ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 1, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

PAEDERUS ALFIERI EXTRACT INDUCES APOPTOSIS IN HUMAN MYELOID LEUKEMIA K562 CELLS

AMER ALI ABD EL-HAFEEZ[1]*, OSAMA MOHAMED RAKHA[2]

[1]Department of Cancer Biology, Pharmacology and Experimental Oncology Unit, National Cancer Institute, Cairo University, Cairo, Egypt.
[2]Department of Economic Entomology, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt.
Email: amer.ali@nci.cu.edu.eg

Received: 16 June 2016, Revised and Accepted: 08 July 2016

ABSTRACT

Objective: The rove beetle *Paederus alfieri* Koch. (Coleoptera: Staphylinidae) is well-known among natural enemies in Egypt as an important predator of agricultural insect pests, it used as an essential agent in the integrated pest management programs. Recent studies have revealed that *Paederus* may have anti-proliferative effect; however, its mechanisms remain unclear. The aim of the present study is to investigate the anticancer effect of *P. alfieri* extract (PAE) on K562 human myeloid leukemia cancer cells and elucidation of its mechanism.

Methods: Human myeloid leukemia K562 cells were treated with PAE at different concentrations. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was evaluated using flow cytometry analysis. The expressions of Bcl-2, Bax, active caspase-3, t-Akt, and p-Akt were evaluated by western blotting.

Results: PAE has a dose-dependent antiproliferative effect against K562 cells. The half maximal inhibitory concentration was estimated as 212±2.3 ng/ml. Flow cytometric analysis showed that PAE induces apoptosis in a dose-dependent manner in K562 cells. We also investigated the molecular mechanism of PAE-induced apoptosis. PAE downregulated Bcl-2 and upregulated Bax and cleaved caspase-3 proteins. Furthermore, the levels of p-Akt are dose-dependently decreased in response to PAE, whereas the total Akt protein levels remained constant during PAE treatment.

Conclusion: Taken together PAE-induced apoptosis in human myeloid leukemia K562 cells by modulating PI3K/Akt pathway. Our findings suggest that may be PAE is a good extract for developing anticancer drugs for human myeloid leukemia cancer treatment.

Keywords: *Paederus alfieri,* Pederin, K562, Apoptosis, PI3K/Akt pathway.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i1.13513

INTRODUCTION

Cancer is one of the most life-threatening diseases with many different types. Chronic myelogenous leukemia is a lethal malignancy characterized by uncontrolled growth and resistance to apoptosis [1]. To improve the survival rate, intensive efforts have been made to find new anticancer agents, and many attentions have been drawn to insect medicines due to their wide range of biological activities. The genus *Paederus* including about 600 species is found in all tropical and temperate climates. Beetles belonging to the genus *Paederus* causing an acute vesicating dermatitis have been reported from many parts of the world [2]. In Egypt, the rove beetle *Paederus alfieri* Koch. is an efficacious predator of several insect pests attacking a wide variety of cultivated crops moreover, this natural enemy plays a substantial role in the biological control programs. A recent study has revealed that *Paederus* may have an antiproliferative effect [3]. However, the effects of *Paederus* on human myeloid leukemia cancer cells and its mechanism remain unclear. The most important chemical which is found in this insect's hemolymph is pederin. Other chemicals such as cantharidin, pederon, and pseudopederin seem to play negligible roles in *P. alfieri* extract (PAE) cytotoxic effects [4]. Pederin is a symbiotic compound which is produced by a kind of bacteria which has close relationship with *Pseudomonas aeruginosa* [5]. This compound has a non-protein structure [6] and cytotoxic properties which could kill cancerous cells in plants, rats, and mice [4]. According to macromolecular synthesis studies using radioactive precursors, pederin interferes with protein and DNA synthesis and eventually blocks, their production but does not affect RNA synthesis [7]. This compound also induces cell fusion in human skin fibroblasts, *in vitro* [8]. Pederin, as the main cytotoxic component of PAE, has tremendous side effects when used systemically in human and despite the numerous studies indicating its antitumor activity, no investigation has been performed concerning the antitumor mechanism of PAE on cancerous cell lines. The objective of the present study is to investigate the antitumor effects of PAE on K562 human chronic myeloid leukemia and to elucidate its antitumor mechanisms. The PI3K/Akt signaling pathway is a critical transduction pathway which plays an important role in regulating cell proliferation and apoptosis [9]. Various types of cancer, including chronic myeloid leukemia, were reported to aberrantly activate this pathway [10]. Recent studies have shown that some anticancer drugs could induce apoptosis accompanying the down-regulation of Akt [11,12]. In the present study, we observed that PAE induces apoptosis in chronic myeloid leukemia K562 cells. PAE-induced apoptosis was associated with inhibition of Bcl-2 and p-Akt expressions and activation of bax and caspase-3. This is the first report to show that PAE induces apoptosis in K562 cells via modulation of PI3K/Akt and may PAE be a good extract for developing anticancer drugs.

