

RESEARCH COMMUNICATION

Induction of Apoptosis in the Human Leukemic U937 Cell Line by *Kaempferia parviflora* Wall.ex.Baker Extract and Effects of Paclitaxel and Camptothecin

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Abstract

Kaempferia parviflora Wall.ex.Baker is a Thai medicinal herb that has high antioxidant and anti-inflammatory activities. Apoptotic effects of the herbal extract alone and in combination with chemotherapeutic drugs, paclitaxel and camptothecin, were here studied in the human promonocytic leukemic U937 cell line. *K. parviflora* extract suppressed cell proliferation and decreased cell viability in a dose- and time-dependent manner as assessed using the trypan blue exclusion assay. Staining of extract-treated cells with propidium iodide and examination under a fluorescence microscope showed condensed nuclei and apoptotic bodies. Mitochondrial transmembrane potential (MTP) decreased after treatment and the number of cells with decreased MTP also increased. Furthermore, activation of caspase-3 was found in herbal extract-treated cells. When the extract was combined with paclitaxel, an additive effect on U937 cell apoptosis was obtained, whereas camptothecin exerted an antagonistic effect.

Key Words: U937 cells - *Kaempferia parviflora* - paclitaxel - camptothecin - caspase-3

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Introduction

Kaempferia parviflora Wall.ex.Baker (or “Krachai Dum” in Thai) is a member of the Zingiberaceae family. Bioactive ingredients in *K. parviflora* include 5,7-dimethoxyflavone (anti-inflammatory), 5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone (antimalarial), and 3,5,7,4'-tetramethoxyflavone (antifungal) (Yenjai et al., 2004). Alcoholic infusions of its rhizomes have been used as a tonic for rectifying male impotence, body pains and gastrointestinal disorders such as colic disorder, peptic and duodenal ulcers (Rujjanawate et al., 2005). They also inhibit aggregation of human platelets (Yenjai et al., 2004) and have antioxidant activity (Kusirisin et al., 2009).

The aim of this study was to investigate the apoptotic effects of a *K. parviflora* ethanolic extract, alone and in combination with the chemotherapeutic drugs, paclitaxel and camptothecin, on U937, a human cell line established from a diffuse histiocytic lymphoma that displays many monocytic characteristics and is lymphoblastoid-like (Clement and Lehmeier, 1983). *K. parviflora* extract induced U937 cell apoptosis via mitochondria and caspase-3 pathway, which was enhanced by paclitaxel but antagonized by camptothecin.

Materials and Methods

Chemicals

RPMI-1640 and fetal bovine serum were obtained from Gibco-BRL, New York, NY, USA, and propidium iodide (PI), 3,3'-dihexyloxycarbocyanine iodide (DiOC₆), paclitaxel (PAC) and camptothecin (CAM) from Sigma, St. Louis, MO, USA. Caspase-3 fluorometric protease assay including DEVD-AFC was obtained from Biosource International, Inc, Camarillo, CA, USA.

Plant materials 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) was deposited in the Center of Research and Development of Herbal Health Products, Khon Kaen University, Thailand.

Rhizomes were washed thoroughly in tap water, shade-dried and grounded into powder. The rhizome powder was extracted with 95% ethanol, evaporated in vacuo at 45-50 °C and freeze-dried to obtain a dry extract (5% yield). The extract was dissolved in dimethylsulfoxide (50 mg/ml), then diluted 1:100 in RPMI-1640 medium and used to treat cells at the indicated concentrations.

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Cell culture and treatment

Human promonocytic leukemic U937 cells (a gift from Prof. Watchara Kasinrerak) were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂. Preconfluent (growth phase) U937 cells (1x10⁶) were incubated with *K. parviflora* extract and determined for cell proliferation and cell viability by trypan blue exclusion assay at 24, 48 and 72 h. U937 cells treated with *K. parviflora* Wall.ex.Baker ethanolic extract for 24 h were then processed for flow cytometry analysis (Beckton Dickinson, USA) and fluorescence microscopy examination following PI staining. For measurement of mitochondrial membrane potential (MTP), 40 nM DiOC₆ were added for 15 min at 37°C and then the cells were subjected to flow cytometry (Xiang et al., 1996).

