

The augmentation in apoptosis and cell cycle-associated transcriptional gene expression in A549 cells induced by *Polyspora huongiana* leaves methanol extract

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Abstract: According to the WHO, in 2020, lung cancer was recorded as the second most common new case of cancer in the world, and it causes the most deaths among cancer types. The side effects during the treatment are considerable, so novel therapies are being proposed and research broadened. Research on medicinal herbs, including tea, in cancer treatment always brings breakthrough discoveries. Although *Polyspora huongiana* was described many years ago, its scientific data on the ability to treat lung cancer have still been mystified. The cytotoxicity assay and the wound healing evaluation combination were performed to investigate the cytotoxicity of the *P. huongiana* leaf extract on lung cancer cells, A549. The flow cytometry assay and the transcriptomic level measurement were experimented with to clarify the action primarily. The results showed that the extract inhibited cell growth with an IC₅₀ of 195.80 ± 25.00 µg/mL, and the apoptotic cells were observed. The apoptosis and cell cycle-regulated gene escalation were recorded in A549 under treatment with the *P. huongiana* extract.

INTRODUCTION

Cancer is a health problem of global concern. According to statistics from the World Health Organisation (WHO) (Sung *et al.*, 2021). Global cancer statistics 2020: GLOBOCAN, in 2020, cancer was the cause of about 10 million deaths. The death rate and new detection of lung cancer are among the top cancer cases. Among the types of lung cancer, four common types account for more than 90% of lung cancer cases: small cell carcinoma, large cell carcinoma, adenocarcinoma, and squamous cell carcinoma (Wahbah *et al.*, 2007). Lung cancer is also classified based on epithelial origin. It is divided into two types: non-small cell lung cancer (NSCLC) and small cell (SCLC), of which the number of cases diagnosed with NSCLC accounts for about 80% (Sechler *et al.*, 2013). Several mechanisms have been proposed as the causes of NSCLC, such as inactivating mutations in the p53 tumour suppressor gene, increasing

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mutations of EGFR, and suppressing the PI3K/AKT pathway (Cooper *et al.*, 2013). Currently, Chemotherapy, radiotherapy, or a combination of Chemotherapy and radiotherapy treats NSCLC; the choice of treatment regimen depends on the overall disease stage (Zarogoulidis *et al.*, 2013). The issue of reduced response and drug resistance is a concern in the treatment of NSCLC, which leads to decreased treatment effectiveness and high relapse rates (Sechler *et al.*, 2013). Moreover, the chemotherapy produced by chemically synthesised drugs has side effects for cardiovascular and renal impairment. It induces blood cell counts, so the current trend is to focus on research using herbal compounds to affect the molecular mechanisms of cancer cells (Bahar *et al.*, 2022; Carr *et al.*, 2008).

