

RESEARCH COMMUNICATION

Pharmacological Activity of *Kaempferia parviflora* Extract against Human Bile Duct Cancer Cell Lines

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Abstract

A crude ethanol extract of *Kaempferia parviflora* Wall. Ex Baker and a purified compound, 5,7,4-trimethoxyflavone (KP.8.10), were evaluated for pharmacological effects on human cholangiocarcinoma cell lines (HuCCA-1 and RMCCA-1). The cells were incubated with various concentrations of extract for various time periods and metabolic activity (MTT assay) was assessed for cell viability. The results showed a dose-dependent effect of both crude ethanol extract and the pure compound. CC50s for the crude extract on HuCCA-1 and RMCCA-1 cells were 46.1 µg/ml and 62.0 µg/ml, respectively. Values for the pure compound could not be determined because of solubility problems. Interestingly, *K. parviflora* ethanol extract and KP.8.10 at low concentrations (10-20 µg/ml and 2.5-5 µg/ml, respectively) markedly reduced rhHGF-induced invasion by HuCCA-1 and RMCCA-1 cells across matrix-coated transwell plates. Higher concentrations of *K. parviflora* ethanol extract (60 and 80 µg/ml) and KP.8.10 (20 µg/ml) dramatically changed the cellular morphology and caused death in both cell types. KP.8.10 further exhibited progressive action via caspase-3 mitochondrial enzyme activation, enhancing cellular toxicity in a time-dose dependent fashion. Therefore, 5,7,4-trimethoxyflavone appeared to be a bioactive component of *K. parviflora* extract capable of exerting anti-cancer action. The results suggested a benefit of this edible plant in prevention and treatment of cholangiocarcinoma.

Key Words: Anti-proliferation - anti-invasion - apoptosis - cholangiocarcinoma cell lines - KP.8.10

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Introduction

Kaempferia parviflora Wall. Ex Baker, a plant in the *Zingiberaceae* family, is considered one of the health-promoting herbs of Thailand. Especially, it is well recognized as a pain reliever and alleviating of abdominal discomforts. Known chemical constituents of *K. parviflora* include polyphenolic flavonoids (Sutthanut et al., 2007) which have a wide range of biological activities, such as anti-inflammatory, anti-bacterial, anti-mutagenic, anti-oxidant, and anti-thrombotic effects (Bors and Saran, 1987; Robak and Gryglewski, 1988; Fotsis et al., 1997). The anti-inflammatory effect has been emphasized for a purified compound, 5,7-dimethoxyflavone (Panthong et al., 1989). Potent anti-bacterial and anti-fungal activities have been pointed out for the compounds 3,5,7,4-tetramethoxyflavone and 5,7,4-trimethoxyflavone (Yenjai et al., 2004). In addition, current investigation indicates that the compounds 5,7-dimethoxyflavone and 3,5,7,3',4-pentamethoxyflavone, as well as *K. parviflora* crude extracts have profound inhibitory action in a dose dependent manner to modulate multidrug resistance in cancer cells (Patanasethanont et al., 2007a; 2007b).

The present study was conducted to evaluate whether *K. parviflora* and its pure compound, 5,7,4-trimethoxyflavone (KP.8.10), might possess anti-cancer potential effect against the most common liver cancer type in Thai

populations, the cholangiocarcinoma (CCA). The disease usually causes a high mortality rate and the epidemiologic incidence trends to cover the Asian and Western countries (Patel, 2001; Taylor-Robinson et al., 2001; Khan et al., 2002; Okuda et al., 2002;). Several risk factors defined to associate with development of cholangiocarcinoma are biliary-tract inflammation, primary sclerosing cholangitis, liver-fluke infestation, hepatolithiasis, and viral infection (Kubo et al., 1995; Watanapa and Watanapa, 2002; Shaib et al., 2005). Diagnosis requires integration of clinical, biochemical, pathological and X-rays examination, meaning delay. Treatment is limited to surgery but this is mostly infeasible. So, the prognosis of patients is poor.