PI staining and fluorescence microscopy

Treated U937 cells were cytospun onto glass slides and air dried. Cells were fixed with absolute methanol for 10 min at -20°C, washed twice with phosphate-buffered saline (PBS) and air dried. PI (200 µg/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, Japan) (Nicolletti et al., 1991).

Caspase-3 activity determination

U937 cells treated with ethanolic rhizome extract for 24 h were pelleted and resuspended in 50 µl of cell lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS ({3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate})) and incubated on ice for 10 min. A 50 µl aliquot of dithiothreitol (10 mM) was added, followed by (5 µl) of DEVD-AFC substrate (50 µM) and then the cells were incubated at 37°C for 1 h in the dark. Fluorescence of the samples were measured using a fluorescence plate reader (Bio-Tek Instruments, Inc., USA) at 400 nm excitation and 505 nm emission (Jaeschke et al., 1998).

Statistical analysis

Duplicate tests were performed in 3 independent experiments and analyzed by Kruskal Wallis analysis. If the p value by Kruskal Wallis analysis was less than 0.05 then each value was compared to control using Mann-Whitney U test, with a p value < 0.05 considered as significant.

Results and Discussion

Apoptotic effects of the *K. parviflora* ethanolic extract

The ethanolic extract of *K. parviflora* was cytotoxic to U937 cells in a dose- and time-dependent manner, with IC₅₀ values at 24, 48 and 72 h of 92, 70 and 61 µg/ml, respectively (see Figure 1). The morphology of treated cells exhibited condensed nuclei and apoptotic bodies (Figure 2).

The effect of *K. parviflora* ethanolic extract on MTP of U937 cells depended on the concentration used in the

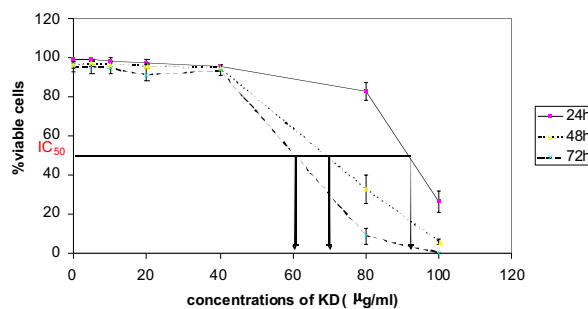


Figure 1. Dose- and Time-dependent Effects of *K. parviflora* Extract on U937 Cell Viability. U937 cells were treated with *K. parviflora* alcoholic extract at concentrations indicated and IC₅₀ value was determined using trypan blue exclusion test. KD: *Kaempferia parviflora* extract

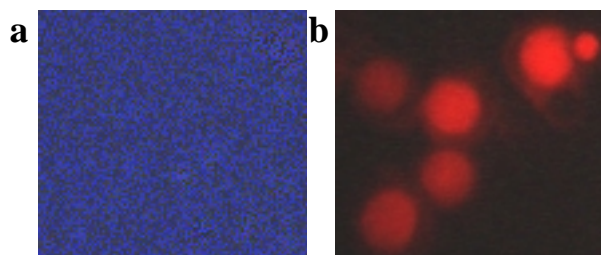


Figure 2. Fluorescent Micrographs of U937 Cells Stained with Propidium Iodide. Control U937 cells (A) and treated with 100 µg/ml *K. parviflora* extract (B) were fixed in absolute methanol and stained with propidium iodide and examined under fluorescence microscope, magnification x 400. An apoptotic body is illustrated at the top right