METHODS

PAE preparation

The insect collection was carried out at the experimental farm of the Faculty of Agriculture, Kafrelsheikh University, Egypt from clover fields. Sampling took by the aid of sweeping net (of muslin, 30 cm in diameter and 55 cm depth with wooden hand, 70 cm long) after that transferred to the Economic Entomology Department laboratory in plastic bags to make identification and insect extract. The extract method followed Samani *et al.* (2014). Briefly, about 162 adult insects (*P. alfieri* Koch.)

were collected, grinded, and mixed in ethanol (99%). The mixture was put in a shaking incubator (37°C) for 2 hr. Then, the homogenate was centrifuged and after decanting, the supernatant was transferred into a petri dish and left to dry, overnight. The dried extract was dissolved in DMSO and diluted by IMDM to prepare different concentrations.

Cell culture and reagents

Human chronic myeloid leukemia (K562) cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was cultured in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich), containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life technologies) in a humidified atmosphere with 5% CO_2 at 37°C. PAE was prepared at Entomology Department Laboratory, Faculty of Agriculture, Kafrelsheikh University, Egypt. Cisplatin was taken from (Sigma-Aldrich Company, Egypt). All chemicals used in this study were of the analytical or cell-culture-grade.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was measured using the MTT assay. K562 cells were plated at a density of 1×10^4 cells per well in 96-well plates overnight and then treated with different concentrations of PAE (1, 10, 25, 50, 100, 250, 500, and 1000 ng/ml) or either cisplatin (15 µg/ml) as a positive control or DMSO as a negative control. After 24 hr treatment, 20 µl of MTT solution (2 mg/ml in phosphate-buffered saline [PBS]) were added to each well and the cells were cultured for another 4 hr at 37°C. Then, the medium was totally removed, and 150 µl DMSO was added to solubilize MTT formazan crystals. Finally, the plates were shaken, and the optical density was determined at 570 nm using ELISA plate reader (Model 550, Bio-Rad, USA). At least, three independent experiments were performed. Percentage of cell inhibition was determined as (1−[OD of treated cells/OD of control cells])×100.

Apoptosis analysis

Apoptosis of K562 cells induced by PAE was analyzed by FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the Annexin V/propidium iodide (PI) staining kit (BioLegend Inc., San Diego, CA, USA), according to the manufacturer's instructions. Briefly, K562 cells were treated with 10, 50 or 250 ng/ml PAE or either 15 µg/ml cisplatin or DMSO for 24 hr. After harvesting, the cells were stained with FITC-labeled annexin V antibody and PI for apoptosis assay in annexin V binding buffer (BioLegend). Quantitative analysis of the FACS data was done using FlowJo software (Flowjo, Ashland, OR, USA).