The cholangiocarcinoma cell lines used in this study were derived from Thai patients with chronic parasitic infection (HuCCA-1 cell) and non-parasitic infection (RMCCA-1 cell). Previous characterization of the cells indicates that HuCCA-1 cells are low in invasive potential and sensitive to hepatocyte growth factor induction (Sirisinha et al., 1991; Imai et al., 2005; Pongchairerk et al., 2005), but RMCCA-1 are highly invasive cells (Rattanasinganchan et al., 2006).

Materials and Methods

Compound preparation

Dried ethanol extract and 5,7,4-trimethoxyflavone

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(KP.8.10), of *K. parviflora* were prepared at the Center for Research and Development of Herbal Health Promotion, Faculty of Pharmaceutical Science, Khon Kaen University. They were dissolved in dimethylsulphoxide (DMSO; Sigma) to make stock solutions (100 mg/ml, and 10 mg/ml), filtered, and stored at -20°C . For working solutions, they were further diluted with HamF-12 media to the desired concentrations. For each preparation, the final concentration of DMSO was limited at 0.2% minimum.

Cell viability assays

HuCCA-1 and RMCCA-1 cells were grown to monolayer and maintained in HamF-12 medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. The cells (1.0×10^5 cells/ml) were plated in 96-well plates in 100 μl of completed medium and cultured overnight in 37°C , 5% CO_2 incubator. Cells were treated with various concentrations of crude ethanol extract or KP.8.10 compound and further incubated for 48h. Cell viability was determined by MTT assay using In Vitro Toxicity Assay Kit MTT based (Sigma). Percentage of cell survival was calculated and the CC50 was determined.

Each experiment was performed in triplicate, the data were collected and evaluated by mean \pm standard error of the mean (S.E), using one-way analysis of variance (ANOVA).

Cell invasion assays

HuCCA-1 or RMCCA-1 cells (5×10^4 cells) were plated in Matrigel[®]-coated transwell (Costar, 8 μm pore sized) plates and incubated overnight at 37°C in 5% CO_2 incubator. The cells were treated with 10 and 20 $\mu\text{g/ml}$ (ethanol extract) or with 2.5 and 5 $\mu\text{g/ml}$ (KP.8.10 compound), respectively, in 200 μl of serum-free medium containing 0.1% BSA for 30 min. The same concentrations of the extracts, with or without 20 ng/ml of rhHGF, were added into the lower chamber. The number of invaded cells was counted after 24 or 48 h incubation, under the microscope (40x magnification), for 6 random fields per membrane and averaged.

Cell proliferation and morphological study

Cells were seeded (1.0×10^5 cells/ml) on the cover slips and cultured overnight at 37°C , 5% CO_2 incubator. HuCCA-1 cells were treated with 60 and 80 $\mu\text{g/ml}$ of ethanol extract for 24 hour. RMCCA-1 cells were treated with 10 and 20 $\mu\text{g/ml}$ of KP.8.10 compound for 9, 12, 18 and 24 h. At the end of exposure times, live cells were examined under the phase contrast microscope before fixing and staining with hematoxylin and eosin (H&E).

Apoptotic detection and mechanism

Treated cells were fixed in cold methanol and stained with fluorescence dye (DAPI, 1 $\mu\text{g/ml}$). Apoptotic nuclei were identified by fluorescence microscope. The proportion of apoptotic nuclei were calculated from counting a total of 500 nuclei and expressed as percentage of positive cells.

To determine the mechanism involved in apoptosis, RMCCA-1 cell (1.0×10^6 cells) were cultured overnight before treatment with 20 $\mu\text{g/ml}$ of KP.8.10 compound for 12-24h. The treated cells were lysed in buffer from ApoAlert[®] Caspase Colorimetric Assay Kit (Clontech), and processed for Caspase-3 enzyme activity assay following the manufacturer's protocol. The negative control was performed in parallel without adding the substrate. The experiments were performed three times and the data were evaluated by mean \pm S.E, using one-way analysis of variance (ANOVA).

Results

Cell viability effect

The crude ethanol extract of *K. parviflora* showed a dose-dependent effect on survival of both CCA cell lines. The CC50 on HuCCA-1 and RMCCA-1 cells were 46.13 $\mu\text{g/ml}$ and 61.97 $\mu\text{g/ml}$, respectively (48h treatment). The pure compound (K.P8.10) at the testing concentration (2.5-20 $\mu\text{g/ml}$) has shown slightly decreased in RMCCA-1 cells viability (Figure 1b). The CC50 could not be defined since the compound was insoluble at higher concentration.

