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Original Research Article

Pseuderanthemum palatiferum (Nees) Radlk extract induces apoptosis via reactive oxygen species-mediated mitochondria-dependent pathway in A549 human lung cancer cells

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Abstract

Purpose: *To investigate the capacity of aqueous Pseuderanthemum paltiferum leaf extracts (PPA) to induce apoptosis in A549 human lung cancer cells and the possible mechanisms of action.*
Methods: *Human lung cancer A549 cells were cultured in the presence of PPA (0 - 1000 µg/mL). Cell viability was assessed by MTT assay while morphological alterations in the cells were observed by Hoechst 33342/PI double staining. Intracellular reactive oxygen species (ROS) levels and subsequent changes of mitochondrial membrane potential were also investigated. Involvement of caspase-3 activation in the apoptotic pathway was determined.*
Results: *PPA inhibited the growth of A549 cells in a concentration- and time-dependent manner. Major phenotypic apoptotic cell death was evidenced in microscopic images. Furthermore, treatment of A549 cells with PPA resulted in a significant increase in the production of ROS accompanied by attenuation of mitochondrial membrane potential, thus inducing the activation of caspase-3 activity ($p < 0.05$).*
Conclusion: *PPA exerts anti-cancer activity by suppression of cell viability and induction of ROS-mediated mitochondrial dependent apoptosis in A549 cells, and may be a potential candidate for the development of a therapeutic agent for lung cancer.*

Keywords: *Pseuderanthemum palatiferum, Apoptosis, Reactive oxygen species, Mitochondria, Lung cancer*

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INTRODUCTION

Lung cancer causes significant devastating morbidity and mortality among men and women worldwide [1]. It is clinically categorized into small cell and non-small cell lung cancer (NSCLC) subtypes. Incidence of non-small cell lung cancer (NSCLC) revealed nearly 80 % among other subtypes. Surgical resection, radiation and chemotherapy are the targeted

therapies for NSCLC, however the survival rate of those patients remains low [2]. To date, cisplatin (cis-diamminedichloroplatinum (II); CDDP) and CDDP-based combinations have been the first line chemotherapeutic agents targeting NSCLC. However, its efficiency is often accompanied by a number of side effects including nausea and vomiting, neurotoxicity, renal toxicity, tumor resistance and secondary malignancies [3,4]. Therefore, new approaches are needed for the development of therapeutic drugs with low toxicities and fewer side effects to overcome cancer more effectively. A large number of anti-cancer therapeutic agents derived from plant sources have been found to be potent in the treatment and prevention of cancers [5,6].

Pseuderanthemum palatiferum (Nees) Radlk. (*P. palatiferum*), a member of the Acanthaceae family, has been traditionally used as a medicinal plant to treat several diseases. The phytochemical investigation of its leaves has revealed the presence of n-pentacosan-1-ol, β-sitosterol, stigmasterol, β-sitosterol 3-O-β-glucoside, stigmasterol 3-O-β-glucoside, kaempferol 3-methyl ether 7-O-β-glucoside, apigenin 7-O-β-glucoside [7,8]. Studies of its leaf extracts have reported their pharmacological effects to be anti-diabetic [9, 10], anti-hypertensive [11], anti-lypolytic and α-amylase inhibitory [12], anti-microbial [13], anti-inflammatory and antioxidant [14,15]. A recent study revealed that *P. paltiferum* exerted an anti-cancer effect on colon cancer [16]. However, there has been no report addressing the effect of *P. palatiferum* on lung cancer. Therefore, this study was undertaken to investigate the anti-cancer effect of *P. palatiferum* leaf extract on the induction of apoptosis in human NSCLC cells (A549) and its possible mechanisms of action.

