Bovine serum albumin protein assay kit (Thermo Scientific). Aliquots of the lysates (20 µg of protein) were boiled for 5 min and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with primary antibodies, Bax 1: 500 (633801, BioLegend), Bcl-2 1: 500 (633501, BioLegend), p53 1: 200 (SC-126, Santa Cruz), or α-tubulin 1: 1000 (2144S, Cell Signaling Technology). The membranes were further incubated with horseradish peroxidase-conjugated secondary anti-mouse or antirabbit antibody (anti-rabbit IgG, HRP-linked Antibody 1: 2000 (7074S, CST) and anti-mouse IgG, HRP-linked Antibody 1: 2000 (7076, CST)). Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Merck). For chemiluminescence detection, the ImageQuant LAS 500 system (Cytiva, GE Heathcare Life Sciences, US) was used [17].

2.7. Statistical analysis

All experiments were repeated at least three times (Mean ± Standard deviation). The IC_{50} value was determined from the curve of log dose-response (X-axis) and percentage of cytotoxicity (Y-axis) calculated from the absorbance values. From this sigmoidal curve, IC_{50} (X value) was calculated by setting Y value as 50% using R version 4.2.2. One-way analysis of variance was used to determine the difference between the experimental groups. Dunnette test was used to compare treatment groups with controls ($p < 0.05$ is statistically significant).

3. Results

3.1. PPRE induces cytotoxicity in A549 human lung cancer cells

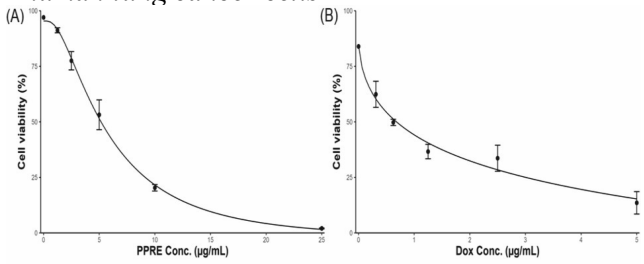

Fig. 1. PPRE induced cytotoxicity within A549 cancer cells. Fig. A and B shows percent cell viability of A549 cancer cells after PPRE and Doxorubicin (Dox) treatment after 48 h, respectively (Mean ± SD, $n = 3$).

The cytotoxic and antiproliferative effects investigated by PPRE on A549 human lung cancer cells by using MTT assay. The results affirmed that PPRE treatment dose from 1.25 µg/mL to 25µg/mL

inhibited the cell growth and proliferation of A549 cells dose-dependently (Fig. 1). The inhibition of A549 cells after treatment with PPRE at the concentration of 5 µg/mL was found to be 46.83% ± 6.70 compared with untreated control cells while Doxorubicin had inhibitory percentage of 50.25% ± 1.41 at 0.625 µg/mL (~ 1.15 µM). The IC50 value of PPRE and Doxorubicin on A549 cancer cells was 5.07 ± 0.25 µg/mL and 0.64 ± 0.07 µg/mL (~1.18 µM), respectively.

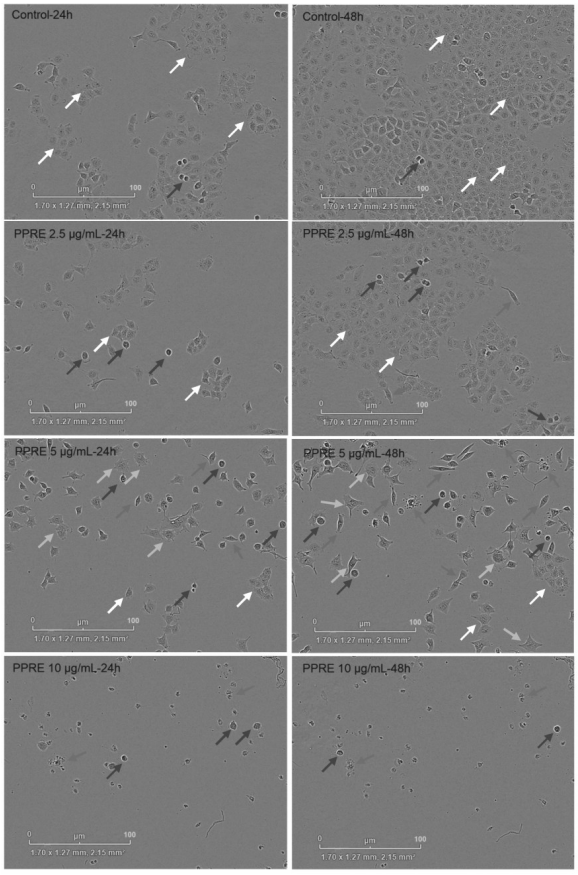

Fig. 2. Morphology of A549 cancer cells treated with PPRE at different concentrations. Representative images of A549 cells with and without PPRE treatment after 24 and 48 h, scale bar = 100 µm. White, red, yellow, orange, and blue arrows indicate cell, shrinkage, swelling, cell debris, and withering of cell organelles