Polyspora hongiana Orel, Curry & Luu is a species of the Theaceae family, first discovered in 2010 in Bidoup-Nui Ba National Park, Vietnam (Orel *et al.*, 2012). This tea is an endemic species found only in Vietnam and holds significant value in terms of biodiversity. *P. huongiana* belongs to the genus *Polyspora* of the Theaceae family. *Polyspora* has more than 40 species that mainly grow in Asia, such as Vietnam, Malaysia, China, Sri Lanka, Indonesia, Thailand, Laos, Myanmar, India, and the Philippines (Nguyet Hai Ninh *et al.*, 2020). *P. huongiana* is a woody plant; the height of a mature tree does not exceed 10 m; The leaves are thin, hard, narrowly elliptical, about 11.0 - 12.5 cm long, and 4.0 - 4.8 cm wide; Flowers grow singly and are dark pink to red, with five to seven petals. Stamens are arranged in a circle with a diameter of 2.0 - 4.0 cm, and are yellow. The flowering period of *Astragalus* is around January; the fruit ripens from February to April and falls around April (Nguyet Hai Ninh *et al.*, 2020; Orel *et al.*, 2012). *P. huongiana* is discovered in small groups scattered in thick, high-canopy forests with excellent climate conditions. The species distribution area is less than 3000 km². It is classified as a vulnerable group according to the classification of the Red List of tea species published by the Botanic Gardens Conservation International (Beech *et al.*, 2021, 2022). The phytochemicals and biological activities of this species have not yet been determined, so *P. huongiana* is a potential subject for research. Studies on plants of the Theaceae family show the presence of natural compounds with high biological activity in antioxidant, antibacterial, anticancer, and anti-inflammatory processes, as well as positive effects on human health, typically green tea (*Camellia sinensis*), which is rich in polyphenols (Namita *et al.*, 2012). Polyphenols have antioxidant properties, fight free radicals formed in the body, inhibit the proliferation of some types of cancer cells through inducing apoptosis, and help improve health problems in blood pressure, protecting the vascular system (Vauzour *et al.*, 2010). Among these polyphenol groups, Catechins make up about 70% of the total polyphenol content and about 1/3 of the dry content in green tea, and catechins include epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), in which the commonly known compound is EGCG (Lorenzo & Munekata, 2016; Musial *et al.*, 2020). Mechanisms preventing cancer of catechin are proposed in several ways, such as decreasing Bcl-2 protein family levels, affecting cell cycle arrest, increasing accumulation of cell G0/G1 block, and enhancing apoptosis through increasing activity of caspase protein (Beltz *et al.*, 2006). In addition, Catechin from green tea (*Camellia sinensis*) is exhibited to inhibit the viability of the A549 cell line and reduce *Bcl-XL* mRNA expression (Sonoda *et al.*, 2014). However, depending on the phytochemical composition, there are differences in the level of impact, so it is necessary to evaluate the bioactivity of this endemic tea species. This study was carried out to assess the effect of *P. huongiana* leaf extract on inhibition of proliferation and the expression of genes related to cell cycle disruption and apoptosis induction in non-small cell lung cancer (A549) cells.

METHOD

Plant Extraction

P. huongiana leaves were harvested by Mr Truong Quang Cuong, an ecological officer at Bidoup National Park, Vietnam. Mr Phung My Trung further identified the plant. The voucher specimen (No. 210622PHU) is deposited at the International Research Centre, Bidoup–Nui Ba

National Park, Vietnam. The leaves were doubly washed with tap water and drained before being fully oven-dried at 40 °C. The dried sample was ground into a powder, separated into tags, and stored at -20 °C until extraction. One hundred grams of tea powder was macerated with 300 millilitres of absolute methanol in continuous shaking for 24 hours before the filtrate was recovered. The residue powder was soaked with methanol again three more times. The vacuum evaporation process using a rotary evaporator (Eyela, USA) was applied to obtain dry crude extract. It was dissolved in DMSO (Sigma-Aldrich, USA) to make a stock extract of 250 mg/mL, abbreviated as PHE. The PHE was stored at -20 °C until use.

Cell Preparation and Cell Culture Conditions

A549 cells (ATCC, USA) were thawed by being incubating at 37 °C in a water bath (Mettler, Germany) for 30 seconds and then immediately diluted in a triple volume of thawing solution consisting of 50% Dex40 10% (Protide Pharmaceuticals, USA), 25% human albumin 10% (Roche, USA), and 25% DPBS (Stemcell, Singapore). The cells were collected by centrifuging and DPBS double washing. Cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 (Roswell Park Memorial Institute Medium (Thermo Scientific, USA) supplemented with 1% Penicillin-streptomycin (Sigma-Aldrich, USA) and 10% Fetal Bovine Serum (Sigma-Aldrich, USA) at 37°C, 5% CO₂ in a CelCulture incubator (Esco, Singapore). The initial cell density was 10⁵ cells/mL, and subcultured after 72 hours using TriPLE enzyme (ThermoScientific, USA).

Cytotoxicity Investigation

A volume of 100 µL cancer cells was seeded at a density of 10⁵ cells/mL and incubated for 24 hours at 37 °C, 5% CO₂ in a CelCulture incubator (Esco, Singapore). 100 µL medium supplemented with the PHE in different concentrations was added to each well. The cells were incubated for 72 more hours. The cell viability was determined by using (3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide (MTT - Sigma-Aldrich, USA). The cultured medium was removed from the plates, and then 200 µL of the basal RPMI harbouring 10% MTT was added to each well. After incubating at 37 °C, 5% CO₂ for 3 – 4 hours, the medium was removed, and the precipitation was dissolved by 100 µL Dimethyl sulfoxide (DMSO – Protide Pharmaceuticals, USA). The absorbance was measured at 570/630 nm. DMSO at a final concentration of 0.1% was used as a negative control. The following formulation computed the cell proliferation: % Cell viability = (OD_{sample}/OD_{control}) x 100%.

