Cytotoxic Effect of Kaempferol on Human Cholangiocarcinoma Cells

ฤทธิ์ฆ่าเซลล์ของแคมป์เฟอรอลในเซลล์มะเร็งท่อน้ำดีมนุษย์

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ABSTRACT

Cholangiocarcinoma (CCA) arises from intra- and extra-hepatic biliary epithelial cells. The incidence of CCA is highest in Northeast of Thailand. At present, there is no effective pharmacological treatment due to CCA has low response and is prone to acquired resistant. Thus, searching for novel and effective natural products used for prevention or treatment of CCA is necessary. In the current study, we examined weather kaempferol (KFR) has cytotoxic effect on human CCA cells. KFR had potent cytotoxicity on four CCA cell lines, KKU-100, KKU-M055, KKU-M156, and KKU-M214, with IC50 values of 25.62±3.24, 41.90±6.24, 25.93±3.18, and 44.56±3.69 μM at 48 h, determined by Sulforhodamine B assay. Furthermore, KFR was associated with induction of apoptosis and necrosis of KKU-100 cells, demonstrated by AO/EB fluorescent dye staining. These data revealed anticancer effect of KFR against CCA cells.

บทคัดย่อ

มะเร็งท่อน้ำดีเกิดขึ้นจากเซลล์เยื่อบุผนังของท่อน้ำดีทั้งภายในและภายนอกตับ มีอุบัติการณ์สูงสุดในภาคตะวันออกเฉียงเหนือของไทย ซึ่งปัจจุบันการรักษามะเร็งท่อน้ำดียังขาดประสิทธิภาพ เนื่องจากมะเร็งมีการตอบสนองต่ำและมักดื้อต่อยาเคมีบำบัด ดังนั้นการค้นหาสารธรรมชาติเพื่อใช้ป้องกันหรือรักษามะเร็งท่อน้ำดีจึงจำเป็น งานวิจัยนี้มุ่งศึกษาความเป็นพิษของแคมป์เฟอรอลต่อเซลล์มะเร็งท่อน้ำดีมนุษย์ การศึกษาด้วย Sulforhodamine B assay พบว่าแคมป์เฟอรอลมีพิษสูงต่อเซลล์มะเร็งท่อน้ำดี 4 ชนิด ได้แก่ KKU-100 KKU-M055 KKU-M156 และ KKU-M214 โดยมีค่า IC50 ที่ 48 ชม. เท่ากับ 25.62±3.24 41.90±6.24 25.93±3.18 และ 44.56±3.69 ไมโครโมลาร์ นอกจากนั้นการศึกษาด้วยเทคนิค AO/EB fluorescent dye staining พบว่าแคมป์เฟอรอลชักนำให้เซลล์ KKU-100 เกิดการตายแบบ apoptosis และ necrosis การศึกษาครั้งนี้ได้แสดงให้เห็นว่าแคมป์เฟอรอลสามารถต้านมะเร็งท่อน้ำดีได้

Key Words: Kaempferol, Cholangiocarcinoma, Cytotoxic effect
คำสำคัญ: แคมป์เฟอรอล มะเร็งท่อน้ำดี พิษต่อเซลล์

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Introduction

Cholangiocarcinoma (CCA) is a malignant cancer arising from neoplastic transformation of cholangiocytes, the epithelial cells lining of intra-hepatic and extra-hepatic bile duct (Lazaridis and Gores, 2005; Patel, 2006). The incidence and mortality rates of intrahepatic CCA are increasing worldwide (Khan et al., 2005; Patel, 2006). Strikingly, CCA is highly prevalent in Northeast of Thailand, where *Opisthorchis viverrini*, a trematode that lives in the biliary system, is endemic (Sriplung et al., 2005). The molecular mechanism of *O. viverrini* infection-associated CCA is reported to be chronic inflammation leading to DNA damage triggered by free radical production (Yongvanit et al., 2012). Flukes also can cause liver and bile duct injury leading to periductal fibrosis in association with CCA development (Sripa et al., 2007). The prognosis is generally poor because most patients present at advanced disease and early diagnosis is difficult (Khan et al., 2005). Additionally, most chemotherapeutic agents are unambitiously effective on CCA and drug resistance is the major obstacle in the treatment. Thus, it is an urgent need to search for novel treatments for CCA.