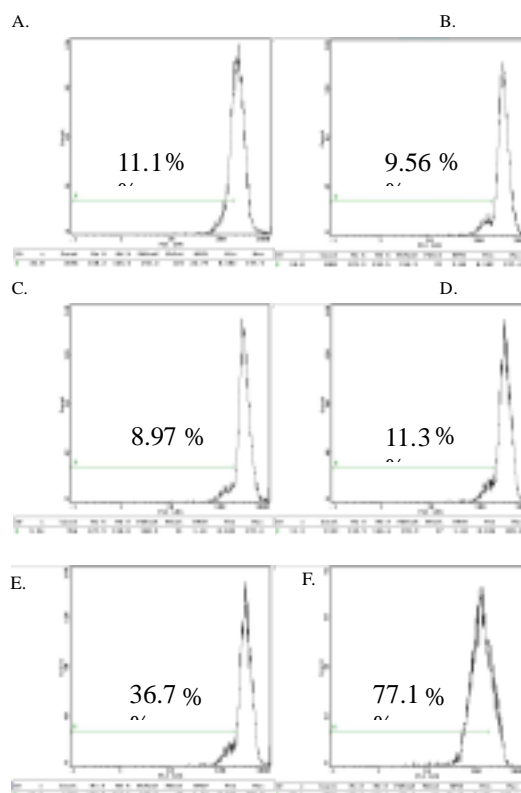


Figure 3. Reduction of Mitochondrial Transmembrane Potential of U937 cells Treated with *K. parviflora* Extract. Mitochondrial transmembrane potential decreased as the concentrations of the extract increased (A = 0, B = 10, C = 20, D = 40, E = 80 and F = 100 µg/ml). The cells were stained with 40 nM 3,3'-dihexyloxycarbocyanine iodide for 15 min and processed by flow cytometer. Area under the line is percent of cells with decreased MTP

treatment. At low concentrations (10 and 20 µg/ml) there was a decrease in the number of cells with decreased MTP compared with untreated control cells, whereas at higher concentrations (80 and 100 µg/ml) there was an increase in a dose-dependent manner (Figure 3). These results indicate that ethanolic extract of *K. parviflora* had an anti-apoptotic role at low concentrations but caused apoptosis at high dose. At the high doses (20-100 µg/ml) *K. parviflora* ethanolic extract activated caspase-3 in a dose dependent manner.

Combined effects of paclitaxel or camptothecin

Among the flavone derivatives isolated from *K.*

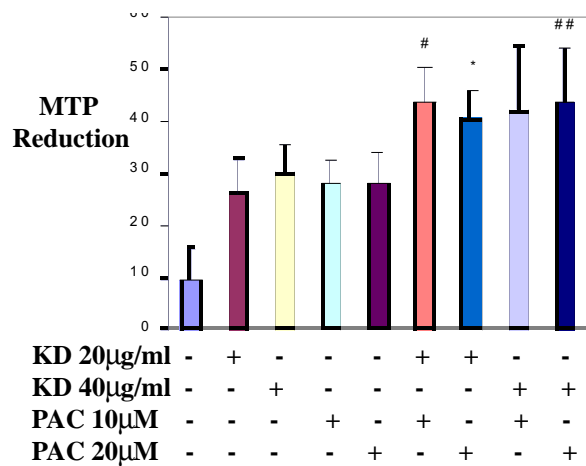


Figure 4. Effect of *K. parviflora* Wall.ex.Baker Rhizome Ethanol Extract in Combination with Paclitaxel on Percentage of U937 Cells with Reduced MTP. U937 cells were treated with *K. parviflora* extract (KD) alone or paclitaxel (PAC) alone or combined at the concentrations indicated and MTP was measured. (*) indicates significant difference from KD (20 µg/ml), p < 0.01; (#) significant difference from PAC (10 µM), p < 0.05; (##) significant difference from PAC (20 µM), p < 0.05