Wound-Healing Evaluation

Cells were seeded at 5 × 10⁴ in 24-well plates and incubated for 24 hours for adhesion. A line scratch was made on the surface of each well, and the medium supplemented with the PHE ranging from 25 to 100 µg/mL was added into each well. The cells were observed every 24 hours; the wound recovery was computed using ImageJ software. DMSO at a final concentration of 0.1% was used as a negative control.

PI and Annexin V-Stained Cell Analysis

Cells were seeded at 10⁵ cells/mL in 6-well plates and incubated for 24 hours for adhesion. The PHE extract was supplemented into the wells to achieve the 100 µg/mL concentration. DMSO at a final concentration of 0.1% was used as a negative control. After a 24-hour incubation, the cells were harvested and doubly washed with DPBS (Stemcell, Singapore). Cells were stained with PI and Annexin V according to the protocol of ANNEX100B (Bio-Rad, USA). The status of cells was analysed using the BD Accuri C6 Plus Instrument (Becton, Dickinson and Company, USA).

Transcriptional Change Evaluation

1.5 mL A549 biomass at 2 × 10⁶ cells/mL density was seeded in 6-well plates. After 24h of cell adhesion formation, a 1.5 mL medium containing PHE at 100 µg/mL was supplemented to each well and incubated for 24 hours. DMSO at a final concentration of 0.1% was used as a

negative control. Cells were collected and extracted RNA using TRIzol™ Reagent (Sigma-Aldrich, USA). The transcriptomic level of selected genes related to the apoptosis pathway and cell cycle was analysed by performing RT-qPCR with the SensiFAST™ Real-Time PCR Kits (Meridian Bioscience, USA) and PCR thermal cycler instrument (DNA-Technology, Russia). The primers used were detailed and listed in Table 1. The GADPH was chosen as a housekeeping gene for analysis. The gene expression was calculated by a $2^{-\Delta\Delta C_t}$ formulation, where ΔC_t was defined as the difference between the C_t value of the target gene and that of the reference gene and $\Delta\Delta C_t$ as the difference between ΔC_t values of treated and control samples.

Table 1. Primers list for analysis.

Gene	(5' – 3') Sequence	Reference
<i>TP53</i>	TGTGGAGTATTTGGATGACA GAACATGAGTTTTTTATGGC	(Lee et al., 2022)
<i>pRB</i>	ACTCCGTTTTTCATGCAGAGACTAA GAGGAATGTGAGGTATTGGTGACA	(Burkhart et al., 2010)
<i>Bcl-XL</i>	TTGGACAATGGACTGGTTGA GTAGAGTGGATGGTCAGTG	(Kumar et al., 2017)
<i>Bcl-2</i>	AAGATTGATGGGATCGTTGC GCGGAACACTTGATTCTGGT	(Shivakumar & Armitage, 2006)
<i>Fas</i>	CACACTCACCAGCAACAC TCCTTTCTCTTCACCCAAAC	(Oehm et al., 1992)
<i>Survivin</i>	GTTGCGCTTTCCTTTCTGTC TCTCCGCAGTTTCCTCAAAT	(Kim et al., 2019)
<i>Caspase 3</i>	GAAGTGGACTGTGGCATTGA CCTTTGAATTTTCGCCAAGAA	(Perveen et al., 2020)
<i>Caspase 8</i>	CTGCTGGGGATGGCCACTGTG TCGCCTCGAGGACATCGCTCTC	(Jana et al., 2017)
<i>Caspase 9</i>	GGTGATGTCGGTGCTCTTGA CGACTCACGGCAGAAAGTTCA	(Quan et al., 2023)
<i>CDK-1</i>	GGGTCAGCTCGCTACTCAAC AAGTTTTTGACGTGGGATGC	(Balasubramani et al., 2019)
<i>CDK-4</i>	CATTGGGGACTCTCACACTCTC ATGGCTACCTCTCGATATGAG	(Quan et al., 2023)
<i>CDK-2</i>	TCATGGATGCCTCTGCTCTCAC TGGAGGACCCGATGAGAATGGC	IDT, Inc.
<i>GADPH</i>	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	(Quan et al., 2023)

Data Analysis

All experiments were conducted at least three times with technical duplicates. The data is expressed as mean $\pm$ SD. The data analysis was computed in GraphPad Prism version 9.0.0. The students' T-test and one-way ANOVA were compared with the alpha value of 5%.