Western blotting analysis

After treatment of K562 cells with PAE (10, 50 and 250 ng/ml) or either cisplatin 15 µg/ml (positive control) or DMSO (negative control) for 24 hr, K562 cells were harvested, washed twice with chilled PBS, and lysed with ice-cold lysis buffer containing 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 µg/ml aprotinin, 5 µg/ml pefabloc SC (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), a protease inhibitor cocktail, 1% phosphatase inhibitor cocktail, and 50 mM Tris–HCl (pH 7.4). The cell lysates were kept on ice for 30 minutes after gently vortex and then centrifuged at 14,000 g for 15 minutes at 4°C. The supernatants were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel immediately after protein extraction, or otherwise, the supernatant was stored at −80°C until use. The protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. For western blotting analysis, 30 µg protein-weight of cell lysate were loaded onto a 15% SDS-PAGE gel. Proteins separated on a SDS-PAGE gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was incubated in blocking buffer containing 3% non-fat milk powder, 1% bovine serum albumin (Sigma-Aldrich), and 0.5% Tween-20 in PBS for 1 hr. Subsequently, the PVDF membrane was incubated with anti-Bax monoclonal antibody (mAb, clone no. D2E11), anti-Bcl-2 polyclonal antibody (pAb), anti-cleaved caspase-3 (mAb, 5A1E), anti-Akt (pAb), antiphospho-Akt (ser 473, mAb, D9E) or anti β Actin mAb (clone no. 8H10D10) (Cell Signaling Technologies, Danvers, MA, USA) for overnight, and followed by HRP-conjugated anti-rabbit IgG (Cell Signaling Technologies) or HRP-conjugated anti-mouse IgG (Becton Dickinson Co, Durham, NC, USA) for 1 hr with agitation at room temperature. Finally, the specific bindings to the each primary antibody were detected on an X-ray film (Konica Minolta Medical Imaging, Wayne, NJ, USA) with ECL prime immune-detection reagent (GE Healthcare, Little Chalfont, UK).

Statistical analysis

Data are represented as mean±standard deviation. Student's t-test was performed to determine the statistical significance between PAE and DMSO. Statistical significant was defined as $*p<0.05$ or $**p<0.005$. The half maximal inhibitory concentration (IC50) of PAE was calculated by Graphpad prism 5 (Version 5.01, GraphPad Software Inc., San Diego, CA, USA). The data appeared on figures are representative data for three independent experimental results.

RESULTS

PAE inhibited K562 cell proliferation

The cytotoxicity of PAE in K562 cancer cell line was first determined by MTT assay. As shown in Fig. 1, PAE exhibited dose-dependent growth inhibition against K562 cancer cells; the IC50 about 212±2.3 ng/ml. The cytotoxicity effect of PAE against K562 was higher than the positive control cisplatin. This result indicated that PAE has anti-proliferative effect against K562.

PAE induced apoptosis in K562 cells

To examine whether cells undergo apoptosis, untreated, PAE-treated or cisplatin-treated K562 were stained with annexin V and PI. Flow cytometry analysis of stained cells can distinguish cells into four groups, namely viable (annexin V− PI−), early apoptosis (annexin V+ PI−), late apoptosis (annexin V+ PI+) and necrotic (annexin V− PI+) cells. As shown in Fig. 2a and b, PAE exposure at different concentrations (10, 50 and 250 ng/ml) resulted in a higher population of the early apoptotic population (3.50±0.7% to 23.4±1.8% in K562) compared to control (3.07±0.2%). This result showed that PAE induced apoptosis in K562 cells.

PAE downregulated the anti-apoptotic and upregulated the apoptotic proteins expression

To investigate the mechanism underlying apoptosis induced by PAE, we tested the effect of PAE extract on the anti-apoptotic Bcl-2 and apoptotic (Bax and cleaved caspase-3) protein levels by western blotting. As shown in Fig. 3, Western blotting analysis revealed that PAE treatment leaded to decrease in Bcl-2 levels and increase in Bax levels

