RESEARCH COMMUNICATION

Ethanolic Rhizome Extract from *Kaempferia parviflora* Wall. ex. Baker Induces Apoptosis in HL-60 Cells

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Abstract

***Kaempferia parviflora* Wall. ex. Baker is a Thai herb containing many flavonoids that have anti-inflammatory, anti-allergic and antioxidant activities. The objective of this study was to demonstrate apoptotic effects of *Kaempferia parviflora* Wall. ex. Baker rhizome ethanolic extract on HL-60 cells *in vitro*. The extract suppressed HL-60 cell growth and decreased cell viability in a dose- and time-dependent manner. Apoptotic cell death was demonstrated by changes in cell morphology, externalization of phosphatidylserine on the cell surface, loss in mitochondrial transmembrane potential and activation of caspase 3. Apoptosis induced by *K. parviflora* Wall. ex. Baker rhizome ethanolic extract was enhanced by treatment with paclitaxel or doxorubicin, and inhibitors of Akt, PI3-K and MEK.**

Key Words: *K. parviflora* Wall.ex.Baker - apoptosis - HL-60 cells - caspase-3 - Akt - mitochondria

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Introduction

Kaempferia parviflora Wall. ex. Baker is a member of the ginger family (Zingiberaceae). In folk medicine of Suriname and Southeast Asia, rhizome is used for treatment of swelling, wounds and diarrhea colic disorder, and as an aphrodisiac (Yenjai et al., 2004). Rhizomes of fingerroot or *K. parviflora* Wall. ex. Baker are used as food ingredients and made into wine (Nagahara et al., 2002). Alcoholic infusion of *K. parviflora* Wall. ex. Baker rhizome has been used as a tonic for body pains and gastrointestinal disorders, such as colic and peptic and duodenal ulcers (Yenjai et al., 2004).

Apoptosis is a major form of cell death and relevant to chemotherapy- and/or radiation-induced cell death in human hematological malignancies. An increase of the apoptotic threshold in leukemia and lymphoma causes treatment resistance (Ghia and Nadler, 1997). Medicinal herbs are of interest to intervene as an additional cancer treatment for a successful therapy. The aim of the present study was to demonstrate cytotoxicity of ethanolic extract of *K. parviflor*a Wall. ex. Baker on human promyelocytic leukemic (HL-60) cells and to investigate the mechanism of cell death.

Materials and Methods

Chemicals

RPMI-1640 and fetal bovine serum were obtained from Gibco-BRL, New York, NY, USA, annexin V-FITC kit from Roche, Indianapolis, IN, USA, and propidium iodide (PI), 3,3'-dihexyloxacarbocyanine iodide ($DiO_6(3)$), paclitaxel and doxorubicin from Sigma, St. Louis, MO, USA. Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol-2(R)-2-O-methyl-3-O-octadecylcarbonate, PI-3K inhibitor wortmannin and LY294002 and MEK inhibitor PD98059 were obtained from Calbiochem, Darmstadt, Germany. Caspase-3 fluorometric protease assay kit was from Biosource International, Inc, Camarillo, CA, USA.

Plant material and extraction

K. parviflora Wall. ex. Baker rhizomes were collected in January, 2005 from Loi province, Thailand. The plant was authenticated by Dr. Bungorn Sripanidkulchai, and a voucher specimen (HHP-BS-KP1) has been deposited at the Center of Research and Development of Herbal Health Products, Khon Kaen University, Thailand. Rhizomes were washed thoroughly in tap water, shade-dried, powdered and extracted with 95% ethanol, which was then evaporated *in vacuo* at 45-50 ºC and freeze-dried to obtain a dry extract (5% yield). The dry extract was dissolved in dimethylsulfoxide (DMSO) (50 mg/ml), which was then diluted 1:100 in RPMI-1640 medium and added to the cells at the indicated concentrations.

Cell culture and treatment

Human promyelocytic leukemic HL-60 cells were

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cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 mg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2. Preconfluent (growth phase) HL-60 cells ($1x10^6$ cells) were incubated with *K. parviflora* Wall. ex. Baker extract and cell proliferation (counting by using hematocytometer) and cell viability (by trypan blue exclusion test) were determined after 24, 48 and 72 h. HL-60 cells treated with *K. parviflora* Wall. ex. Baker extract for 24 h were then processed for flow cytometry analysis and PI staining for examination under fluorescence microscope.