Over many decades, natural products derived from plants have traditionally been the main source of active medicinal compounds. Research in this field has exponentially increased since numerous biological compounds found in plants have strong evidence of beneficial health effects. Recently, plant-derived polyphenols and phytochemicals have been paying attention for their chemopreventive and chemotherapeutic properties against CCA. For example, a major component from turmeric, curcumin showed antiproliferation and apoptosis-inducing effect in CCA cells. These growth suppressive effects of curcumin were related to inhibition of NF-ΚB and STAT-3 signaling pathways (Prakobwong et al., 2011; Suphim et al., 2010). Quercetin and epigallocatechin-3-gallate (EGCG) are polyphenolic compounds that could suppress STAT-3 signaling and decrease inflammation in CCA cells (Senggunprai et al., 2014). A natural isothiocyanate, phenethyl isothiocyanate (PEITC) induced apoptosis of CCA cells through interruption of glutathione and mitochondrial pathway (Tusskorn et al., 2013). A flavonoid luteolin inhibited proliferation of CCA cells by suppression of the JAK/STAT3 signaling pathway (Aneknan et al., 2014). Data obtained from these studies provide the potential use of plant-derived polyphenols and phytochemicals as promising chemopreventive and chemotherapeutic agents for CCA.

Kaempferol is one of naturally occurring polyphenolic flavonoids that ubiquitously exists in a variety of vegetables and fruits, such as tomatoes, hop, red grapes, grapefruit, strawberries (Hertog et al., 1993; Somerset and Johannot, 2008) and gingko biloba leaves (Kang et al., 2010). Over the years, kaempferol is known for its health promoting effects such as reducing risk of arteriosclerosis, alleviating the cardiovascular disorder, and also serving as antioxidant and anti-inflammatory. It is used as herbal medicine and also available as drugs for medical purpose. To date, kaempferol is not known for any serious toxicity or being harmful to the human body.

Kaempferol exhibits an antiproliferative activity in several *in vitro* studies and can effectively induce apoptosis in various human cancer cell lines, such as non-small cell lung cancer (Leung et al., 2007), leukemia (Marfe et al., 2009), prostate cancer

(De Leo et al., 2006), and colon cancer (Mutoh et al., 2000). Yoshida et al. (2008) reported kaempferol could strengthen the toxic effect of TRAIL-base (tumor necrosis factor-related apoptosis-inducing ligand) anti-cancer agent in colon cancer cells. However, the potential anticancer effect of kaempferol in CCA has not been validated.

Objective of the study

The current study aims to evaluate the cytotoxic effect of kaempferol on human CCA cells.

Materials and Method

Materials

Kaempferol (KFR), sulforhodamine B (SRB), acridine orange (AO) and ethidium bromide (EB) were obtained from Sigma Chemical (St. Louis, MO, USA). Reagents for cell culture were from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell line and cell culture

Four cell lines used in this study were KKU-100, KKU-M055, KKU-M156, and KKU-M214 cells, which kindly obtained from Professor Dr. Banchob Sripa. All CCA cells were routinely cultured in Ham's F12 media, supplemented with sodium bicarbonate, 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), at pH 7.3, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS), and maintained under an humidified atmosphere containing 5% CO_2 at 37°C. Cells were subcultured every 3 days using 0.25% trypsin-EDTA.