EXPERIMENTAL

Chemicals and Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichloro-fluorescein diacetate (DCFH-DA), Hoechst 33342 and N-acetylcysteine (NAC) were purchased from Sigma Chemical (MO, USA). Propidium iodide (PI) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were obtained from Cell signaling (MA, USA). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12K medium, L-glutamine, Fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco-BRL (NY, USA). Tetramethyl rhodamine ethyl ester (TMRE) and polyclonal rabbit anti-active caspase-3 antibody (1:200; ab2302) were purchased from Abcam (MA, USA). Caspase-Glo 3/7 was purchased from Promega (WI, USA). Cisplatin was purchased from Calbiochem (CA, USA).

Preparation of PPA extract

P. palatiferum leaves were purchased from Hoan-Ngoc Thailand Herb Garden, Bangkok, Thailand. The plant was authenticated by Ms Thepwalee Khananthong a taxonomist, and a voucher specimen (no. 187517) deposited at Forest Herbarium, Bangkok, Thailand. The dried powder of *P. palatiferum* leaves was subjected to aqueous extraction in ultrapure water at 1:10 ratio and the extractions were carried out in triplicate. Thereafter, the solution was filtered through Whatman filter paper No. 1 and the filtrate was then lyophilized to achieve a powder of *P. palatiferum* aqueous extract (PPA). The percent yield of PPA was 17.37. The extract was then kept at -20 ºC until use. For all experiments, PPA was diluted with culture medium to the final concentrations of 100 to 1000 µg/mL.

Phytochemical evaluation

The PPA extract was subjected to quantitative tests for the detection of phytochemical constituents such as phenolic compounds, flavonoids, proanthocyanidins and tannins. These tests were carried out according to standard procedures [17].

Cell culture

Human non-small cell lung cancer A549 cells were purchased from Cell Lines Service (CLS, Eppelheim, Germany) and cultured in Ham's F-12K medium. Normal human lung fibroblast MRC-5 cells were obtained from American Type Culture Collection (ATCC, Virginia, USA) and cultured in DMEM medium. All media were supplemented with 2 mM L-glutamine, 10 % FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 ºC with a 5 % CO_2 incubator atmosphere. The culture medium was renewed every 2 days and subcultured when 80 % confluent.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine the cytotoxic activity of PPA extracts on A549 cells. Cells were plated in 96-well plates at a density of 1 x 10^4 cells/well and incubated for 24 h. The cells were treated with various concentrations of PPA ranging from 0 to 1000 µg/mL for 24, 48, 72 h, respectively. After

treatment, the medium was removed and the cells were then incubated with 5 mg/mL MTT reagent for 1 h at 37ºC. The media was then aspirated and dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The absorbance was measured at 570 nm using the ELISA plate reader (BioTek, Highland Park, USA). The IC_{50} values were calculated by using CompuSyn software (ComboSyn Inc, Paramus, USA). The data were presented as percentage of cell viability and calculated based on the following formula: % Cell viability = 100 – [(O.D. $_{control}$) – (O.D. $_{sample}$) / (O.D. $_{control}$)] x 100

Morphological studies of cell lines

The alteration of cell morphology was examined with an inverted microscope (Nikon ECLIPSE Ti, Kanagawa, Japan) after A549 cells were exposed for 72h to PPA and cisplatin at IC_{50} dose and compared to the control.

Hoechst 33342/PI staining assay

Briefly, 1 x 10^4 cells were grown on 6-well culture plates and incubated with PPA for 72 h**.** The cells were subsequently washed with PBS and then re-suspended in 10 µg/mL Hoechst 33342. Propidium iodide was counter stained to the cells to final concentrations of 2 µg/mL for 30 min in darkness. The cells were then examined under the inverted fluorescence microscope (Nikon ECLIPSE Ti, Kanagawa, Japan) using excitation 360 nm and emission 465 nm.

Measurement of intracellular ROS production

The intracellular ROS level was determined using a spectrophotometric method. We plated A549 cells and they were allowed to attach for 24 h and then treated with PPA for 2 and 4 h in the presence or absence of 5 mM NAC prior to incubation with 20 µM DCFH-DA for 1 h in darkness. The fluorescent 2', 7'-dichloro-fluorecein (DCF), which was oxidized from DCFH to DCF by the ROS within the cells, was measured by a fluorescence microplate reader (BioTek, Highland Park, USA) at excitation 485 nm and emission 530 nm.