RESULTS

P. huongiana Extract Inhibits A549 Cell Growth

The cell proliferation decreased under the effect of PHE; the impact was recorded to be dose-dependent, as the more concentrated PHE, the more lethal activity observed (Figure 1). The influence was documented to be unchanged with less than 100 $\mu\text{g/mL}$ PHE. In other words, the results indicated the inhibitory impact of PHE on A549 at low dosages. The cell viability dramatically decreased with the PHE dosage above 100 $\mu\text{g/mL}$. The non-linear regression depicted the IC₅₀ in the anti-lung cancer cells A549 of PHE was 195.80 ± 25.00 $\mu\text{g/mL}$.

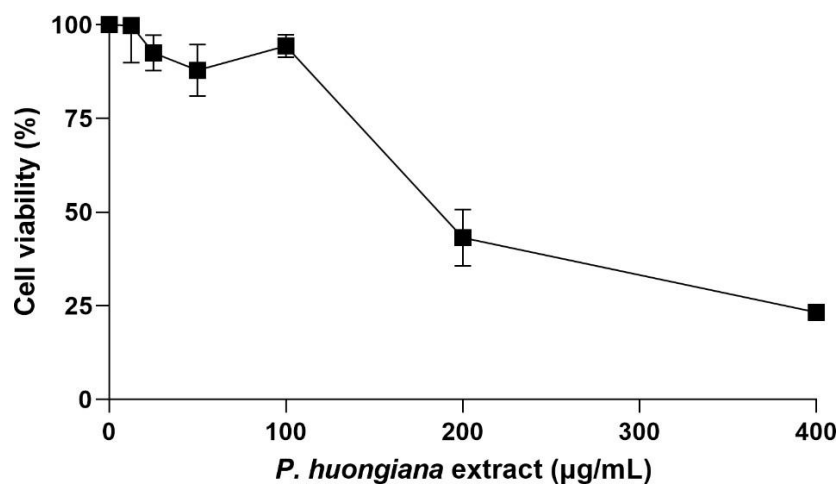


Figure 1. The lethality of the *P. huongiana* total extract on A549 cells.

P. huongiana Retards the A549 Wound Recovery

In the presence of PHE, the ineffective healing wound is expressed (Figure 2A). The cell-free area's digitalisation reflected the cells' healing capacity under treatment; the PHE presentation inhibited the recovery of the cells. Almost 90% of the free zone was usurped by cells after 72 hours, but the recovery percentage was 68.06 ± 5.92 and 57.19 ± 0.40 for the presence of 50 µg/mL and 100 µg/mL PHE (Figure 2B). The PHE treatment significantly decreased the cells' invasion, p -value = 0.0003. The wound closure rate ($\mu\text{m}^2/\text{h}$) was calculated as 7164 ± 180.10 , 7025 ± 507.4 , 4132 ± 638.40 , and 5073 ± 483.70 for the negative control and the treatments with 25, 50, and 100 µg/mL, respectively. The results suggested that the PHE exhibited the strongest inhibitory effect on A549 migration at a concentration of 50 µg/mL.