Cytotoxicity assay

Sulforhodamine B (SRB) was used to measure the effect of kaempferol on the proliferation of CCA cell lines. In brief, CCA cells were seeded onto 96-well cultured plates for overnight, then media was renewed with fresh media containing KFR at different concentrations for 48 h. Assay is performed at the endpoint of treatment to determine amount of protein remaining in each well. Cells were fixed by the addition of 10% trichloroacetic acid (TCA) and placed in 4°C for at least 1 h. After incubation, TCA was removed and all wells were carefully washed with deionized (DI) water. Then the plate was allowed to air dry and 0.4% SRB in 1% acetic acid was added into each well for 30 min. Cells were rinsed with 1% acetic acid and air dried at room temperature. Finally, the adhered cells were solubilized with 10 mM Tris base (pH 10.5) solution and the absorbance reading was made using a microplate reader with filter wavelength of 540 nm. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. The IC50 concentration (50% inhibition of cell growth values) was calculated from the dose-response curves.

Acridine orange / ethidium bromide (AO/EB) staining

KKU-100 cells were cultured in 96-well plates and allowed to attach overnight. On the next day, the medium were removed and the cells were treated with various concentrations of KFR for 48 h. Then, cell were washed once with PBS and stained with AO/EB. The cells were examined using a Nikon Eclipse TS100 inverted microscope with excitation and long pass emission filters of 480 and 530 nm, respectively. The fluorescent images were taken at

predetermined areas with Nikon Coopix digital camera. The numbers of viable, apoptotic and necrotic cells were enumerated.

Statistical analysis

All results are presented as the mean±SEM. Statistical comparison between control and treatment group was performed with Student's t-test. Results are considered to be statically significant at $p<0.05$. The IC50 values calculations and statistical analyses were performed using the Prism 5 program (GraphPad Software, San Diego, CA, USA).

Results and Discussion

KFR decreased CCA cell viability

Several previous studies have reported KFR has promising anti-proliferation activities and been known to potently induce cytotoxicity in tumor cells. KRF treatment also induces apoptosis in several human cancer cell lines, including prostate cancer (De Leo et al., 2006), lung cancer (Leung et al., 2007), esophageal cancer (Zhang et al., 2008), and colon cancer cells (Yoshida et al., 2008). In this study, the cytotoxic effect of KFR on CCA cells were observed in four human CCA cell lines established from primary tumors of liver fluke-associated CCA patients including KKU-100 and KKU-M055 (poorly differentiated CCA), KKU-M156 (moderately differentiated CCA), and KKU-M214 (well-differentiated CCA), using SRB assay.

The results showed that KFR has potent cytotoxic effect on all CCA cells in a dose-dependent manner. The IC50 concentrations calculated from the dose-response curves of 48 h KFR treatment were 25.62±3.24, 41.90±6.24, 25.93±3.18, and 44.56±3.69 μM for KKU-100, KKU-M055, KKU-M156, and KKU-M214, as shown in Figure 1 A-D. The results reveal KFR induced antiproliferation in all CCA cells with any origin and/or different histological type of primary tumors. Thus, KFR may be useful as a growth-suppressive agent for CCA, a cancer having high tumor heterogeneity. Among these four CCA cells, KKU-100 has the lowest IC50 value, therefore, it was selected as a representative CCA cell line for investigating the cytotoxic mechanism of KFR.

KFR induced apoptosis and necrosis of KKU-100 cells

To examine the cell killing effect of KFR, KKU-100 cells were stained with the AO/EB fluorescent dye. The results showed that the KKU-100 cells were undergone apoptosis and necrosis after incubation with KFR for 48 h (Figure 2). The relatively large proportion of KKU-100 cells was apoptotic after the KRF treatment in a concentration-dependent manner. In addition, at high dose (80 μM), KFR could also induced necrotic cell death. Thus, the growth-suppressive activity of KFR in CCA cells is mediated partly by its inducing apoptosis action. These data prompt us for the future studies investigating the molecular mechanisms underlying apoptosis-inducing activity of KFR and also the role of KFR on cell cycle regulation in CCA.

Conclusion

In conclusion, our findings demonstrate that KFR play a role on cell growth and cell death of CCA cells. The results show that KFR exhibits potent cytotoxic activity against CCA cells. It effectively induces apoptosis in KKU-100 cells. In order to prove that KFR is a potential agent for the treatment of CCA, the further studies investigating the molecular mechanisms underlying apoptosis-inducing activity of KFR are warranted.

