Pelargonidin Inhibits Cell Growth and Promotes Oxidative Stress-Mediated Apoptosis in Lung Cancer A549 Cells

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Research Article

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Abstract

Background: Lung cancer has the worst prognosis with an average 5-year survival rate of only 10-20%. Lung cancer has the highest prevalence rate and a second most common cause of cancer-associated mortalities worldwide. Objective: The present study was planned to explore the anticancer effects of pelargonidin against the lung cancer A549 cells via analyzing oxidative stress-mediated apoptosis.

Methodology: The viability of both control and pelargonidin-treated A549 cells was analyzed using the MTT cytotoxicity assay at different time periods. The levels of endogenous ROS generation, mitochondrial membrane potential ($\Delta \psi_m$), and apoptosis was assessed using corresponding fluorescent staining assays. The levels of oxidative stress biomarkers including TBARS, SOD, CAT, and GSH in the cell lysates of control and pelargonidin-treated A549 cells was examined using the assay kits.

Results: The pelargonidin treatment at diverse concentrations were substantially suppressed the viability of lung cancer A549 cells with an IC50 range of 20 $\mu$M for 48 hr. The further treatment with 20 and 40 $\mu$M effectively promoted the endogenous ROS production and depleted the $\Delta \psi_m$ levels in the A549 cells. The outcomes of the dual staining and DAPI staining assays witnessed the occurrence of increased apoptosis in the pelargonidin-treated A549 cells. The pelargonidin also boosted the TBARS and reduced the CAT, SOD, and GSH levels thereby promoted the oxidative stress-regulated apoptosis in the A549 cells.

Conclusion: In summary, the findings results of the current study demonstrated an anticancer activity of pelargonidin on A549 cells. The pelargonidin treatment substantially decreased the growth and encouraged the oxidative stress-regulated apoptosis in A549 cells. Therefore, it was evident that the pelargonidin could be employed as an effective anticancer candidate to treat the lung cancer.

Introduction

Lung cancer is a foremost cause of cancer-associated mortalities worldwide, accounting for around 27% of all cancer-related fatalities. Lung cancer has the worst prognosis of all tumor forms, with a 5-year survival rate of only 10–20% on average [1]. According to estimates, 2.20 million new cases of lung cancer occur each year, making it one of the most prevalent. The report by Global Cancer Observatory highlighted the world prevalence of lung cancer was 2.21 million worldwide in 2020 and it may increase to 3.63 million new incidences by 2040. Furthermore, the death rate of lung cancer is reported to rise from 1.80 million to 3.01 million incidences [2]. Both men and women are equally at risk for developing lung cancer [3].

The lungs are a major secondary location for cancers that originate in other organs, such as the breast, skin, pancreas, and liver. Tobacco use is directly responsible for 75% of lung cancer incidences, while the remaining 20% are accountable to secondhand smoke and air pollution [4]. High alcohol consumption, cigarette use, secondhand smoke, exposure to radon gas, infections such the Human Papillomavirus (HPV), and inflammatory disorders are the most frequent risk factors associated to rising cases and fatality rates of lung cancer [5].
Apoptosis is a crucial cell death mechanism in the eradication of tumor cells. This cell death process can be activated by several cascades, especially both the intrinsic and extrinsic signaling cascades can induce this mechanism [6]. Several signaling pathways contribute to inducing cancer cell apoptosis, which is an effective cancer treatment. Several anticancer medications work by controlling the body’s response to oxidative stress, which has been shown to have an effect on tumor growth. Patients with cancer may sometimes need multiple therapies administered in order to decrease dosage dependency and ultimately eradicate the disease [7]. Lung cancer is notoriously resistant to therapy, both initially and as the disease progresses. Incorrect apoptotic signaling can play a role in chemotherapy and radiation resistance [8].

Despite its prevalence, this form of cancer is currently only discovered in the later stages of the disease, when most treatments have lost their efficacy. As a result, people with this disease have a life expectancy of no more than five years following diagnosis [9]. The existing anticancer medications (chemotherapy) are extremely toxic to both malignant and the non-malignant cells to other parts of the body. In addition, it shows several adverse effects including hepatotoxicity, nephrotoxicity, and cardiotoxicity, and is linked to antioxidant defence system abnormalities, cardiomyopathy, arrhythmias, and myocarditis. Moreover, they have many drawbacks, such as low selectivity, low adsorption, and high drug resistance [10].

Thus, finding new anticancer medicines that cause only tumor cell death has gained significant interest. Furthermore, it is still challenging to develop chemotherapeutic medications that can target cancer cells without harming healthy cells [11]. Hence, researchers are continually developing new anticancer medications by chemical synthesis or the isolation of chemicals from natural sources that are more deadly to cancer cells while causing less harm to healthy cells [12].