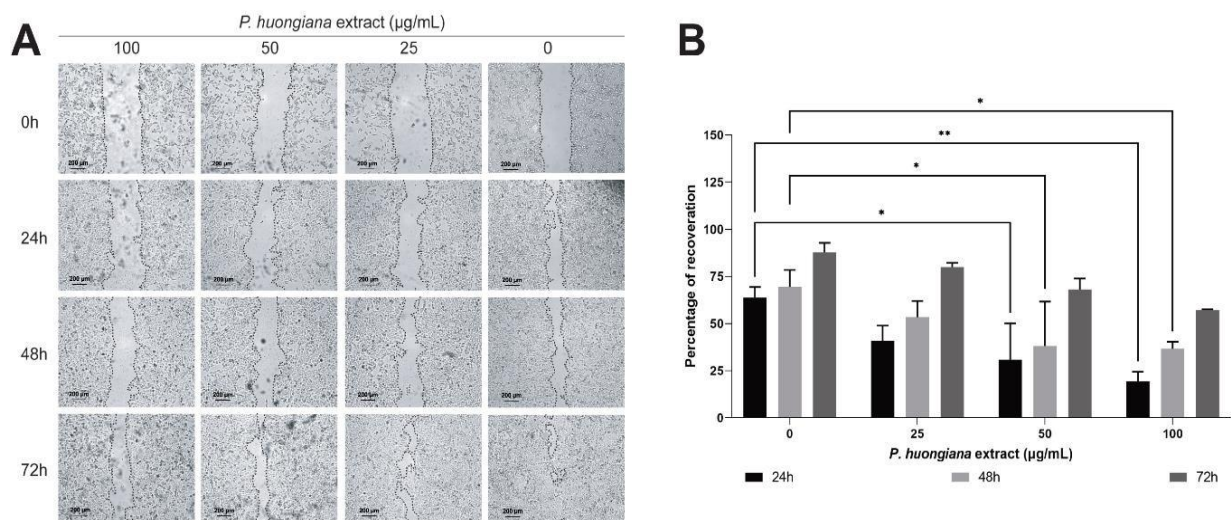


Figure 2. *P. huongiana* inhibits the wound recovery of A549 cells. (*) p -value < 0.05; (**) p -value < 0.01.

P. huongiana Induces Apoptosis in A549 Cells

PI (read by PE detection) and Annexin V (read by FitC detection) dyes were used on cells exposed to PHE for 24 hours to check cell status. The results are shown in Figure 3. The quartile representing apoptotic cells was denoted as Q2; the number of events displayed in this area increased from 12.6% to 33.3% after 24 hours of PHE impact (Figure 3).

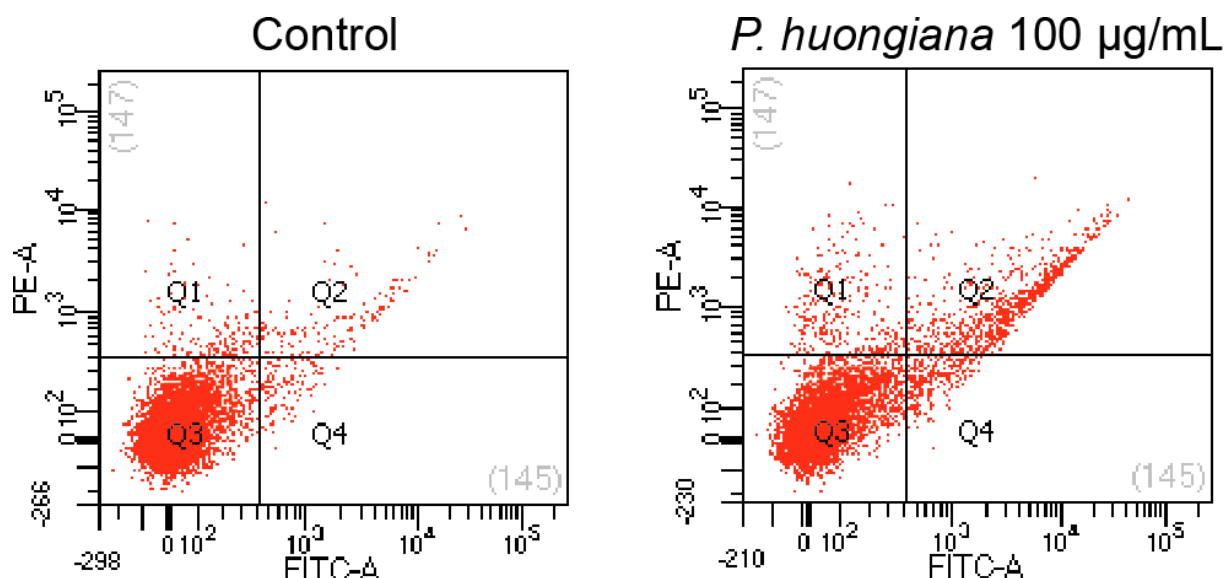


Figure 3. *P. huongiana* induces apoptosis in A549 cells.

