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Apoptosis-Inducing Effect of Pogostemin A Isolated from the Aerial Parts of *Pogostemon auricularius* Against the Human Lung Cancer Cells

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Abstract: The extracts or compounds isolated from *Pogostemon* spp. (Lamiaceae) showed various potential biological activities such as antimicrobial, analgesic, anti-inflammatory, antioxidant, as well as cytotoxicity. In our previous studies, pogostemin A (new compound), isolated from the aerial of *Pogostemon auricularius*, showed strong cytotoxic activities. Herein, our aim of the study was to investigate apoptosis induction activity of pogostemin A on SK-LU-1 human lung cancer cells. Pogostemin A was isolated from the aerial parts of *P. auricularius* growing in Vietnam. Apoptosis, as the type of cell death, was assessed morphologically by Hoechst staining activity and evaluation of the cell surface phosphatidylserine (PS) expression through annexin V/PI staining. Overall, the obtained data suggests that pogostemin A induced apoptosis in lung cancer cells at a concentration of 20 μg/mL, with the percentages of both early and late apoptotic cells found to be 14.9 % and 12.6 %, respectively, meanwhile, the negative control showed that only 9.8 % early apoptotic cells and 5.5 % late apoptotic cells. Pogostemin A, a new meroterpenoid with pyrone-sesquiterpenoid hybrid skeletons induced apoptotic cell death in lung cancer cells by activation of caspase 3.

Key words: *Pogostemon auricularius*; Lamiaceae; caspase 3; Hoechst staining.

Introduction

Pogostemon Desf., which is a large genus in the Lamiaceae family, includes about 94 species distributed mainly in tropical and subtropical regions all over the world [1,2]. A variety of species of this genus has been used as traditional medicine in many countries, mostly in Asian countries, for the treatment of diarrhea, stomach ache, rheumatism, headache, fever and wounds [3]. Recent findings suggest that some extracts or compounds isolated from *Pogostemon* Desf. have various potential biological activities such as antimicrobial, analgesic, anti-inflammatory, antioxidant, and cytotoxicity [4,5].

P. auricularius is an annual herb which is found in many countries, particularly in tropical and subtropical countries such as India, Sri Lanka, Bangladesh, China and Southeast Asia [6,7]. This herb has a wide variety of uses, but primarily it is used as an antiseptic, analgesic and anti-inflammatory agent. In the Tanchongya-Kongmain tribe of Bangladesh, the leaf juice of *P. auricularius*

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is given orally to treat tetanus [8]. In Indonesia, many people use the leaves of this herb to treat stomach ache and urinary problems in children. In Malaysia, the whole of this plant has been used to cure diarrhea and rheumatism [9,10]. According to traditional Chinese medicine, *P. auricularius* is used to treat high temperature in both children and the elderly [7,9]. In addition, many people in India use this herb to treat snakebites. The leaf juice is, also, used to treat fever [3]. The phytochemical studies of this species have led to the isolation of diterpenes [11,12].

Our previous papers showed that herbs known as natural anti-cancer drugs in ethnomedicine can really have biological activity against various cancer cells [13-17]. For example, the essential oil of *Pinus sylvestris* showed the stronger cytotoxic effect to both negative and positive breast cancer cell lines (both IC_{50} = 29 µg/ml) than the Scots pine extract (IC_{50} = 42 and 80 µg/ml, respectively) [13]. The anticancer activity of pine shoots was known in Estonian ethnomedicine [18].

In the previous work [19], the new triterpene pogestem and the new phloroglucinol derivative pogostemon D were isolated from the aerial parts of *P. auricularius*. Also, we reported the isolation, structural elucidation of three new meroterpenoids, pogostemins A - C [16], and three new phloroglucinols, pogostemonons A - C [20], and evaluated the cytotoxicity of the isolated compounds against five human cancer cell lines, including lung adenocarcinoma (LU-1), oral epidermoid carcinoma (KB), liver hepatocellular carcinoma (Hep-G2), colon adenocarcinoma (SW-480), and gastric adenocarcinoma (AGS). Pogostemin A (Figure 1) showed strong cytotoxic activities against the tested cell lines with IC_{50} values ranging from 7.21 ± 1.09 to 12.76 ± 0.88 µg/mL [16].