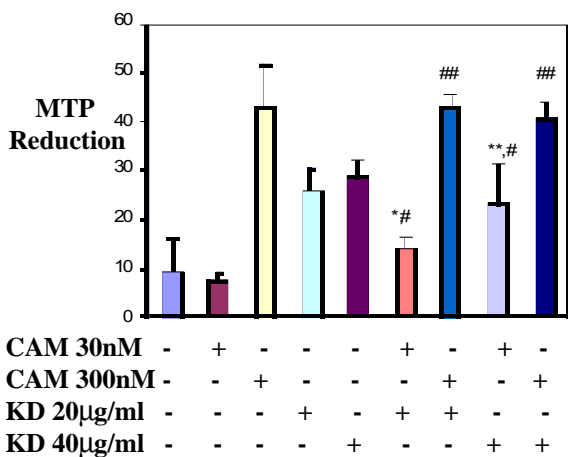


Figure 5. Effect of *K. parviflora* Wall.ex.Baker Rhizome Ethanol Extract in Combination with Camptothecin on Percentage of U937 Cells with Reduced MTP. U937 cells were treated with *K. parviflora* extract (KD) alone or camptothecin (CAM) alone or combined at the concentrations indicated and MTP was measured. (*) indicates significant difference from KD (20 µg/ml), p < 0.01; (#) significant difference from PAC (10 µM), p < 0.05; (##) significant difference from PAC (20 µM), p < 0.05

parviflora rhizome, 5,7-dimethoxyflavone exhibited maximal stimulatory effect on the accumulation of doxorubicin in A549 cells (Patanasethanont et al., 2007). The accumulation of doxorubicin was increased by four flavone derivatives without 5-hydroxy group, but not by two other flavone derivatives with 5-hydroxy group. In addition, 5,7-dimethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone decreased resistance of A549 cells to doxorubicin. These findings indicate that extracts and flavone derivatives from *K. parviflora* rhizome suppress multidrug resistance protein function, and therefore may be useful as modulators of multidrug resistance in cancer cells (Patanasethanont et al., 2007). This led us to investigate the effects of the extract in combination with chemotherapeutic drugs on U937 cells.

Paclitaxel (10 and 20 µg/ml) increased percent U937 cells with decreased MTP, which was enhanced when *K. parviflora* ethanolic extract (20 and 40 µg/ml) was combined (Figure 4). Our previous study demonstrated that paclitaxel enhances human promyelocytic leukemic HL-60 cell apoptosis induced by *K. parviflora* ethanolic extract (Banjerdpongchai et al., 2008). It has been reported that paclitaxel induces apoptosis of HL-60 cells via caspase-3 activation (Lu et al., 2005).

On the other hand, although camptothecin at low dose (30 nM) had no effect on U937 cells, but when combined with *K. parviflora* ethanolic extract (20 and 40 µg/ml), an antagonism was observed (Figure 5). At high dose (300 nM) camptothecin was able to increase percent U937 cells with decreased MTP, this result was not attenuated by *K. parviflora* ethanolic extract (20 and 40 µg/ml). Camptothecin, which induces an unusual type of DNA damage by trapping cellular topoisomerase I on chromosomal DNA in the form of drug-enzyme-DNA cleavable complexes, inhibits DNA synthesis and specifically kills S-phase cells. Cotreatment of L1210 cells with aphidicolin, which is an inhibitor of replicative DNA polymerases, completely abolishes camptothecin cytotoxicity, suggesting the involvement of DNA replication in camptothecin cytotoxicity.

It has been proposed that the collision between moving replication forks and camptothecin-stabilized topoisomerase I-DNA cleavable complexes results in fork arrest and possibly fork breakage, which is lethal to proliferating cells (Hsiang et al., 1989). Dosage of camptothecin influenced the effect on *K. parviflora* ethanolic extract-induced apoptosis differently, which may be caused by the number of complex-collision. Furthermore, the cytotoxic mechanism of paclitaxel is also proposed that the microtubule disassembly in M-phase is blocked. The pro- or anti-apoptotic effect of both chemotherapeutic drugs is phase-dependent of the cell cycle. The consumption of *K. parviflora* extract in cancer patients should be performed with careful awareness of these combined effects.

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