P. huongiana Modifies the Apoptosis and Cell Cycle Gene Expression

The gene expression was augmented in the presence of PHE (Figure 4). The elevation tendency of the genes was also divided into three groups, including the group of genes related to the extrinsic pathway of apoptosis (*Caspase 8* and *Fas*), the group of genes in the intrinsic way (*Caspase 9*, and anti-apoptosis genes: *Bcl-XL*, *Bcl-2*), and the downstream genes (*Survivin* and *Caspase 3*), p -value = 0.0036. The increased amount of mRNA of the *CDK-2* was recorded as the most preponderant, p -value = 0.0122, while the amounts of the *TP53* and *CDK-4* was dwarfed, p -value = 0.0384.

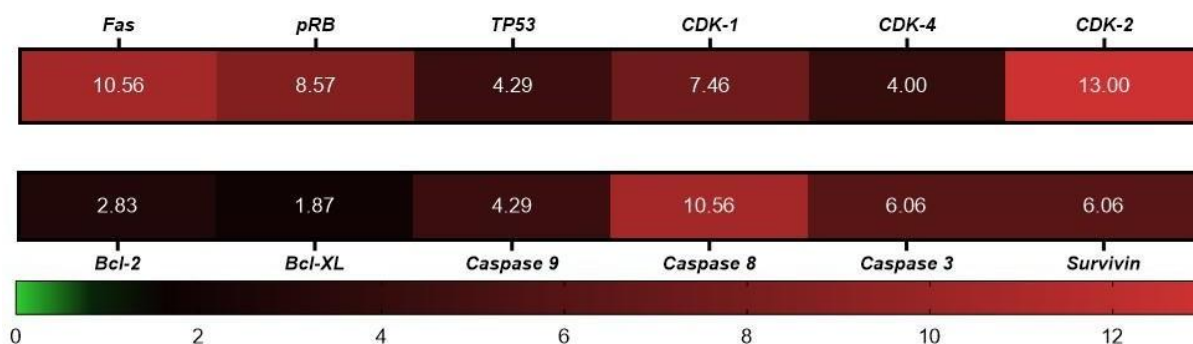


Figure 4. The transcriptomic expression changes in A549 under the effect of PHE.

DISCUSSION and CONCLUSION

Lung cancer is one of the most prevalent cancer types nowadays (Padinharayil *et al.*, 2023). Beings had faced this issue for decades before the productivity treatment was applied. However, the restriction during treatment reduces the effectiveness of therapies but somehow enhances the development of new treatment material discovery. Thus far, scientific evidence suggests that Theaceae plays a promising role in alternative treatments for lung cancer (Monteiro Lde *et al.*, 2014). Up to now, the lethal effect of tea on lung cancer cells has been in short supply, and this impact induced by the *Polyspora* genus has been utterly blurred. In previous report of *Polyspora* interconnected species, *Camellia* genus, and anti-proliferation on A549 cells, sharply revealed such species as *Camellia nitidissima* (IC₅₀ of leaf extract ranged from 50 µg/mL), *Camellia Oleifera* (IC₅₀ of seed-derived saponin was 18.5 µM), and *Camellia sinensis* (IC₅₀ of leaf extract was 112 µg/mL) (Lu *et al.*, 2009; Wang *et al.*, 2022; Zhou *et al.*, 2014). On the other hand, Vietnamese endemic tea plants exhibited anticancer effects in previous reports on

HepJ5 and K562 (Linh *et al.*, 2023; Xuân *et al.*, 2023). Recently, the study on *P. huongiana* successfully identified the structures of 54 volatile compounds present in the leaves, which provides a basis for the observed antioxidant and Gram-positive antibacterial activities (Tan *et al.*, 2024). In this study, the inhibitory activity against A549 cells was described. Although the observed effect was associated with a relatively high IC₅₀ value ($195.80 \pm 25.00 \mu\text{g/mL}$) and no cytotoxicity was detected at the commonly expected threshold of $100 \mu\text{g/mL}$, the wound-healing results further support the notion that, even at non-cytotoxic concentrations, the investigated extract may still slow cellular motility. The study was considered the first record of *P. huongiana* anti-proliferation activity on human alveolar basal epithelial adenocarcinoma cells, and this work played an essential role in promoting the expansion of in-depth research on this tea plant.