In the present study, we report the apoptosis-inducing activity of pogostemin A.

Materials and methods

General experimental procedures

The human lung cancer SK-LU-1 cell lines were purchased from the American Type Culture Collection (ATCC®CCL185™). The cell culture medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Trypsin EDTA were purchased from the Grand Island Biological Company (GIBCO, Invitrogen). Dimethyl sulfoxide (DMSO), hoechst 33342, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The cell culture flasks and 96-well plates were from Corning Inc. (Corning, USA). The ELISA Plate Reader (Bio-Rad, California, USA) was used to measure the absorbance of the color caused by the reagent in cytotoxicity assay. Flow cytometry analysis apoptosis was done with the NovoCyte flow cytometer (Acea Bioscience Inc., San Diego, CA 92121, USA).

Biological material

Pogostemin A was isolated from the aerial parts of *Pogostemon auricularius*. This plant was collected from Quang Tri province, Vietnam (N16°44'38.9" E107°14'51.1") in May 2017 and was identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Vietnam.

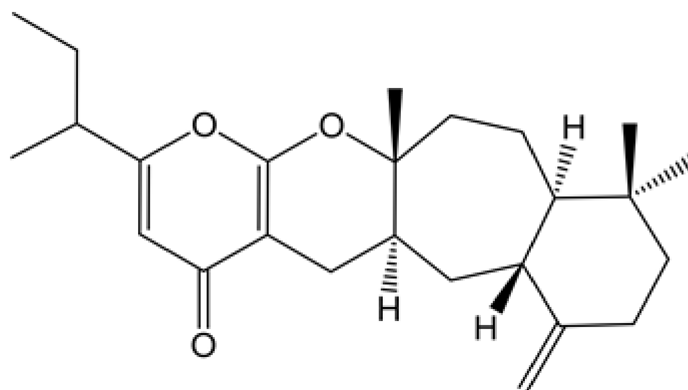

Figure 1. Structure of pogostemin A

Cell culture

The human lung cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-Glutamine, and 10 % fetal bovine serum (FBS). Cells were maintained at 37ºC with 5 % CO_2 in a humidified chamber. These cells were sub-cultured every 3-5 days with the ratio of (1:3) and incubated at 37°C under humidified 5 % carbon dioxide atmosphere [21].

Hoechst staining

SK-LU-1 cells were seeded on the 35-mm plate and incubated overnight before treatment with different concentrations of pogostemin A, including 5 mg/mL, 10 mg/mL, and 20 mg/mL. Camptothecin and DMSO were used as positive and negative controls, respectively. After 24 h of incubation, cells were fixed with 4 % formaldehyde for 30 min, then washed with PBS, and followed by staining with Hoechst 33342 (0.5 mg/mL) for 10 min. The cells were observed under the inverted fluorescence microscope (Zeiss) using the 350-nm excitation wavelength and 461-nm emission wavelength. The cells with fractionated or intensely fluorescent nuclei were determined in at least a total of 200 cells count for each concentration [22,23].

Caspase 3 activity

The caspase 3 activity was determined by Caspase 3 colorimetric kit (Biovision, Chester Springs, PA, USA). Briefly, after treatment with different concentrations of pogostemin A, positive and negative controls for 24 h, cells were harvested and washed twice with PBS. Then, cells were lysed using lysis buffer for 10 min and determined the caspase 3 activity by adding 50 µL of cell lysis which contained 50 µg of cell proteins, 50 µL of 2X Reaction Buffer, and 5 µL of 200 µM DEVD-pNA to each well of 96-well plates and incubated at 37°C for 1-2 h. The absorbance density of samples was read by ELISA reader at 405 nm. Fold-increase of caspase 3 activity of the compound was determined by comparing the data with the level of the untreated control Data is represented as fold-increases of caspase 3 activity of the compound by comparing the data with the level of the untreated control. [24,25].