Measurement of change in mitochondrial membrane potential (MMP)

The alterations of MMP were quantified by Abcam's TMRE mitochondrial membrane potential assay kit. Cells were treated with or without PPA and then incubated with TMRE for 20 min at 37º C in the dark. Mitochondrial membrane potential was evaluated under fluorescence microplate reader using 549/575 nm wavelength (BioTek, Highland Park, USA).

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase-Glo 3/7 luminescence assay according to the manufacturer's guidelines. A549 cells were cultured into 96-well plates at a density of 1 x 10^4 cells/well and treated with PPA and cisplatin at their respective IC_{50} values. At the end of the period of treatment, the cells were incubated with 75 µL of Caspase-Glo reagent for 1 h in darkness. The luminogenic substrate containing the DEVD sequence cleaved by caspase-3 was then measured with the luminometer (BioTek, Highland Park, USA).

Western blot analysis

Following treatment with PPA (100 and 250 µg/mL) and cisplatin (1.95 µg/mL), the total proteins of A549 cells were extracted with RIPA buffer and centrifuged at 14,000 rpm for 20 min at 4 ºC. Then, the supernatants containing proteins were collected and protein concentration was determined using Bradford protein assay. Total proteins were loaded onto 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separated and transferred onto a nitrocellulose membrane. The membranes were blocked with 5 % bovine serum albumin (BSA) and were probed with anti-active caspase-3 and anti-β-actin primary antibodies with gentle agitation overnight at 4 ºC. After washing three times with TBST buffer, the corresponding HRP-conjugated secondary antibody was added and incubated for 1 h at room temperature. The blots were visualized with enhanced chemiluminesence detection and quantified by densitometry using Image J software.

Statistical analysis

The results are expressed as mean ± standard error of mean (SEM, n = 3). The data were analyzed by one-way ANOVA using SPSS 13 software. Statistically significant differences from the control was established when $p < 0.05$.

RESULTS

Phytochemical profile of PPA

The preliminary phytochemical screening of PPA extracts was evaluated and revealed the presence of phenolic compound, flavonoid, proanthocyanidin and tannin contents. The concentration of the total phenolic compounds was 33.46 ± 0.62 milligrams of gallic acid

Table 1: Contents of phenolic compounds, flavonoid, proanthocyanidin and tannin in PPA

Sample	Phenolic compounds (mg GAE/100 g dry extract)	Flavonoid content (mg quercetin/100 g dry extract)	Proanthocyanidin (A500) in 100 µg/mL	Tannin (% pyrogallol equivalent)
PPA	33.46±0.62	17.66±0.46	0.12±0.01	4.10±0.13

Data are shown as mean ± SEM (n = 3)

equivalent per 100 g of dry extract. The total flavonoids were 17.66 ± 0.46 milligrams of quercetin equivalent per 100 g. The proanthocyanidin content was determined to be 0.12 ± 0.01 whereas tannin was present at 4.10 ± 0.13 % pyrogallol equivalent, respectively (Table 1).