Moreover, alterations in the morphology of PPRE treated A549 cancer cells were studied by performing microscopy after 24 and 48 h treatment. At both times, the number of A549 cancer cells was significantly reduced by PPRE treatment compared with the control in a concentration-dependent manner. A549 cells had morphological alterations such as cell shrinkage, swelling, cell granulation, and withering of cell organelles (Fig. 2), the number of cells with this phenomenon appeared more at the concentration of 5 µg/mL PPRE compared to the concentration of 2.5 µg/mL PPRE. In particular, there seemed to be no viable cells after 24 and 48 h of PPRE treatment at 10 µg/mL. These findings suggesting PPRE mediated cytotoxicity in A549 cancer cells.

3.2. PPRE regulates pro-apoptotic proteins in A549 human lung cancer cells

The role of cellular elements such as Bax, Bcl-2, and p53 in apoptosis has been acknowledged. Bax and Bcl-2 are two crucial downstream factors involved in p53-dependent apoptosis [21],[23]. To further identify the mechanism cytotoxicity effect of PPRE on A549 cells, several key markers associated with apoptosis in A549 cells were initially examined after 24 h of treatment with 4.5, 5.0, and 5.5 µg/mL PPRE. Result revealed that a significant increase in the expression of the pro-apoptotic marker (Bax) in PPRE-treated cells compared to the control (Fig. 3D), while the expression of the anti-apoptotic protein (Bcl-2) was reduced significantly (Fig. 3C). On the other hand, Bax/Bcl-2 ratio was profoundly increased in cells treated with PPRE (Fig. 3E). Taken together, these findings provide evidence that PPRE can induce apoptosis in A549 cells, which is associated with the deregulation of Bcl-2/Bax signaling pathway. The regulation of expression of p53 is implicated in the induction of apoptosis. However, the PPRE did not significantly downregulate the expression of p53 compared with the control (Fig. 3B).

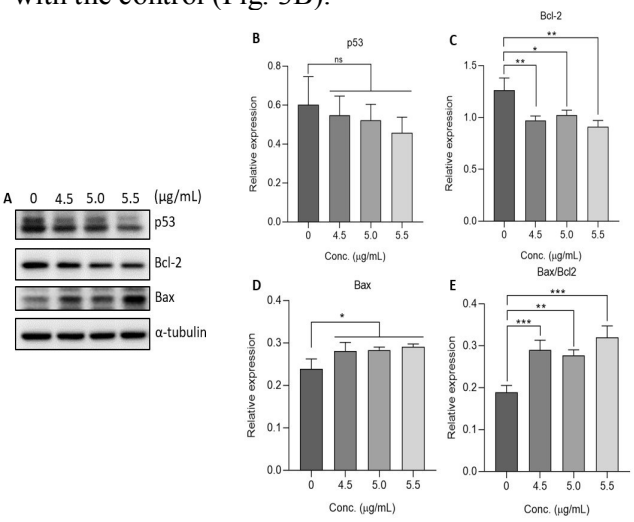

Fig. 3. Effects of PPRE on pro-apoptotic protein expression (p53, Bcl-2, Bax) in A549 cancer cells. (A) Representative images of protein bands analyzed by SDS-PAGE with appropriate primary antibodies combination, (C-D) Relative expression levels of the target proteins, (E) Bax/Bcl-2 ratio (Mean ± SD, $n = 3$). Values were corrected for the expression of the housekeeping protein alpha tubulin. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with untreated group.

4. Discussion

Medicinal plants and natural components are used more and more widely in health care and disease treatment [6]. Some species of the genus *Paris* are well known for their good anti-tumor properties [7],[8]. The genus *Paris* includes several species, such as *P. polyphylla* var. *chinensis*, *P. polyphylla* var. *yunnanensis*, and *P. vietnamensis*, all of which contain very rich phytochemicals, especially the steroid saponin, main active components, has outstanding anticancer effect. *P. polyphylla* var. *yunnanensis* saponins has been shown to inhibit cell proliferation, induce apoptosis and cell cycle arrest to control the growth of cancer cells [8],[12,[13]. Steroidal saponins from *P. polyphylla* var. *chinensis* significantly reduced survival, induced apoptosis and autophagy in A549 lung cancer cells [18]. Steroidal saponins of *Paris vietnamensis* reversed temozolomide resistance in glioblastoma cells *via* inducing apoptosis through ROS/PI3K/Akt pathway [19]. In the present study, the *P. polyphylla* var. *yunnanensis* rhizome ethanol extraction was be investigated for its effects on A549 lung cancer cell proliferation and apoptosis, contributing a piece of scientific data for current and future studies.