Cancer invasion effect

24 h treatment with ethanol extract of *K. parviflora* (10-20 $\mu\text{g/ml}$) showed significantly decreased ($p < 0.001$) rhHGF-induced HuCCA-1 cells invasion. Also, 24 h treatment with compound KP.8.10 (2.5-5 $\mu\text{g/ml}$) exhibited significantly reduced ($p < 0.05$) in RMCCA-1 cells self-invasion. Extended-time of treatment (48h) did not further inhibit RMCCA-1 cell invasion (Figure 1).

Anti-cancer proliferation and cell death induction

Incubation of the cells with ethanol extract of *K. parviflora* and KP.8.10 significantly reduced cell growth

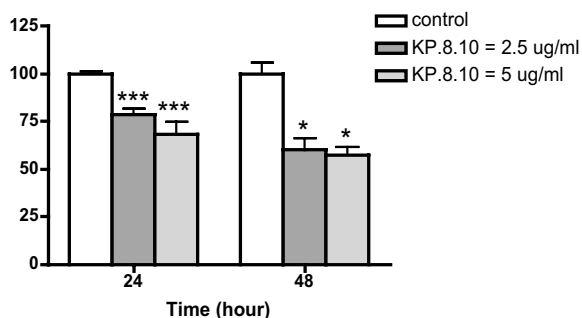


Figure 1. Effect of KP.8.10 Compound on RMCCA-1 Cell Invasion. (* $p < 0.05$, *** $p < 0.001$)

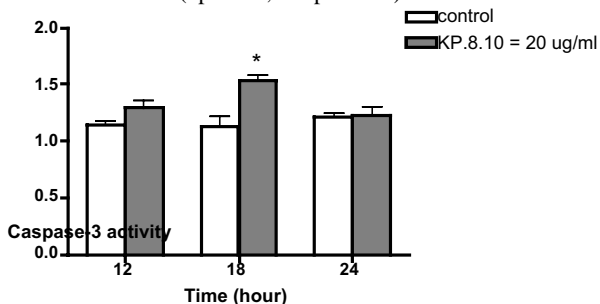


Figure 1. Effect of KP.8.10 Compound on RMCCA-1 Caspase-3 Activity. (* $p < 0.05$)

and proliferation. As demonstrated, a dramatic loss of HuCCA-1 cells was observed after 24h treatment with the crude extract. Similarly, a continuously decreased number of RMCCA-1 cells after treatment with the pure KP. 8.10 compound was showed in a time dependent manner. RMCCA-1 cells displayed membrane extrusion, vacuole formation, cell retraction and detachment in stepwise fashion, which indicated the process of cell death.

Apoptotic induction and mechanism of action

To determine whether cell death affected by *K. parviflora* could possibly involved apoptotic induction, HuCCA-1 and RMCCA-1 cells were treated with high dose ethanol extract (60µg/ml) and KP.8.10 compound (20µg/ml) for 24h. By nuclear staining (DAPI), the two treated cells presented apoptotic nuclei and certain amount of nuclear fragmentation, respectively. The crude extract of *K. parviflora* could induce apoptosis while its pure compound had a deleterious effect to the cell suggesting that KP.8.10 was a major bioactive compound to induce apoptosis and death of these cancer cells.

Semi-quantitative amount of cells with chromatin condensation and nuclei fragmentation was determined from KP.8.10 treated RMCCA-1 cells (10 and 20 µg/ml) after 9-48h treatment. The number of cell death was slightly increased after 9-18h and markedly increased after 24-48h treatment with 20 µg/ml of KP.8.10. This was clearly demonstrated that the pure compound of *K. parviflora* exerted its effect in a time-dose dependent action.

To identify the possible mechanism involved in apoptotic induction, RMCCA-1 cells were incubated with 20 µg/ml of KP. 8.10. Caspase enzyme activity inside the cells were evaluated after 12, 18 and 24 h treatment by using ApoAlert Caspase Colorimetric Assay Kit. The level of caspase-3 activity was significantly increased at 18 h after the treatment (Figure 2), indicating that KP. 8.10 activated via caspase mediator.