Pelargonidin is major type of anthocyanidin, which is extensively found in the various fruits, vegetables, and berries [13]. Pelargonidin has been found to be exhibit an excellent biological properties such as anti-genotoxic [14], anti-inflammatory [15], neuroprotective [16], anti-diabetic [17], antihypoxic [18], antithrombotic [19], and anti-atherosclerosis [20] properties. Furthermore, the pelargonidin has reported to show an excellent antitumor effects against several tumor cells [21, 22]. However, the anticancer properties of pelargonidin against the human lung cancer was studied yet. Therefore, the current study was dedicated to explore the anticancer properties of the pelargonidin against the lung cancer A549 cells via promoting the oxidative stress-regulated apoptosis.

**Materials and methods**

**Chemicals**

The pelargonidin, fetal-bovine serum (FBS), antibiotics, dimethyl sulfoxide (DMSO), etc., were procured from Sigma-Aldrich, USA. The assay kits were procured from Thermofisher, USA.

**Cell culture collection and maintenance**
The A549 cells were acquired from ATCC, USA, and were cultivated in an incubator containing 5% CO2 using DMEM media with 10% FBS. After 80% confluence, cells were harvested for use in further fluorescence staining and biochemical tests.

**MTT assay**

The MTT test was used to determine the effect of pelargonidin on the growth of A549 cells. Using 96-well plate, the cells were cultured for 24 hrs. Following that, cells were exposed to pelargonidin for 24 hrs at a dosages of 1, 5, 10, 20, 40, 60, and 120 µM for 24, 48, and 72 hr. Following treatment, 20 µl of MTT reagent along with 100 µl of DMEM was added to each well for 4 hrs. Followed by the dissolving of the generated formazan depositions using DMSO (100 µl), the absorbance was taken at 570 nm using an microplate reader.

**Dual staining**

A dual staining method was employed to find the apoptosis in both untreated control and pelargonidin-treated A549 cells. A 24-well plate was utilized to cultivate the cells, and then they were treated with 20 and 40 µM pelargonidin for 24 hrs. To find out the apoptosis in A549 cells, 100 µg/ml of an AO/EB dye combination was mixed to each well, and the plates were maintained in the dark for 5 min. The produced fluorescence was examined with a fluorescence microscope.

**DCFH-DA staining**

DCFH-DA staining was used to evaluate the ROS generation in both control and pelargonidin-exposed A549 cells. After 24 hours of growth in a 24-well plate, the cells were treated with 20 and 40 µM pelargonidin. Then, 10 µl of DCFH-DA dye was mixed to each well for 10 min. Using a fluorescent microscope, the strength of the produced fluorescence was assessed, which is relevant to the developed ROS level.

**DAPI staining**

Using the DAPI staining technique, the nuclear structure of apoptotic cells in pelargonidin-treated A549 cells was examined. The 24-well plate was used to grow the cells for 24 hr and then treated with pelargonidin at 20 and 40 µM for 24 hr. The control and treated cells were then stained with DAPI (200 µg/mL) for 15 min after being fixed for 30 min with the paraformaldehyde (4%). After that, a fluorescence microscope was used to examine the developed fluorescence to detect the nuclear damage and apoptosis in A549 cells.

**Rhodamine-123 (Rh-123) staining**

The changes in Δψm level was analyzed by Rh-123 staining technique in pelargonidin-treated A549 cells. After the 24 hr of growth in the 24-well plate, the cells were exposed to the 20 and 40 µM of pelargonidin for 24 hr at 37°C. Rh-123 dye (10 µg/ml) was then utilized to stain the cells for 30 mins before they were examined using fluorescent microscopy.
Detection of oxidative stress marker levels

The control and pelargonidin-treated A548 cells were obtained and homogenized using the cell lysis buffer to prepare the cell lysate. The prepared cell suspension was centrifuged at 3000rpm for 4 min and the resulting supernatant was gathered in order to analyze the oxidative stress biomarker levels. The levels of TBARS, SOD, CAT, and GSH in the cell lysates of both control and pelargonidin-treated A549 cells were examined using the corresponding assay kits by strictly following the guidelines provided by the kit’s manufacturer (ThermoFisher, USA).

Statistical analysis

The obtained values are statistically assessed using SPSS software, and the final data was given as a mean ± SD of triplicate measurements. One-way ANOVA and DMRT assays was employed to analyze the values and the significance was set at p < 0.05.