For measurement of mitochondrial transmembrane potential, HL-60 cells were incubated with 40 nM $DiO_6(3)$ at 37ºC for 15 min and then were washed twice in phosphate-buffered saline (PBS). Cells were then resuspended in PBS and subjected to flow cytometric analysis (Xiang et al., 1996).

HL-60 cells were treated with LY294002 or wortmannin for 1 h before incubating with the ethanolic extract. Then cells were incubated with $DiO_6(3)$ as described above and fluorescence in cells were measured (with excitation at 490 nm and emission at 513 nm) using a fluorescence plate reader (Bio-Tek Instruments, Inc., USA).

Flow cytometry

Treated cells were sedimented at 200 x g and the cell pellet was resuspended in 100 µl of binding buffer provided by the annexin V-FITC reagent kit. Annexin V-FITC (2 µl) and PI (2 µl) were then added together with 900 µl of binding buffer. Analysis was conducted using FACScan (Beckton Dickenson, USA). Cells that are stained with annexin V-FITC are designated as apoptotic, whereas cells that are stained with both annexin V-FITC and PI are considered necrotic (He et al., 2005).

PI staining and fluorescence microscopy

Treated HL-60 cells were cytospun onto glass slides. After air drying, cells were fixed with absolute methanol for 10 min at -20˚C, washed twice with PBS and air-dried. PI (200 mg/ml) was applied to the fixed cells for 10 min at room temperature. After washing with PBS and drying, slides were mounted with 90% glycerol and examined under a fluorescence microscope (Olympus, Germany) (Nicolletti et al., 1991).

Caspase-3 activity determination

Treated cells were pelleted and resuspended in 50 µl of cell lysis buffer provided by caspase-3 activity kit and incubated on ice for 10 min. A 50 µl aliquot of dithiothreitol (10 mM) was added followed by 5 µl of DEVD-AFC (50 mM). Cells were incubated at 37ºC in the dark and fluorescence determined with excitation at 400 nm and emission at 505 nm in a plate reader (Bio-Tek Instruments, Inc., USA).

Statistical analysis

Experimental results were expressed as means + SEM. Comparisons between samples were evaluated with one-way ANOVA. Statistical significance was determined using Mann-Whitney U test (two-sided) with SPSS/PC V11.0 software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Ethanol extract of *K. parviflora* Wall. ex. Baker (5-100 mg/ml) suppressed HL-60 cell growth and decreased viability in a dose- and time-dependent manner (Figure 1). Fifty-percent inhibitory concentrations (IC_{50}) at 24, 48 and 72 h were 25.5, 18.5 and 14.5 mg/ml, respectively.

Loss of HL-60 viability induced by *K. parviflora* Wall. ex. Baker ethanol extract was due to apoptosis as manifested by the presence of annexin V stained cells (Figure 2). The ethanol extract induced apoptosis in a dose-dependent manner (Table 1), but treatment with 100 µg/ml resulted in 30.04 ± 4.54% necrosis. HL-60 apoptotic

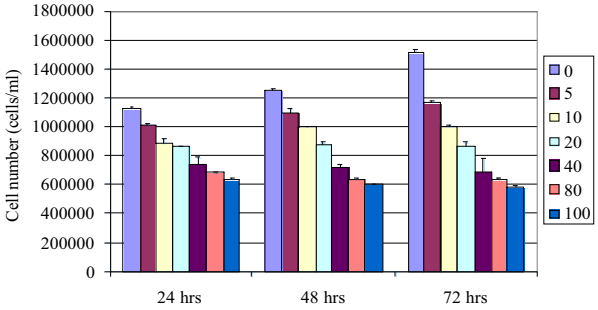

Figure 1. Dose- and Time-response of *Kaempferia parviflora* Wall. ex. Baker Rhizome Ethanol Extract on HL-60 Cell Proliferation. Treatment was with extract at various concentrations (5-100 µg/ml) for 24, 48, and 72 h. Mean and SEM of 3 independent experiments in duplicate are shown.