Uncontrolled cell proliferation and avoidance of programmed cell death are prominent features of cancer and its resistance to treatment; therefore, targeting apoptosis-related pathways is considered a powerful tool in cancer treatment [20]. Apoptotic genes/proteins regulate apoptosis by the action of their pro- and anti-apoptotic factors, in which Fas, caspase, and Bcl-x family proteins are important proteins. Many drug components have anticancer activity *via* the Bax/Bcl-2 pathways *in vitro* [21]. However, there are differences in the expression of apoptotic genes related to the clinical and pathological features of tumor progression and metastasis. Interestingly, protein expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax has both been associated with longer survival in NSCLC patients [22].

The study results revealed that *P. polyphylla* var. *yunnanensis* rhizome extract could significantly inhibit A549 cellular proliferation in dosage-dependently. Moreover, this study also demonstrated the effect of *P. polyphylla* var. *yunnanensis* rhizome extract on A549 cellular apoptosis through apoptosis-associated protein expressions including p53, Bax, and Bcl-2. The appearance of shrinking, swelling, and withering cells was observed in PPRE-treated A549 cancer cells for 24 hours. Additionally, the increased expression of Bax protein and decreased expression of Bcl-2 protein by PPRE treatment resulted in cellular apoptosis. On the other hand, the Bax/Bcl-2 ratio was markedly increased when A549 cancer cells were treated with the extract, however further insights into the cell signaling pathways, for example Bax/Bcl-2 activation and interactions, is necessary to be able to draw reasonable conclusions in conjunction with existing data. Therefore, further *in vivo* studies should be encouraged.

In carcinogenesis, p53 can regulate cell growth, DNA repair, angiogenesis, and apoptosis by participating in various cell signaling pathways. P53 controls the transcription of proapoptotic members of the Bcl-2 family, for instance Bax, Bid, Noxa, and Puma, resulting in apoptosis induction [23]. The expression of the tumor suppressor gene p53 was analyzed. Notably, *P. polyphylla* var. *yunnanensis* rhizome extract indistinctly downregulates the expression of p53 while increased its downstream signaling (Bax). This is similar to previously published results showed that rhizoma *Paridis* saponins induced apoptosis in NSCLC A549 cells through decreased the expression of p53, Bcl-2 and increased the expression of Bax [24]. Although the majority of previous studies showed that increased p53 expression positively contributes to anticancer activity, loss/reduction of p53 expression in some cancer cell lines altered cell cycle responses and did not prevent cell death [25]. NSCLC is a heterogeneous disease associated with multiple gene variants, expression imbalances, and disturbances of many biological functions, including cell cycle, apoptosis, angiogenesis, and immune escape. Therefore, repeated assessments and further studies should be performed to better assess the effects of the extract.

The present study showed that *P. polyphylla* var. *yunnanensis* rhizome extract could suppress proliferation and apoptosis of the A549 cancer cells by regulating the expression of p53, Bax, and Bcl-2. Therefore, in accordance with the present experimental results, a pathway including p53, Bax, and Bcl-2 was speculated that can be affected by *P. polyphylla* var. *yunnanensis* rhizome extract (Fig. 4). In this pathway, *P. polyphylla* var. *yunnanensis* rhizome extract could directly or indirectly affect the target-regulated expression of p53, subsequently change the expression of the downstream proteins Bax and Bcl-2, and lead to cell proliferation inhibition and apoptosis, finally inhibiting NSCLC A549 progression. More comprehensively, investigations of the effects of *P. polyphylla* var. *yunnanensis* rhizome extract on other related proteins and different treatment times in A549 cells should be performed for a more precise assessment of the molecular mechanism leading to A549 lung cancer cell apoptosis.

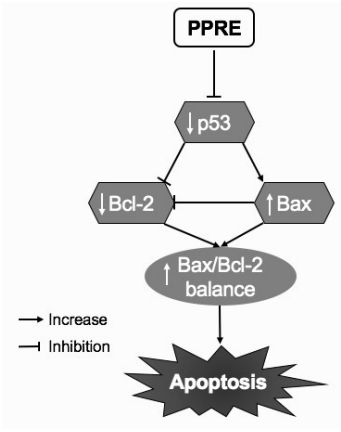

Fig. 4. The proposed mechanism of *P. polyphylla* var. *yunnanensis* rhizome extract (PPRE) treatment for non-small cell lung cancer A549. p53, Tumor protein p53; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

5. Conclusion

Paris polyphylla var. *yunanensis* rhizome extract is able to inhibit the cancer cell proliferation by inducing mitochondria-dependent apoptosis in A549 cancer cells. These results contributed to the understanding of the anticancer activity of *Paris polyphylla* var. *yunanensis* rhizomes and its potential in providing bioactive compounds for treating lung cancer.

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