The manifestations of abnormal cell shape during the experiment suggested apoptosis in A549 cells induced by PHE. Although various scientific studies on apoptosis-inducing agents were carried out on the genus *Camellia*, the works on *Polyspora* have been limited. The increase in the population, simultaneously positive for both dyes, illustrated that the cells followed the apoptosis pathway. The consequences of tea extracts that cause alteration in gene expression have been submitted to and discussed in many academic works, and they have outcomes in considerably different approaches. Whereas the data on *Polyspora* have not been published, the gene expression modification of closely related plant species, such as *Camellia* or *Pyrenaria* (Pat. No. KR102135071B1), has been carried out through several scientific reports (Kim *et al.*, 2022). In this study, the gene expression was investigated indirectly through the gene target's mRNA, and the transcription level was reflected via $2^{-\Delta\Delta C_t}$ values. Apoptosis-related genes are transcriptionally activated at early stages following cellular stress, whereas prolonged treatment may obscure mRNA changes due to cellular adaptation or RNA degradation during late-stage cell death (Healy *et al.*, 2013). Hence, the PI/Annexin V and mRNA level determinations were evaluated after a 24-hour treatment. As shown in Figure 4, the death of A549 cells could be suggested to be extrinsic apoptosis, as indicated by the increase in the mRNA levels of *Fas* and *Caspase 8*. However, further analysis is required to clarify the pathway. The increased expression of signalling factors such as *Caspase 8* is closely related to the increased expression of other intracellular elements, including *Caspase 9* and *Caspase 3*. A slight increase in the expression of the anti-apoptotic members such as *Bcl-2* and *Bcl-XL* might lead to the sequestering of proforms of death-driving cysteine proteases, including *Caspase 9*, causing inhibition of expression. The transcriptional regulation was illustrated as the capacity of numerous compounds belonging to tea, including catechins (Chi *et al.*, 2014; Kim *et al.*, 2022). Tea extracts were demonstrated to include the ability to induce apoptosis in many cancer cell lines, such as leukemia, pheochromocytoma, and hepatoma (Chi *et al.*, 2014; Ly *et al.*, 2013). Survivin plays a dual role in suppressing apoptosis and regulating cell-cycle progression (Chen *et al.*, 2016). Changes in *Survivin* expression may therefore be associated with adaptive survival mechanisms and alterations in cell-cycle regulation rather than direct induction of apoptosis. Although the mechanism of action-reflection was not as directly exhibitable as protein measurement, the mRNA fluctuation data contributed to the initial lore of comprehensive insights into the pathways of action of PHE extract on A549 cells. The changes in the cell cycle were observed to parallel the progression of death through the apoptotic pathway. These findings further support a cautious interpretation of the molecular data. The cell cycle-associated genes were evaluated at the transcriptomic level, and the gene expression indicated the amelioration. The *CDK-2* transcriptomic growth could be intimately related to the rise of the *pRB*, which led to *CDK-2* enhancement at the G1 checkpoint. The cell cycle reorganising has been previously discussed as a biological effect of tea extracts, such as the enhancement of the S phase of *C. sinensis* on U-937 or the enhancement of the G0 phase on K562 and the push of the G2 phase cell population of *C. nitidissima* on HCT116, SW480, and HCT116 (Chen *et al.*, 2021; Yusoff *et al.*, 2021). The inhibitory effect of *P. huongiana* methanol extract on lung cancer was first observed in this study, suggesting an influence on gene expression adjustments

associated with the cell cycle and apoptosis pathways. The data depicted the extract's dose-dependent manner of toxicity; the inception of extrinsic-induced apoptosis was also suggested. The primary scientific knowledge of *P. huongiana* serves as an initial stepping stone to enlargement. Therefore, in-depth research should be earnestly contemplated for a comprehensive data set on this tea species to exploit, use, and conserve effectively.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. Ethics Committee Approval and its number should be given by stating the institution name which gave the ethical approval.

Data Availability Statement

Research data is available and can be sent via e-mail upon request.

Authorship Contribution Statement

NTQ: Methodology, Performance, Visualisation, Software, Formal Analysis, and Writing-original draft; **TTCT:** Performance, Methodology; **BTKL:** Supervision and Validation; **HTC:** Methodology, Supervision, Validation, and Approval.

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