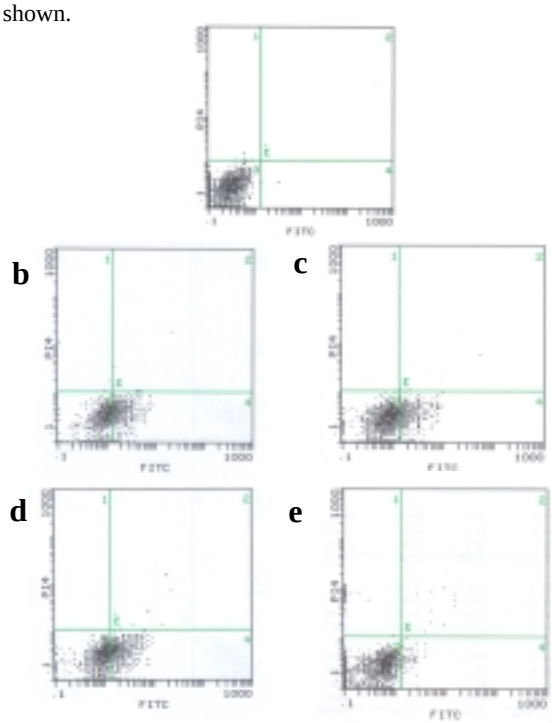

Figure 2. Dot Plot Pattern of HL-60 Apoptotic Cells Treated with of *Kaempferia parviflora* Wall. ex. Baker Rhizome Ethanol Extract. HL-60 cells were treated with 0, 5, 20, 80, and 100 mg/ml of ethanol extract (A-E), respectively, stained with annexin V-FITC and propidium iodide and processed by flow cytometer. The dot plot represents one of three independent experiments.

Table 1. Dose-response effect of *K. parviflora* Wall. ex. Baker Rhizome Ethanol Extract on HL-60 Cell Apoptosis and Necrosis. HL-60 cells were treated with the ethanol extract at the concentrations indicated and percent apoptotic and necrotic cells were detected by flow cytometry using annexin V-FITC and propidium iodide staining.

KPE (µg/ml)	% Apoptosis	% Necrosis
0	1.1 ± 0.21	0.3 ± 0.14
5	32.9 ± 8.48[a]	0.3 ± 0.31
20	43.3 ± 21.64[a]	0.2 ± 0.16
80	65.4 ± 3.33[a]	1.0 ± 0.47
100	4.9 ± 0.16	30.0 ± 4.54[a]

Data are Mean ± SEM from 3 independent experiments in duplicate. [a]significant difference compared to the control

cell death was also confirmed by the presence of condensed nuclei and apoptotic bodies (Figure 3).

The mechanism of HL-60 cell apoptosis involved loss of mitochondrial transmembrane potential (Figure 4) and activation of caspase 3 (Figure 5). Several anti-cancer agents are available for clinical use. Most of these agents, however, can cause adverse effects. If the ethanol extract of *K. parviflora* can interact synergistically with anti-cancer agents to improve their cancer preventive activity,

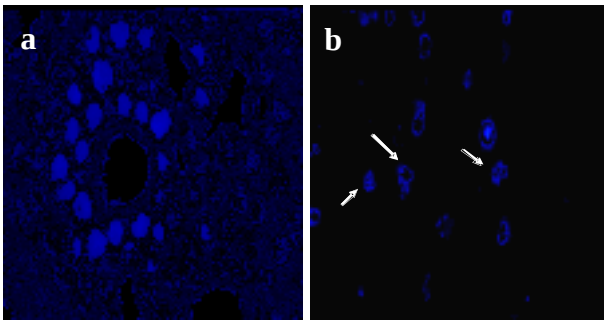

Figure 3. Fluorescence Micrographs of Normal and Apoptotic HL-60 Cells. HL-60 cells without treatment (a) and treated with ethanol extract of *Kaempferia parviflora* Wall. ex. Baker rhizome at 40 µg/ml for 24 h (b) were stained with propidium iodide and examined under fluorescence microscope. Apoptotic bodies are indicated by arrows. Magnification 400x.