Effect of PPA on cell viability

For the determination of cytotoxicity of PPA extract on human non-small cell lung cancer, A549 cells were treated with different concentrations of PPA (0-1,000 µg/mL) for 24, 48, 72 h and cytotoxicity analyzed using MTT assay. The percentage of cell viability was significantly reduced ($p < 0.05$) after treatment with PPA in both a concentration- and a time-dependent manner, and IC_{50} value of 24, 48 and 72 h time course were 727.50 ± 0.09, 391.25 ± 0.05 and 247.50 ± 0.02 µg/mL, respectively, when compared to the control (Figure 1a). Cisplatin, a chemotherapeutic drug targeting lung cancer, was also evaluated in A549 cells. Figure 1 b shows that treatment with cisplatin caused a concentration- and time-dependent reduction of the percentage of cell viability with an IC_{50} at 72 h of 1.95 ± 0.09 µg/mL. Meanwhile, the viability of human normal lung fibroblast MRC-5 was examined to determine whether the cytotoxic effect of PPA was selective for lung cancer cells or not. The result showed that PPA had minimal effect on MRC-5 with an IC_{50} value at 72 h of 923.33 ± 0.24 µg/mL (Figure 1 c). In addition, the percentage of cell viability for MRC-5 was more than 80 % at the IC_{50} dose of A549 cells suggesting its selectivity between normal MRC-5 and cancer A549 cells.

Effect of PPA on the morphology and apoptosis of A549 cells

We further examined the cellular morphological changes of A549 cells after 250 µg/mL PPA treatment. When compared to the control, we observed, under the phase-contrast inverted microscope, that PPA and cisplatin treatment reduced the number of viable cells, which became rounded in shape followed by their detachment from the tissue culture plate (Figure 2a).

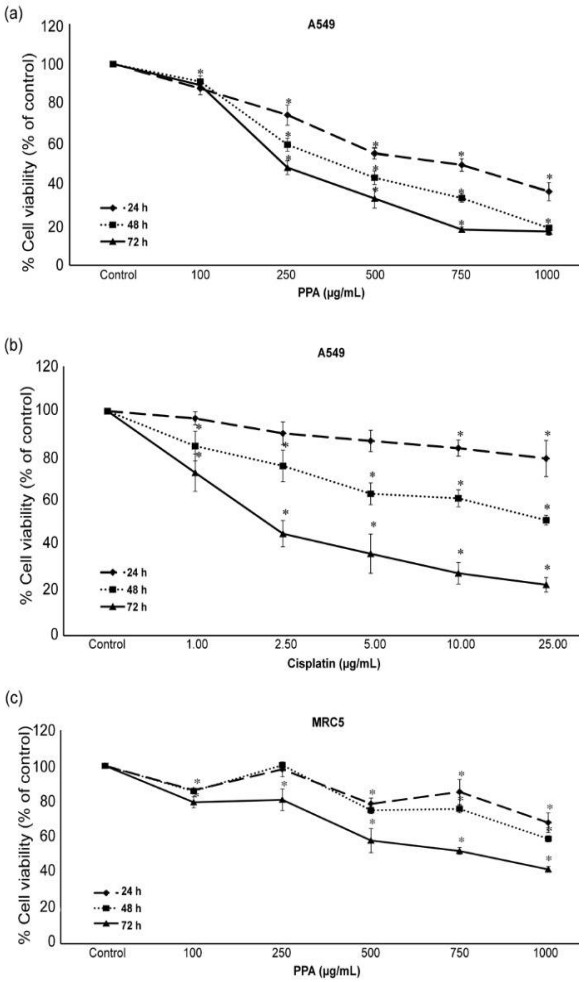

Figure 1: Effect of PPA on A549 and MRC-5 cell viability assessed by MTT assay after 24, 48 and 72 h treatment. (a) Percentage of cell viability after treatment with various concentrations and times of PPA on A549 cells; (b) Percentage of cell viability after treatment with various concentrations and times of cisplatin on A549 cells; (c) Percentage of cell viability after treatment with various concentrations and times of PPA on MRC-5 cells. Data are shown as mean ± SEM of three independent experiments; * $p < 0.05$ versus the control

The mode of apoptotic cell death was examined by Hoechst 33342/PI double staining assay. Hoechst 33342 fluorescent dye was used in order to observe the DNA condensation whereas PI which only enters the impaired cytoplasmic membrane was used to counterstain for dead cells. Figure 2b shows bright nuclear condensation, markedly disrupted membrane integrity with morphological changes such as cell

shrinkage and rounding accompanied by irregularities in cell contour and reduced cell density after treatment with PPA or cisplatin compared to the untreated control.