Results

Effect of pelargonidin on the viability of A549 cells

The growth level of the control and pelargonidin-treated A549 cells was assessed by MTT assay, and the findings were revealed in Fig. 1. The growth of the A549 cells was considerably decreased by pelargonidin treatment at various doses (1-120 µM) when compared to control. The inhibitory concentration 50 (IC50) of the pelargonidin was found to be 20 µM, which inhibited 50% of cell growth (Fig. 1). As a result, an IC50 and high dosage of pelargonidin of 20 and 40 µM, respectively, were selected for the additional experiments.

Effect of pelargonidin on the apoptosis in the A549 cells

Figure 2 displays the findings of an AO/EB dual staining analysis of apoptosis in control and pelargonidin-treated A549 cells. There was a remarkable upsurge in the intensity of yellow and orange fluorescence when A549 cells were exposed to the pelargonidin at dosages of 20 and 40 µM compared to the control. Hence, it was clear that the pelargonidin enhances both early and late apoptosis in A549 cells, as revealed by a significant increase in the intensity of the yellow/orange fluorescence in the treated cells compared to the control (Fig. 2).

Effect of pelargonidin on the ROS generation in the A549 cells

The endogenous ROS generation in the control and pelargonidin-treated A549 cells was detected by DCFH-DA staining, and the outcomes are exhibited in Fig. 3. The outcomes proved that the 20 and 40 µM pelargonidin treatment substantially increased the endogenous ROS production in the lung cancer cells, as evidenced by an upsurge in the green fluorescence in the pelargonidin-treated A549 cells compared to
control (Fig. 3). Hence, it was clear that pelargonidin promotes ROS production thereby facilitate oxidative stress-mediated cell death in A549 cells.

**Effect of pelargonidin on the Δψm level of A549 cells**

Figure 4 shows the results of a Rh-123 staining technique to determine the status of Δψm in the control and pelargonidin-treated A549 cells. As revealed in Fig. 4, an upsurge in green fluorescence was observed in the control cells, demonstrating a healthy and normal Δψm level. However, treatment of the A549 cells with 20 and 40 µM pelargonidin caused a dramatic reduction in Δψm level, as demonstrated by a dull green fluorescence.

**Effect of pelargonidin on the apoptosis in the A549 cells**

Figure 4 depicts the results of DAPI staining, which was used to examine the nuclear morphology of apoptotic cells in both control and pelargonidin-treated A549 cells. Higher rates of apoptosis, chromatin condensation, nuclear damage, membrane rupture, and the production of apoptotic bodies were seen in A549 cells exposed to the 20 and 40 µM of pelargonidin. Furthermore, the apoptotic incidences and loss in cell density in A549 cells were significantly higher in 40 µM pelargonidin treatment compared to 20 µM pelargonidin treatment.

**Effect of pelargonidin on the levels of oxidative stress markers in the A549 cells**

The results of an analysis of oxidative stress biomarkers such as TBARS, CAT, SOD, and GSH in the control and pelargonidin-treated A549 cells is depicted in Fig. 5. A549 cells treated with 20 and 40 µM pelargonidin showed significant increase in TBARS level and decreases in CAT, SOD, and GSH levels (Fig. 7). As a result, it became evident that pelargonidin might induce apoptosis in A549 cells by disturbing the redox balance thereby increasing oxidative stress.

**Discussion**

Lung cancer has the highest incidence rate of all malignancies and is responsible for the second most cause of cancer-associated fatalities [23]. Additionally, only 15% of patients survive for five years after diagnosis. Most patients with lung cancer are diagnosed at the developed stage due to the lack of apparent specific symptoms. More over half of all patients with lung cancer develop metastases. Metastasis from this cancer has been observed in the brain, bones, and liver. Because of this, it is clear that there is a significant medical need in the area of advanced lung cancer treatment [24]. Despite the fact that diagnostic and prognostic markers have been established and current treatment strategies have evolved, chemoresistance is frequently acquired, which has a negative impact on clinical outcomes [25]. Even though immense advancements in the therapies of lung cancer, the prognosis still remain unsatisfactory, with only 20% of the 5-year survival rate [26]. Hence, the need for the exploration of novel and potential alternative drug candidates is of great interest.
Cancer is a condition marked by the unchecked proliferation of cells referred to as neoplastic cells. These cells have their local origin in a primary tissue, and they have the ability to spread via the blood and lymphatic streams to invade any other kind of tissues. In order to the transition of normal cells into neoplastic cells, they must acquire some specific abilities [27]. Lung cancer cells are well known for their rapid growth and risks of distant metastasis [28]. Therefore, the inhibition of growth of lung cancer cells is a initial step towards treating the lung cancer. In this work, the influence of pelargonidin on the growth of lung cancer A549 cells was assessed by MTT assay, and the findings demonstrated the substantial decrease in the growth of A549 cells. This finding proves that the pelargonidin can inhibit the progression of lung cancer via inhibiting its cell growth.