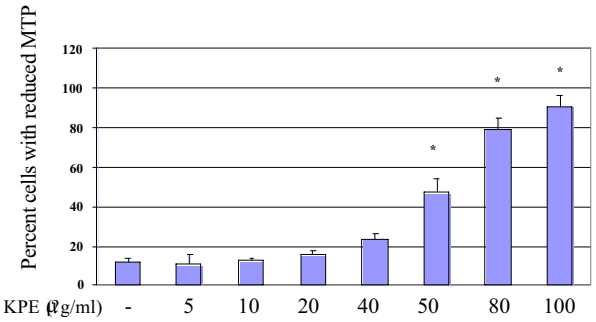

Figure 4. Dose-response of *Kaempferia parviflora* Wall. ex. Baker Rhizome Ethanol Extract on Reduction of Mitochondrial Transmembrane Potential (MTP) of HL-60 cells. HL-60 cells were treated for 24 h with the ethanol extract at the concentrations indicated, incubated with 40 nM 3,3'-dihexyloxacarbocyanine iodide for 15 min and subjected to flow cytometry analysis as described in Materials and Methods. Means and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference compared to untreated control, $p < 0.05$.

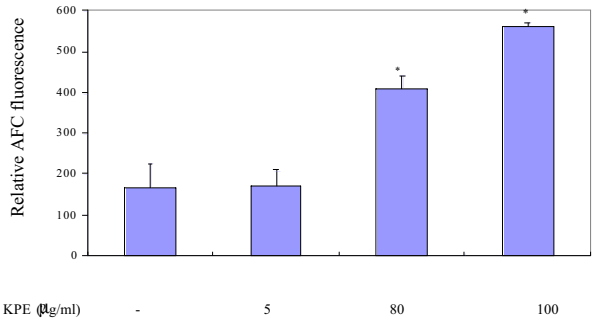

Figure 5. Caspase-3 Activity of HL-60 cells Treated with Ethanol Extract of *K. parviflora* Wall. ex. Baker Rhizome. HL-60 cells were incubated for 4 h with ethanol extract at the concentrations indicated and resuspended in lysis buffer. A reaction solution containing 50 mM DEVD-AFC and 10 µM dithiothreitol was added and fluorescence measured as described in Materials and Methods. Mean and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference compared to untreated control, $p < 0.05$.

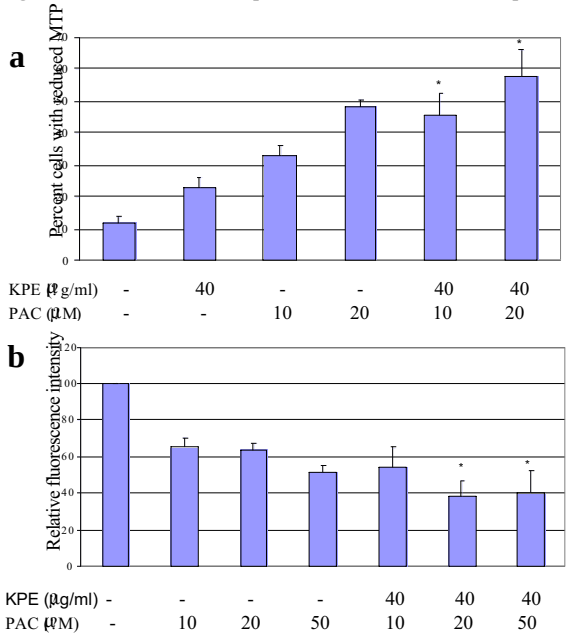

Figure 6. Effect of Paclitaxel on HL-60 Cell Apoptosis Induced by *Kaempferia parviflora* Wall. Ex. Baker Rhizome Ethanol Extract. (a) Percentage of HL-60 cells with decreased mitochondrial transmembrane potential treated with ethanol extract alone and/or paclitaxel by using flow cytometry as described in Materials and Methods. (b) Relative fluorescence intensity of HL-60 cells stained with 40 nM 3,3'-dihexyloxacarbocyanine iodide after treatment with paclitaxel and/or ethanol extract. (*) indicates significant difference compared to Pac alone, $p < 0.05$. Pac, paclitaxel; KPE, *K. parviflora* extract.

it might be possible to reduce the doses of these drugs and thus also reduce their adverse effects. To investigate a possible synergy between the extract and chemical cancer preventive agents, paclitaxel and doxorubicin were chosen, which are clinically used against several types of cancer including breast and ovarian cancer (Thomadaki and Scorilas, 2008; Lu et al., 2008). A synergistic effect was obtained when paclitaxel or doxorubicin was added together with K. parviflora Wall. ex. Baker ethanol extract (Figure 6 and 7). There was an inverse correlation between the decrease in mitochondrial transmembrane potential