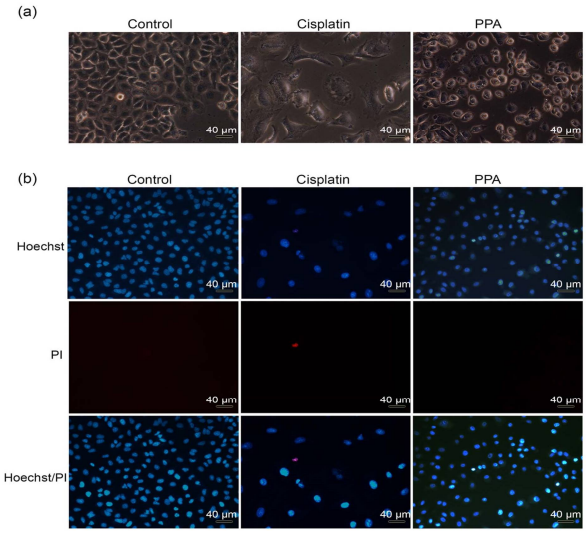

Figure 2: Representative photomicrograph showed the changes of cell morphology and apoptosis of A549 cells after exposure to PPA for 72 h. (a) Inverted microscope examined cell morphology on A549 after treatment with control, 1.95 µg/mL cisplatin, 250 µg/mL PPA; (b) Fluorescence dual staining of Hoechst 33342/PI examined apoptotic nuclei on A549 after treatment with control, 1.95 µg/mL cisplatin, 250 µg/mL PPA. Scale bar: 40 µm

Effect of PPA on intracellular ROS level

We investigated whether PPA could generate intracellular ROS which are known to induce biochemical alterations including apoptosis in the A549 cells. Figure 3 demonstrates 100 and 250 µg/mL PPA treatment significantly increased the percentage of ROS production in A549 cells in a concentration- and time- dependent manner compared to the control group ($p < 0.05$). Additionally, A549 cells were found to have a significantly increased percentage of ROS generation after exposure to cisplatin ($p < 0.05$). To confirm the role of ROS in PPA-induced apoptosis, we used NAC which is the ROS scavenger. Pre-treatment of cells with 5 mM NAC for 2 h prior to treatment with 100 and 250 µg/mL PPA for 2 and 4 h significantly attenuated ROS accumulation as shown in Figure 3 ($p < 0.05$). These results provide the evidence that increased intracellular ROS play a crucial role in PPA-induced apoptosis in A549 cells.

Effect of PPA on MMP

The disruption of MMP plays a major role in the apoptosis mechanism thus we examined, by TMRE assay, whether PPA mediated A549 cell death through this pathway. In the positive control group, cisplatin treatment showed a significant loss of mitochondrial membrane potentials in comparison to the control group ($p < 0.05$). Of note, the percentage of TMRE fluorescence intensity significantly diminished following the treatment with PPA ($p < 0.05$), suggesting the presence of PPA mediated the perturbation of mitochondrial metabolic activity (Figure 4).

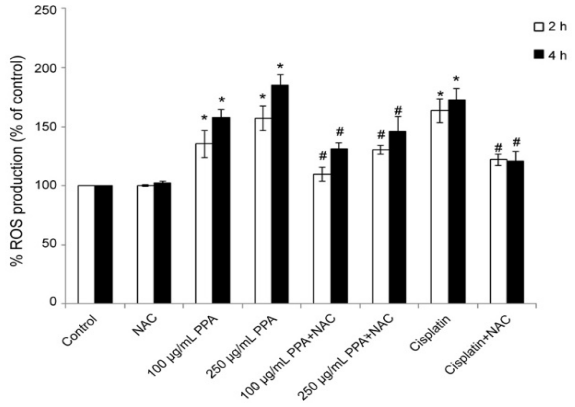

Figure 3: Effect of PPA on ROS production in A549 cells by DCFH-DA assay. Cells were pre-treated for 2 h with or without 5 mM NAC followed by incubation with PPA or cisplatin for 2 and 4 h. Data are shown as mean ± SEM of three independent experiments. *$p < 0.05$ versus the control, # $p < 0.05$ versus the corresponding dose between cells without and with the treatment of NAC