The role of ROS in tumor cell death after chemotherapy and radiation therapy has been widely established [29]. Increased ROS production is characteristic of cancer cells, where it promotes essential cell survival signaling that promotes rapid growth and proliferation. Nevertheless, high ROS levels can induce cancer cell death via several mechanisms [30]. Mitochondria are both the primary sources of ROS generation and the main targets of ROS. The mitochondria of tumor cells are physically and functionally unique from normal cell mitochondria, and they actively contribute to metabolic recombination, marked by ROS generation, to enhance tumor progression [31]. Moreover, DNA, proteins, mitochondrial lipid membranes, and other subcellular compartments can all be severely damaged by an abundance of ROS [32]. Since ROS production can either directly or indirectly affect the sensitivity to commonly used chemotherapeutic medications like cisplatin and radiation agents [33], controlling endogenous ROS status is of critical importance in tumor treatment. Cancer cells enter senescence when ROS levels rise above a lethal threshold, setting off a chain of reactions that kills the cells. A earlier report by the Almahi et al. [34] has found that the hemin has increased the formation of ROS and apoptosis in lung tumor cells. In this work, the current findings demonstrated an upsurge in the endogenous ROS production in A549 cells after treatment with the pelargonidin. These findings provide an suggestion that the pelargonidin can promote the cell death mechanisms in A549 cells via increasing the endogenous ROS production.

Excessive ROS are mainly targets the mitrochondria, which disturbs the mitochondrial function by causing the failure of $\Delta \psi_m$. The reduction in $\Delta \psi_m$ is thought as the initial sign of the apoptotic mechanisms. The mitochondrial membrane becomes more penetrable during apoptosis, allowing pro-apoptotic substances to be released. Generally the caspase enzymes are activated followed by the substantial reduction in the $\Delta \psi_m$ in order to initiate the apoptotic signaling pathway and irretrievable entry into the apoptotic mechanism [35]. The assay of $\Delta \psi_m$ levels in the A549 cells using the Rh-123 staining technique exhibited that pelargonidin disturb and decreases the $\Delta \psi_m$ level. This findings reveal that pelargonidin initiates apoptotic cascade in A549 cells via decreasing the $\Delta \psi_m$ level.

The cell death process known as apoptosis is crucial for normal tissue function and development. The pathogenesis of cancer and therapy resistance are often associated with deregulation of apoptosis [36]. Lung cancer develops when apoptotic signaling is deregulated, which happens when there are abnormalities in cancer suppressor genes [37]. Apoptosis can be triggered by alterations in membrane
integrity, inhibition of cell growth, cytoplasmic shrinkage, and cell agglomeration [38]. It has been widely believed that apoptosis is the fundamental mechanism, which driving the antitumor efficacy of the drugs [39]. Apoptosis is characterized by the production of apoptotic bodies and when the cancer cells undergo apoptosis, they possess array of apoptotic morphological changes including shrinkage, membrane blebbing, rounding, cell damage, nuclear fragmentation, and condensation [40]. In line with this, the present findings of both DAPI and dual staining assays revealed an aberrant morphological alterations with the presence of apoptosis such as cell damage, shrinkage, nuclear damage, and formation of apoptotic bodies in the pelargonidin-treated A549 cells. Hence, it was clear that the pelargonidin effectively promoted apoptosis in the A549 cells, which can be an effective anticancer agent to treat the lung cancer.

Tumor cells fine-tune ROS level and its removal in order to make themselves at comfort. Increased ROS generation in a cell would normally lead to oxidative stress thereby promotes apoptosis, but in the tumor niche, ROS are kept at steady levels by being detoxified by scavengers such as SOD, CAT, and GSH. Inhibiting ROS-induced cell damage may require the removal of substantial levels of ROS by antioxidants. Cellular antioxidants neutralize the vast majority of free radicals and mitigate the severe damage caused by oxidative stress [41]. Due to their high reactivity, ROS play a crucial function in regulating the redox balance inside a cell. In a while, an excess of ROS can lead to oxidative stress, which in turn can degrade lipids, nucleic acids, and proteins, and ultimately lead to cell death [42]. Chemotherapy is extensively employed in clinical settings to treat the cancer patients. Most chemotherapy drugs cause increased ROS production, and several drugs can alter the redox balance in tumor cells [43]. An upsurge in mitochondrial synthesis of ROS and the decrease of the cellular antioxidants are the two major reasons of the cell death by chemotherapeutic medicines. Since cancer cells can be efficiently eradicated by triggering an upsurge in oxidative stress, there has been substantial research into alterations of redox homeostasis in the area of chemotherapy [44]. The outcomes of the present work was demonstrated that the pelargonidin effectively increased the TBARS and reduced the CAT, SOD, and GSH in the A549 cells. Hence, it was clear that the pelargonidin can alter the redox balance and promote oxidative stress-regulated apoptosis in the lung cancer A549 cells.