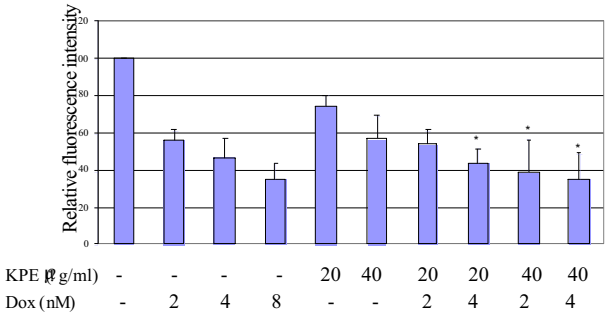

Figure 7. Effects of Doxorubicin on HL-60 Apoptosis Induced by *Kaempferia parviflora* Wall. ex. Baker Rhizome Ethanol Extract. HL-60 cells were treated with ethanol extract at 20 or 40 μg/ml and/or doxorubicin at 2 or 4 nM, incubated with 40 nM 3,3'-dihexyloxacarbocyanine iodide for 15 min and fluorescence measured as described in Materials and Methods. (*) indicates significant difference compared to Dox alone, $p < 0.05$. Dox: doxorubicin; KPE, K. parviflora extract.

ethanol extract that gave rise to apoptosis of human leukemic (HL-60) cells is needed to allow an understanding of the mechanism of action.

In this study, the effects of some survival-death transducers were also examined. PI3-K (survival factor)-mediated apoptosis is found in human leukemic cells, such as acute myeloid leukemic (Karajannis et al., 2006), chronic myelogenous leukemic (Moehring et al., 2005) and chronic lymphocytic leukemic B (Bernal et al., 2001) cells, induced by chemotherapeutic drug, imatinib mesylate (antileukemic drug targeting Bcr-Abl) and antigen-receptor-derived signals, respectively. This brought to the experiments using PI3-K inhibitors as a tool to test the role of PI3-K in the death pathway. When

and increase in the number of cells with decreased mitochondrial transmembrane potential.

Phytochemical screening of *K. parviflora* Wall. ex. Baker alcoholic extract gave positive tests for alkaloids, anthrones, coumarins and flavonoids (Patanasethanont et al., 2007). Yenjai et al. (2004) reported nine flavonoids isolated from K. parviflora Wall. ex. Baker. These flavonoids possess no cytotoxicity against KB (oral human epidermoid carcinoma), BC (breast cancer), and NCI-H187 (human small cell lung cancer) cell lines.

It has been reported that oral administration of *Kaempferia parviflora* Wall.ex.Baker extract at doses of 30, 60, and 120 mg/kg in Sprague-Dawley rats significantly inhibits gastric ulcer formation induced by indomethacin, HCl/EtOH and water immersion restraint stress (Rujjanawate et al., 2005). Tewtrakul and Subhadhirasakul (2008) have recently reported of 7 methoxyflavone derivatives and found that 5-hydroxy-3,7,3',4'-tetramethoxyflavone exhibits high activity against lipopolysaccharide-induced nitric oxide release in RAW264.7 macrophage cells. This compound also inhibits PGE2 release but is inactive on TNF-alpha production. Furthermore, it also possesses high anti-allergic activity against antigen-induced beta-hexosaminidase release as a marker of degranulation in rat basophilic leukemia RBL-2H3 cells (Tewtrakul et al, 2008). Interestingly, ethanol extract of K. parviflora rhizome and its flavone derivatives significantly increase the accumulation of rhodamine 123 and daunorubicin, substrates of P-glycoprotein multi-drug transporter, in LLC-GA5-COL150 cells (porcine kidney epithelial cells transfected with human MDR1 cDNA), but not in parental cells (Patanasethanont et al., 2007). The identity of the compounds present in *K. parviflora* Wall. ex. Baker