Flow cytometry analysis apoptosis

The Annexin V/PI Kit (Invitrogen) was used to evaluate the capacity of the samples to induce apoptotic cell death. Subsequent to treatment for 24 h, SK-LU-1 cells were harvested by trypsin-EDTA and washed with PBS. Cells were then suspended in 100 µl binding buffer and stained with 5 µl fluorochrome-conjugated annexin V (Invitrogen) and 1 µl PI (100 µg/ml). After that, Cells were incubated for 15 min at 37°C, and then supplemented 400 µl binding buffer. A minimum of ten thousand cells were analyzed by using the NovoCyte flow cytometry system (ACEC), in which the *x*-axis was annexin-V intensity and *y*-axis was PI intensity [26,27].

Results and discussion

Based on IC_{50} value of pogostemin A, strong cytotoxic activities against the human lung cancer cells LU-1 (IC_{50} = 12.76 ± 0.88 µg/mL) has been found in our earliar paper [16]. The three different concentrations of pogostemin A (5 µg/mL, 10 µg/mL, and 20 µg/mL) were selected for all the experiments in this study.

Among the types of cell death, apoptosis and necrosis is the most common. Necrosis is a degradation process, meanwhile apoptosis is a controlled physiological process [28]. At this time, the apoptosis pathway is the target of many anti-cancer drug therapies [29]. In the Hoechst staining experiment, the number of cells with apoptotic morphological features, such as cell shrinkage or nuclear chromatin condensation and fragmentation, increased when cells were treated with pogostemin A at each concentration for 24 h compared to the negative control at 5.19 % apoptotic cells. At a concentration of 20 µg/mL, pogostemin A induced apoptotic cell death in 34.3 % of the cells, while only 4.5 % of cells were found to be apoptotic at 5 µg/mL (Table 1, Figure 2).

In order to clearly understand the mechanism by which pogostemin A induced apoptosis, the effects of this compound on caspase 3 activity were examined. Caspase 3 is the one activated by death proteases, catalyzing the specific cleavage of many key cellular proteins [30]. Caspase 3 is also required for some typical hallmarks of apoptosis, such as chromatin condensation and DNA frag-

Table 1. Effect of pogostemin A at different concentrations on nuclear morphology after 24 h of treatment using Hoechst 33342 staining

Apoptotic cells (%)				
Pogostemin A			Camptothecin (5 μM)	Negative control
(5 μg/ml)	(10 μg/ml)	(20 μg/ml)		
4.50	6.54	34.30	28.57	5.19

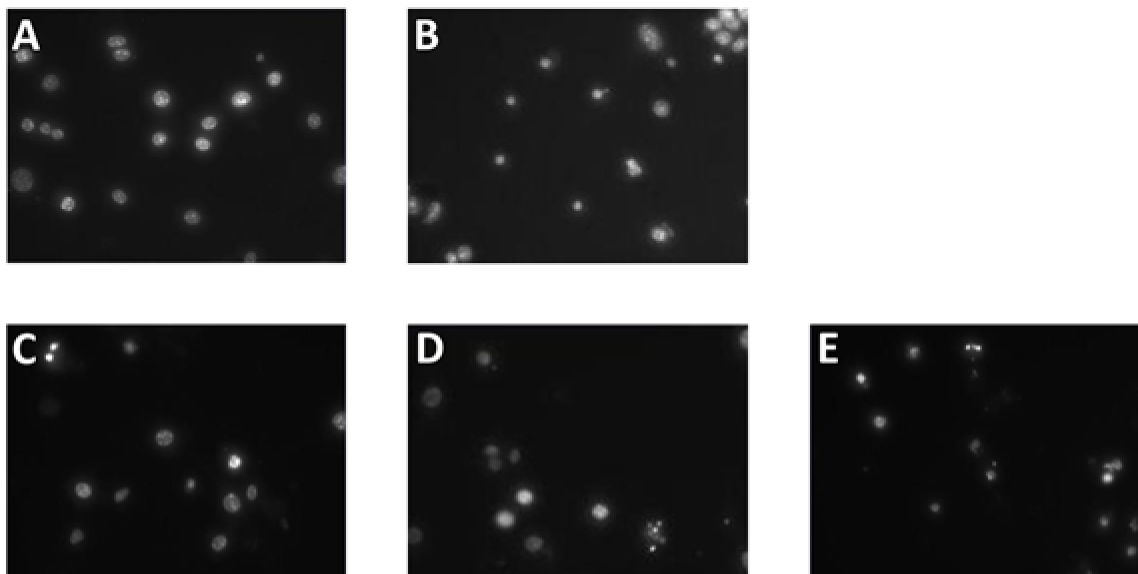

Figure 2. Effect of pogostemin A at different concentrations on nuclear morphology after 24 h of treatment using Hoechst 33342 staining. The SK-LU-1 cells that were treated with 0.5 % DMSO (**A**) showed normal oval shape of nucleases. The reference compound (camptothecin 5μM) also induced apoptosis significantly (**B**). Some cells treated with different concentrations of pogostemin A (5, 10, and 20 μg/mL (**C-E**, respectively), showed condensed or fragmented chromatin