**Conclusion**

In summary, the results of the current study demonstrated an anticancer properties of pelargonidin on A549 cells. The pelargonidin treatment substantially decreased the growth and encouraged the oxidative stress-regulated apoptosis in A549 cells. Pelargonidin promoted the endogenous ROS generation, reduced the Δψm, and promoted apoptosis via altering the redox homeostasis (increase in TBARS and decrease in CAT, SOD, and GSH levels) in A549 cells. Hence, it was evident that the pelargonidin could be employed as an effective anticancer candidate to treat the lung cancer. In addition, the more studies are recommended in the future to explore the underlying molecular pathways attributed to the anticancer effects of pelargonidin in lung cancer.
Declarations

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during this work are available from the corresponding author on request.

Authors’ contribution

All authors listed have significantly contributed to this work. All authors have read and approved the manuscript.

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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References


**Figures**

![Figure 1](image)

**Figure 1**

**Effect of pelargonidin on the viability of lung cancer A549 cells**
The treatment with the diverse concentrations (1-120 μM) of the pelargonidin considerably reduced the cell growth of lung cancer A549 cells for 24, 48, and 72 hr. The IC50 range of pelargonidin against A549 cells was observed at 20 μM for 48 hr, which inhibited the 50% of the cell viability. The data are exhibited as a mean±SD of triplicate assays and each value was evaluated by one-way ANOVA and Tukey's post hoc tests. The data in each bar not sharing a common superscript and differ significantly from control group at p<0.05.

![Effect of pelargonidin on the apoptotic cell death in the A549 cells](image)

**Figure 2**

**Effect of pelargonidin on the apoptotic cell death in the A549 cells**

After an 24h of treatment with 20 and 40 μM of pelargonidin resulted in the increased yellow and orange fluorescence, which evidences the higher occurrences of both early and late apoptotic incidences, respectively in the lung cancer A549 cells. The control cells exhibited bright green fluorescence, which evidences the live cells without apoptosis. After treatment, the developed fluorescent intensity was measured using fluorescent microscope.

![Effect of pelargonidin on the ROS generation in the A549 cells](image)

**Figure 3**

**Effect of pelargonidin on the ROS generation in the A549 cells**

A dull green fluorescence was observed in the control cells, which proves the less ROS production. Whereas, the A549 cells exhibited the bright green fluorescence after treatment with the 20 and 40 μM of
pelargonidin, which witnessing an upsurge in the endogenous ROS production. After treatment, the developed fluorescent intensity was measured using fluorescent microscope.

**Figure 4**

**Effect of pelargonidin on the \( \Delta \psi_m \) level of lung cancer A549 cells**

The control cells demonstrated the bright green fluorescence, which proves the presence of normal and intact \( \Delta \psi_m \) level. However, the A549 cells demonstrated a dull green fluorescence followed by the exposure to 20 and 40 \( \mu \)M of pelargonidin, which proves the substantial reduction in the \( \Delta \psi_m \) level. The control cells revealed higher green fluorescence (normal and intact \( \Delta \psi_m \) level). After treatment, the developed fluorescent intensity was measured using fluorescent microscope.

**Figure 5**

**Effect of pelargonidin on the apoptotic cell nuclear morphology of A549 cells**

After the exposure to the 20 and 40 \( \mu \)M of the pelargonidin, the A549 cells revealed a higher cell damage, nuclear cleavage, decreased cell number, and the development of the apoptotic bodies when compared with control, which evidences the occurrence of increased apoptotic incidences. After treatment, the developed fluorescent intensity was measured using fluorescent microscope.
Figure 6

Effect of pelargonidin on the levels of oxidative stress markers in the A549 cells

The levels of oxidative stress biomarkers was assessed in the cell lysates of control and pelargonidin-treated A549 cells using assay kits. The 20 and 40 µM of pelargonidin treatment caused the considerable increase in the TBARS and decrease in the CAT, SOD, and GSH levels in the A549 cells when compared to the control. The data are exhibited as a mean±SD of triplicate assays and each value was evaluated by one-way ANOVA and Tukey's post hoc tests. The data in each bar not sharing a common superscript and differ significantly from control group at p<0.05.