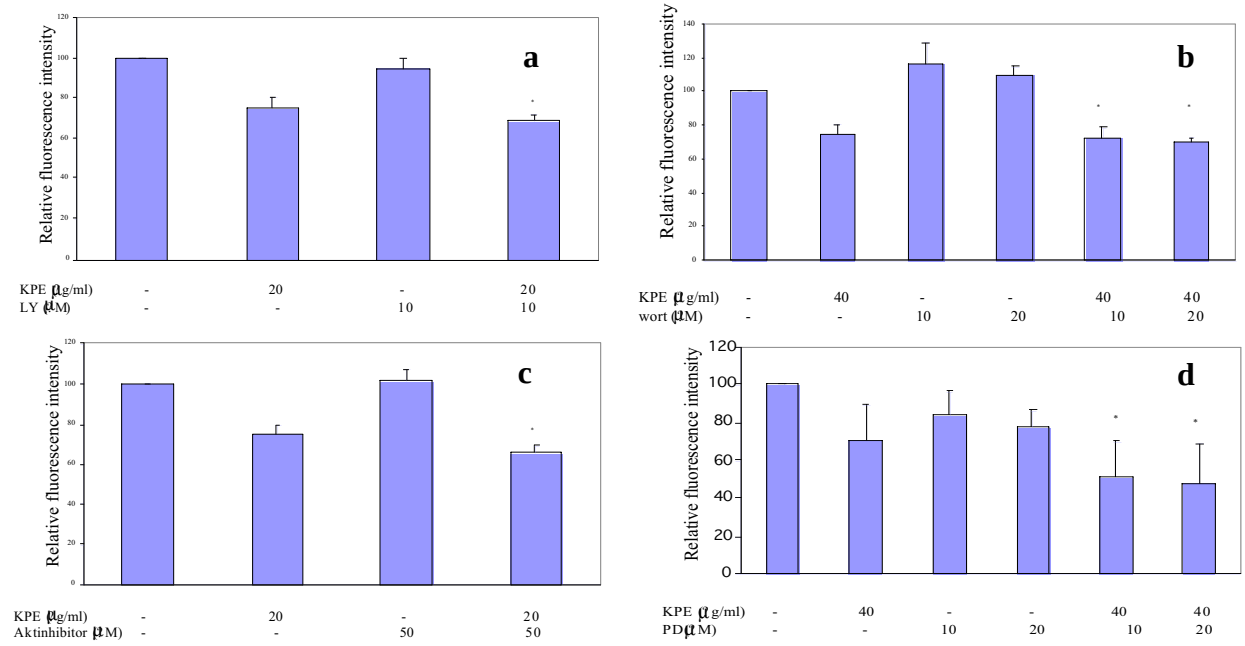

Figure 8. Effects of an Akt Inhibitor, PI3-K Inhibitors (LY294002 and wortmannin), and an MEK Inhibitor (PD98059) on HL-60 Apoptosis Induced by *Kaempferia parviflora* Wall. ex. Baker Rhizome Ethanol Extract. HL-60 cells were incubated with LY294002 (a PI3-K inhibitor) (a), wortmannin (another PI3-K inhibitor) (b), Akt inhibitor (c) and PD98059 (MEK inhibitor) (d) for 1 h then incubated with ethanol extract of *K. parviflora* Wall. ex. Baker rhizome for 24 h. The cells were treated with 40 nM of $DiO_6(3)$ for 15 min and subjected to fluorescence plate reader. (*) indicates significant difference compared to KPE alone, $p < 0.05$. LY, LY294002; wort, wortmannin; PD, PD98059; KPE, *K. parviflora* extract

HL-60 cells were treated with inhibitors (LY294002 or wortmannin) of PI3-K, fluorescence intensity of DiO6(3) also decreased compared to the HL-60 cells treated with the extract alone (Figure 8a and b). To confirm the involvement of PI3-K, inhibitor of Akt (a serine-threonine kinase or protein kinase B, a downstream effector of PI3-K pathway) could also reduce fluorescence intensity compared to HL-60 cells treated with the extract alone (20 μg/ml) (Figure 8c), i.e., the apoptosis pathway induced by *K. parviflora* extract was mediated through PI3-K and Akt in addition to the mitochondrial pathway. MEK inhibitor (PD98059, at 10 and 20 μM) synergistically decreased the fluorescence intensity compared to that of the HL-60 cells incubated with the ethanol extract alone (Figure 8d).

Taken together, apoptosis of HL-60 cell line induced by *K. parviflora* Wall. ex. Baker ethanol extract involved activation of caspase-3 via a mitochondria-mediated pathway. This could be enhanced by inhibitors of Akt, PI3-K and MEK, indicating the involvement of the first two transducers (Akt and PI3-K) in the signaling of death pathway.

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