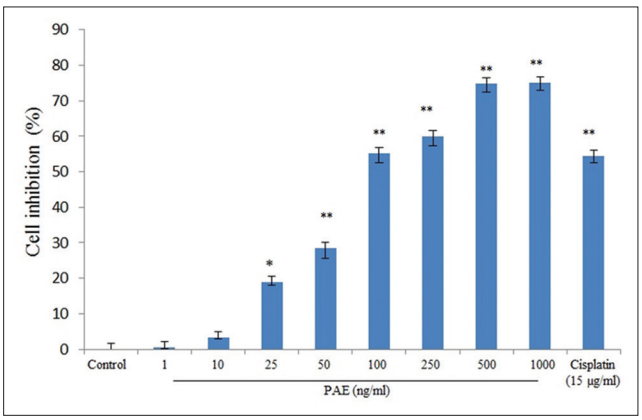

Fig. 1: Anticancer activity of *Paederus alfieri* extract (PAE) against K562 cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for the anticancer activity of PAE against K562 cells revealed that PAE induced dose-dependent cell inhibition. Cisplatin served as a positive control

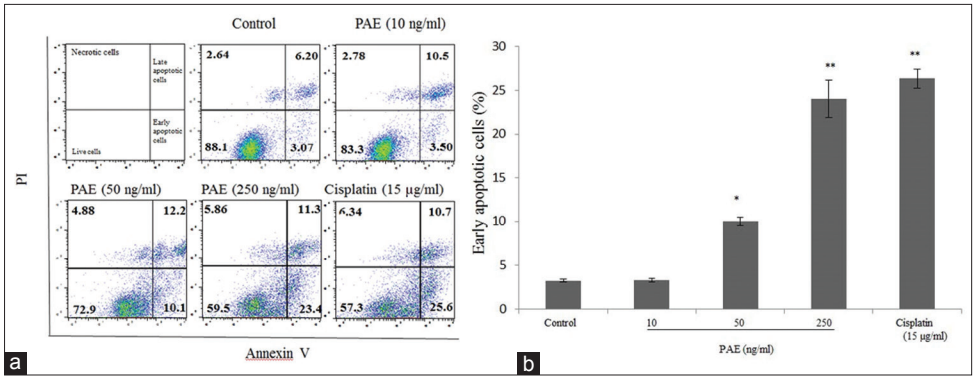

Fig. 2: Flow cytometric analysis of apoptosis induction by *Paederus alfieri* extract (PAE) in K562 cells. (a) Representative cytograms of apoptotic cells in K562 cells incubated in the absence (control) or presence of 10, 50, 250 ng/ml PAE or 15 µg/ml cisplatin as a positive control for 24 hr. Within a cytogram, the lower left quadrant represents the viable cells; the lower right quadrant, early apoptotic cells; the upper right quadrant, late apoptotic cells; the upper left quadrant, necrotic cells. (b) Percentage of early apoptotic cells after 24 hr incubation in the absence (control) or presence of 10, 50, 250 ng/ml PAE or presence of 15 µg/ml cisplatin as a positive control. The values are the means±standard error of the mean of three different experiments. *p<0.05 and **p<0.005, significantly different compared with control treatment

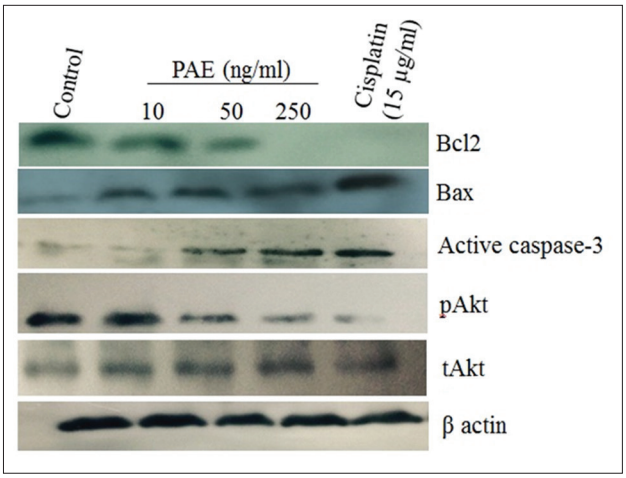

Fig. 3: Effect of *Paederus alfieri* extract (PAE) on the expression of anti-apoptotic, pro-apoptotic and p-Akt/t-Akt marker proteins. Western blotting analysis showed that levels of Bcl-2 and p-Akt were decreased while levels of Bax and active caspase-3 were increased, but levels of t-Akt remained constant following treatment with PAE in a dose-dependent manner in comparison with the vehicle as a negative control. β actin served as a loading control. The experiment repeated for three different times

as compared to control cells in a dose-dependent manner. Furthermore, PAE induced the activation of caspase-3 in a dose-dependent manner.