Discussion

K. parviflora has extensive anti-cancer activities on both types of cholangiocarcinoma cells in a time-dose relationship. KP.8.10 was a bioactive component of *K. parviflora* that exhibited significant inhibitory action on cancer invasion, growth and proliferation, also inducing apoptotic cell death. Although, the inhibitory mechanism of cell invasion was not elucidated here, the 5,7,4'-trimethoxyflavone component was proved to participate. Highly possibility is that the compound decreased the action of matrix metalloproteinases (MMPs) involved in cell invasion (Garbett et al., 1999; Thomas et al., 1999; Upadhyay et al., 1999; Kelly et al., 2000), since several investigators have demonstrated the effects of flavones to suppress MMPs secretion in cancer cells such as epidermal carcinoma cells, A431 (Huang et al., 1999), and breast tumor cell line, MDA-MB231 (Lindenmeyer et al., 2001). A flavonoid, myricetin, also inhibited MMP-2 activity and MMP-2 gene expression in colorectal carcinoma cell invasion (Ko et al., 2005). *K. parviflora* which contains flavonoids, especially flavones and

flavonol, may thus exhibit its effects on either matrix metalloproteinases or serine proteinases resulting in anti-invasion effect. Since the crude ethanol extract here reduced the percent invaded cells more than the pure compound, there might have some other constituents remained in the crude extract of *K. parviflora* that contribute. Each flavonoid may participate directly or indirectly. Although further investigation is required, *K. parviflora* and its pure compound might offer a future therapeutic basis for preventing CCA invasion.

The effect of crude extract and K.P8.10 to induce cholangiocarcinoma cells death were remarkable. Apoptotic cell death could be demonstrated during treatment with the crude extract and irreversible cell death were induced with high doses of 5,7,4'-trimethoxyflavone. The morphological alteration of the cells after treatments revealed a progressive effective results of the compound in a time-dose dependent fashion.

A possible mechanism of apoptosis induced by KP.8.10 was demonstrated here by the enhancement of mitochondrial enzyme caspase-3 activity which signaling and subsequent promoting apoptotic cell death in a common pathway of cytotoxic drug exposure. Accomplishment in this potential pathway could be via increasing of Fas ligand and/or Fas receptor expression, activation of caspase-8, or activation of cell death receptors (Ashkenazi and Dixit, 1998; Takahashi et al., 1999; Wajant, 2003). This further activated caspase-3 to cleave a variety of target proteins in the cells that caused cell morphological and functional changes. It also promoted permeabilization of mitochondrial outer membrane and released of apoptogenic proteins leading to cell death (Slee et al., 1999; Susin et al., 1999; Li et al., 2001). In this study, the apoptotic response of RMCCA-1 cells to KP 8.10 compound was a slightly increasing in caspase-3 activation at 12h, highest at 18 h, and declined within 24 h. This effect was rather slow and quite short action when compared to the other flavones on the other cell lines. For example, ellipticine (isolated from the leaves of the evergreen tree *Ochrosia elliptica* Labill, Apocynaceae) treated-breast cancer MDA-MD-231 cells triggered activation of caspase-3 after 3 h exposure and sustained to maximum at 24 h (Kuo et al., 2005), but in treated-HepG2 cells it was triggered after 12 h exposure and reached maximum at 48 h (Kuo et al., 2006); Apigenin (a commercial flavonoid) treated-human leukemia HL-60 cells induced a rapid rising in caspase-3 activity and maintained for 12 h (Wang et al., 1999); Baicalein, (a flavone extracted from the dried root of *Scutellaria baicalensis*) induced caspase-3 activation in human hepatoma, Hep3B, cells for 4 days (Chou et al., 2003). These results suggested that the time and effect of activated caspase-3 were depended on the flavones subclass and the type of cell lines testing.

In conclusion, flavonoids component in *K. parviflora* extracts possess anti-cancer effects against both types of cholangiocarcinoma cells. The specific pure compound, 5,7,4'-trimethoxyflavone, exhibited a potent anti-invasion effect, apoptosis induction by activation of intracellular caspase-3 mediator and further induced irreversible cell death.

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