mentation in many cell types [31,32]. Therefore, the caspase 3 activity is one of the indicators of apoptosis induction. As the results shown in Figure 3, the caspase 3 activities increased significantly after pogostemin A treatment at 10 and 20 μg/mL ($p < 0.05$). Especially, the activity of 10 and 20 μg/mL concentrations were 1.49 and 1.22 times higher than negative control, respectively. It seems to be interesting that the fold-increase for caspase 3 is higher for the 10 μg/mL concentration than the 20 μg/mL concentration of pogostemin A treated cells. The hypothesis could be that this might be to more of the cells dying at 20 μg/mL than 10 μg/mL, therefore there would be less cells to express caspase 3. Besides, the 5 μg/mL concentration of this compound slightly induced caspase 3 activity compared with negative control but there is no statistical significance.

Besides the change of cell morphology, one of the early events of apoptosis is the loss of phospholipid asymmetry that leads to phosphatidylserine (PS) translocation from the internal of the plasma membrane to the external of the membrane [32]. On the other hand, PS has high affinity with annexin V in the presence of calcium [33]. The translocation of phosphatidylserine occurs before the loss of cell membrane integrity that is induced by the cell death in apoptosis or necrosis process. Therefore, by using the fluorescent prober annexin V-FITC and propidium iodide, the early and late apoptosis can be detected. In flow-cytometry analysis of annexin V-FITC and propidium iodide double stain, four cell populations were distinguished: the live cell at the lower-left quadrant

(annexin V⁻/PI⁻); early apoptosis cells at the lower-right quadrant (annexin V⁺/PI⁻); late apoptosis cells at the upper-right quadrant (annexin V⁺/PI⁺); and necrotic cells at the upper-left quadrant (annexin V⁻/PI⁺). As presented in Figure 4, pogostemin A at 20 µg/mL induced apoptosis af-

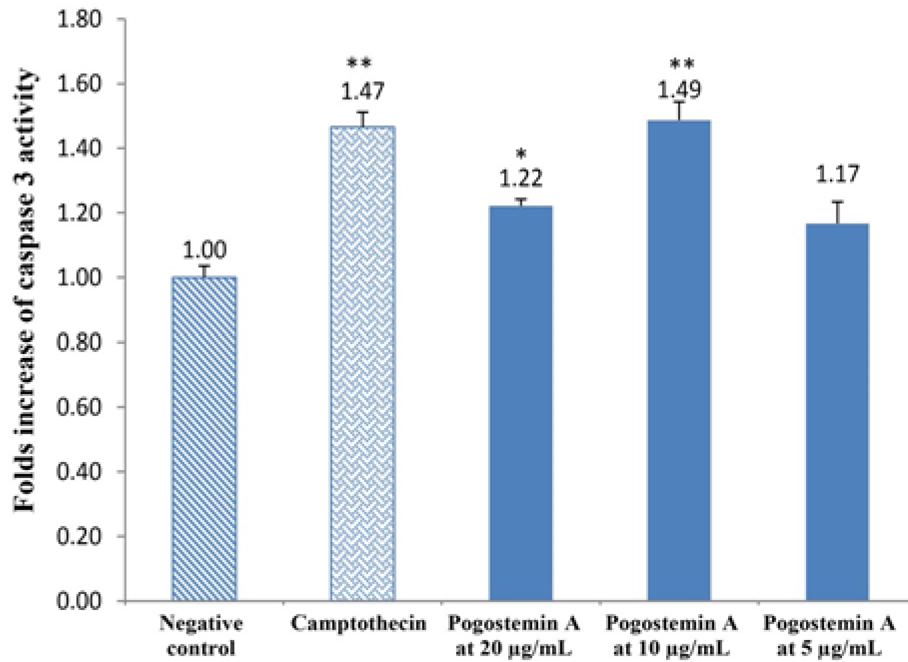

Figure 3. Effect of pogostemin A at different concentrations on the activities of caspase 3 in SK-LU-1 cells. Values represent mean ± SD of three independent experiments (*P<0,05; **P<0,01).