PAE inhibited the phosphorylation of Akt

To better understand the molecular basis of PAE induced apoptosis, we investigated the expression of p-Akt and t-Akt after treatment with PAE (10, 50, and 250 ng/ml) for 24 hr. As shown in Fig. 3, the levels of p-Akt are dose-dependently decreased in response to PAE, whereas the total Akt protein levels remained constant during PAE treatment.

DISCUSSION

The genus *Paederus* including about 600 species is found in all tropical and temperate climates. Beetles belonging to the genus *Paederus* causing an acute vesicating dermatitis have been reported from many parts of the world [2]. Furthermore, the potential antitumor effect of PAE has been reported in a few papers [3]. However, the effects of *Paederus* on K562 (human myeloid leukemia cells) and its mechanism remain unclear. In this study, we tried to evaluate the anticancer activity of PAE on the K562 cells. Our results indicated that PAE has anti-proliferative effect against K562 cells with IC50 about 212±2.3 ng/ml. The induction of apoptosis is common mechanism proposed for the cytotoxic effects of anticancer extract [13]. Apoptosis or programmed cell death is a physiological process in which cells die. Many cancer chemical drugs induce apoptosis to treat cancer [14]. Apoptosis considered as an outcome of a complex interaction between pro- and anti-apoptotic molecules. Proteins of the Bcl-2 family are key regulators of the apoptotic pathway [15]. Bcl-2 family can be divided into two subfamilies: One is an anti-apoptotic protein such as Bcl-2; the other is pro-apoptotic protein such as Bax. Accumulated data have shown that many anticancer agents induced apoptosis by targeting the proteins of Bcl-2 family and the ratio of Bax/Bcl-2 played a critical role in determining whether cells will undergo apoptosis [16,17]. In our study, by examining the effect of PAE on Bcl-2 and Bax, we found that PAE decreased anti-apoptotic Bcl-2 expression and increased proapoptotic Bax expression, leading to up-regulation of the ratio of Bax/Bcl-2. This might be one of the molecular mechanisms through which PAE induces apoptosis. Salakou *et al.* [18] showed that the increase in bax/Bcl-2 ratio may up-regulate caspase-3 activation and increase the apoptosis process. Our results showed that cleaved caspase-3 was increased in PAE treated cells in a dose-dependent manner leading to apoptosis. The PI3K/Akt is one of the most important signaling pathways in regulating apoptosis, and Akt is a major downstream target of PI3K [9]. The PI3K/Akt pathway is highly involved in tumorigenesis [19]. The PI3K/Akt signaling pathway regulates the development and progression of various cancers by elevating the activity of the anti-apoptotic action of Akt, and the phosphorylation of Akt is routinely used as readout for the Akt activation [20]. Asnaghi *et al.* [21] have shown that some anticancer drugs could induce apoptosis accompanying down-regulation of Akt. In our study, we evaluated the effect of PAE on the PI3K/Akt pathways by measuring the protein expression levels of total Akt and phospho-Akt protein. We found that treatment of K562 cells with PAE reduced the protein expression of p-Akt in a dose-dependent manner, whereas the total Akt protein levels remained constant during PAE treatment. In summary, these results indicated that PAE-induced apoptosis possibly by down-regulating Akt signaling in human myeloid leukemia cancer K562 cells.

CONCLUSIONS

Our studies demonstrated that PAE inhibited the growth of human myeloid leukemia cancer K562 cells by inducing apoptosis via modulating PI3K/Akt pathway. This might be the important mechanism of PAE suppressed the growth of the myeloid leukemia cancer cells. Our findings suggest that may be PAE is a good extract for developing anticancer drugs for human myeloid leukemia cancer treatment.

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