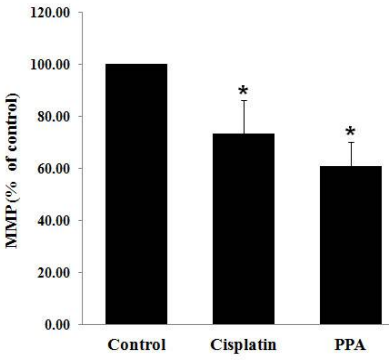

Figure 4: Mitochondrial membrane potential upon exposure to PPA. A549 cells were exposed to the reference drug cisplatin or 250 µg/mL PPA. Data are shown as mean ± SEM (n = 3); * $p < 0.05$ versus the control

Effect of PPA on caspase-3 activity

Caspase-3 plays a critical role in the molecular effector pathway of apoptosis. We thus sought to ascertain whether or not PPA effects the induction of apoptosis via the caspase-3 pathway. As shown in Figure 5a, PPA treatment significantly increased the percentage of caspase-3 activity compared to the untreated

control ($p < 0.05$). Moreover, A549 cells treated with cisplatin at its respective IC_{50} showed a marked increase in caspase-3 activity.

We further confirmed whether the increased percentage of caspase-3 activity is due to the cleavage of procaspase-3 (inactive form) into the corresponding active form. Therefore, the expression level of apoptosis-related active protein (cleaved) caspase-3 was determined by western blot assay. The level of active caspase-3 (17 kDa) was significantly upregulated by 100 and 250 µg/mL PPA treatment compared to the control group (Figure 5b and c). Since the increased levels of active caspase-3 are consistent with luminescence assay, our results suggested the role of PPA induced apoptosis in human lung cancer A549 cells was dependent on the activation caspase-3.

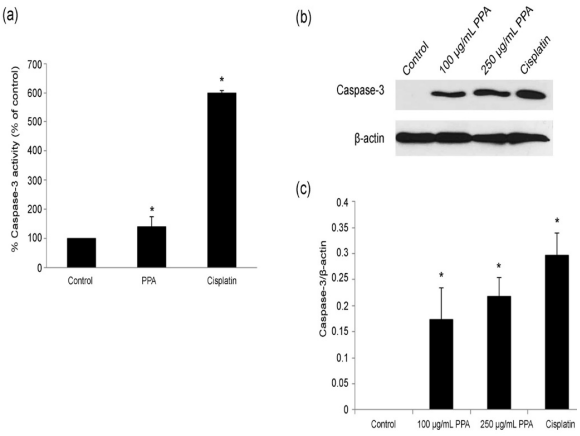

Figure 5: Effect of PPA on caspase-3 activity. (a) The luminescence assay demonstrated the percentage of caspase-3 activity after A549 cells were exposed to 250 µg/mL PPA or the reference drug cisplatin; (b) Expression and (c) Quantification of active caspase-3 protein by western blot analysis. Data are shown as mean ± SEM of three independent experiments. * $p < 0.05$ versus the control

DISCUSSION

The consumption of plant extracts with high contents of antioxidant phytochemical constituents has shown beneficial results and few side effects in several chronic diseases including cancers [5] and in recent years, numerous researches have focused on cancer therapy using plant-based products as a source of chemotherapeutic agents. In the case of cancer therapy, strategies involving the induction of apoptosis have gained great importance. In this study, we therefore aimed to investigate whether *P. palatiferum* leaf extract has an anti-cancer effect through the induction of apoptosis in human NSCLC A549 cells and its possible mechanisms of action.