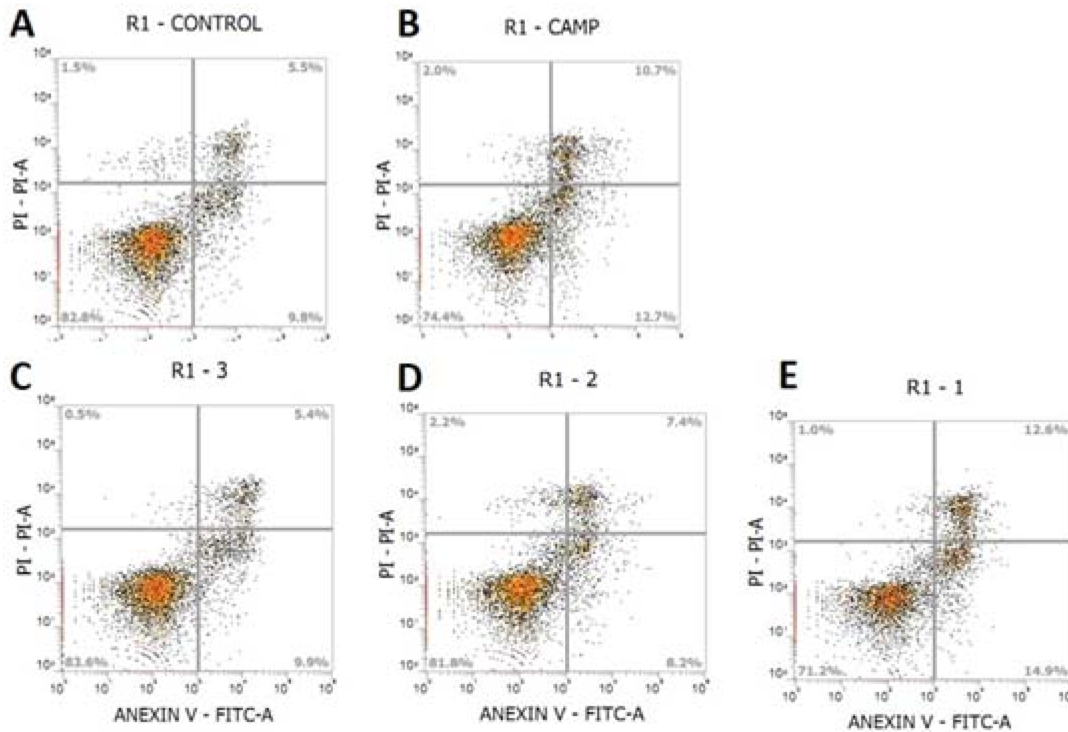

Figure 4. Effect of pogostemin A at different concentrations on induction of apoptosis in SK-LU-1 cells using Annexin V and PI staining. The cells were treated with 0.5 % DMSO (**A**) or camptothecin (**B)** or different concentrations of pogostemin A (5, 10, and 20 µg/mL (**C-E**, respectively).

ter 24 h of treatment; the ratio of both early and late apoptotic cells increased at 14.9 % and 12.6 %, respectively. The negative control showed that only 9.8 % early apoptotic cells and 5.5 % late apoptotic cells. However, at the lower concentration (5 and 10 μg/mL), the difference in both early and late apoptotic cells are not clear when compared with negative controls.

Previously, the content and chemical composition of the essential oil of *P. auricularius* has been studied [34]. As much the essential oil of *Pogostemon cablis* showed cytotoxic and antifungal activities [35], it may be interest to study also anticancer activity of essential oil from *P. auricularius*.

As a result of the current study, pogostemin A, a new meroterpenoid with pyrone-sesquiterpenoid hybrid skeletons induced apoptotic cell death in lung cancer cells by activation of caspase 3.

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