Prior to that, we evaluated the anti-proliferative potential of the PPA extract which was expressed as IC_{50} values on human lung cancer A549 and normal lung fibroblast MRC5 cell lines. The present study demonstrated that PPA exhibited selective cytotoxicity against A549 cells in concentration- and time-dependent manner, with expressed IC_{50} values of 727.50 ± 0.09 at 24 h, 391.25 ± 0.05 at 48 h and 247.50 ± 0.02 µg/mL at 72 h. At the same time, PPA showed limited toxicity to normal lung fibroblast MRC5 cells at the same concentrations. In addition to lung cancer, colon cancer cell proliferations have been inhibited by aqueous and ethanol extracts of *P. palatiferum* [16].

Interestingly, certain properties of the cytotoxic effect observed in this study might be attributable to the presence of high phenolic compounds within the PPA extract as indicated in Table 1 and consistent with the results of previous studies [12,15]. Various bioactivities of phenolic compounds play an important role in anti-carcinogenic properties and also contribute to the induction of apoptosis by inhibiting cancer cell growth, producing reactive oxygen and DNA damage in diverse cancer cells [18,19].

Further investigation elucidated the influence of PPA extract on the induction of apoptosis cell death in A549 cells by both morphological and biochemical assays *in vitro*. During the apoptotic process, the distinct morphological features of apoptotic cells exhibit nuclear fragmentation and chromatin condensation, which originate along the periphery of the nuclear membrane. Thereafter the apoptotic nucleus further condenses in the late stage and eventually breaks up [20]. The microscopic examination of the staining assay revealed changes in the morphological structures of PPA treated cells, when compared to the untreated control cells, correlated to cellular apoptosis.

A number of biochemical characteristics associated with apoptosis were also implicated in determining the possible mechanism of action of PPA triggered lung cancer cell death. It is well established that several anticancer drugs mediate apoptotic properties by activating ROS generation in order to target cancer cells [21]. The ROS take part in the cellular signaling pathway and act as subcellular messengers which trigger intracellular redox status and apoptosis cell death [22]. Findings obtained in this study showed that treatment of A549 cells with PPA extract increased the generation of intracellular ROS similar to the treatment with

cisplatin. These results of ROS burst supported the observations from the cell viability study.

Mitochondria, a major site of ROS production, are often considered as central coordinators of cell death. A variety of apoptotic stimuli including free radicals, radiation and chemotherapeutic agents cause the opening of the mitochondrial permeability transition (MPT) pore and a corresponding loss of MMP [6,22,23]. These changes lead to enhanced ROS generation, and the release of cytochrome C and other apoptogenic proteins into the cytosol. Ultimately, it activates the downstream effector caspase-3 culminating in apoptotic cell death [22]. In order to evaluate the function of mitochondria, we used the positively charged tetramethylrhodamine ethyl ester (TMRE) fluorescence probe to label the active mitochondria.

The MMP of A549 cells treated with PPA was markedly lower compared to the control. It has been reported that principal chemotherapeutic agents, for example cisplatin, influence the disruption of MMP which is in accordance with our present finding [23,24]. This might be associated with the increase in the ROS production in the cells. Consistent with this finding, the exposure of A549 cells to PPA extracts induced an increased activity of central executioner caspase-3 which is involved in the intracellular cleavage of key structural proteins that results in the morphological alterations whose common features include nuclear condensation, cell shrinkage and membrane blebbing implicated in apoptosis cell death [25].

CONCLUSION

The findings of the present investigation indicate that PPA extract inhibits the growth of human NSCLC A549 cells. Its mechanism of action is probably by ROS-mediated mitochondrial pathway to the activation of caspase-3 and the subsequent cytomorphological changes associated with apoptosis cell death. In conclusion, aqueous leaf extract of *Pseuderanthemum palatiferum* (Nees) Radlk is a potential candidate for NSCLC treatment. However, the active compounds responsible for its apoptosis activity should be identified in future studies.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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