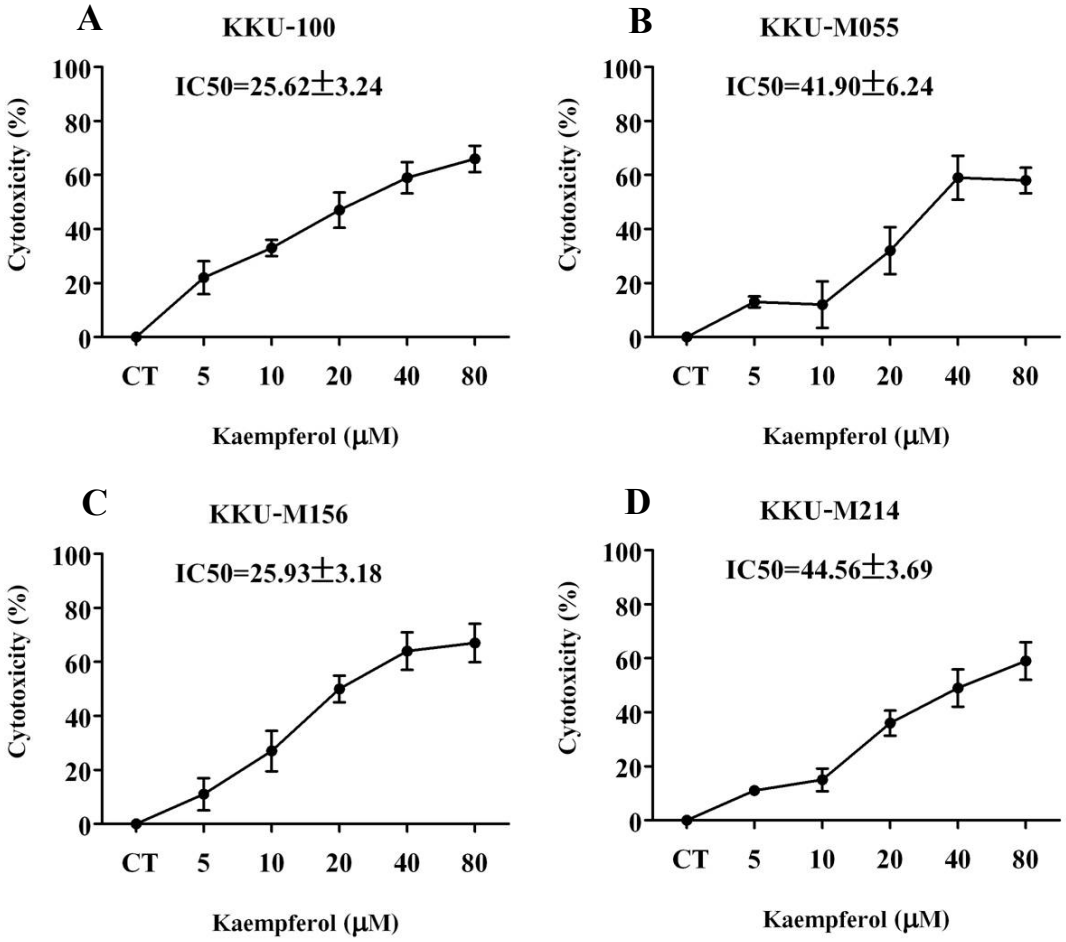

Figure 1 Effect of KFR on the cell viability of CCA Cells. (A) KKU-100 (B) KKU-M055 (C) KKU-M214 and (D) KKU-M156 cells were treated with various concentration of KFR (0, 5, 10, 20, 40 and 80 µM) for 48 h. After treatment, the cell viability was determined by the SRB assay. The results are presented as percentage of control. The data are the mean±SEM averaged from three independent experiments (quadruplet in each experiment) except KKU-100 averaged from five independent experiments.

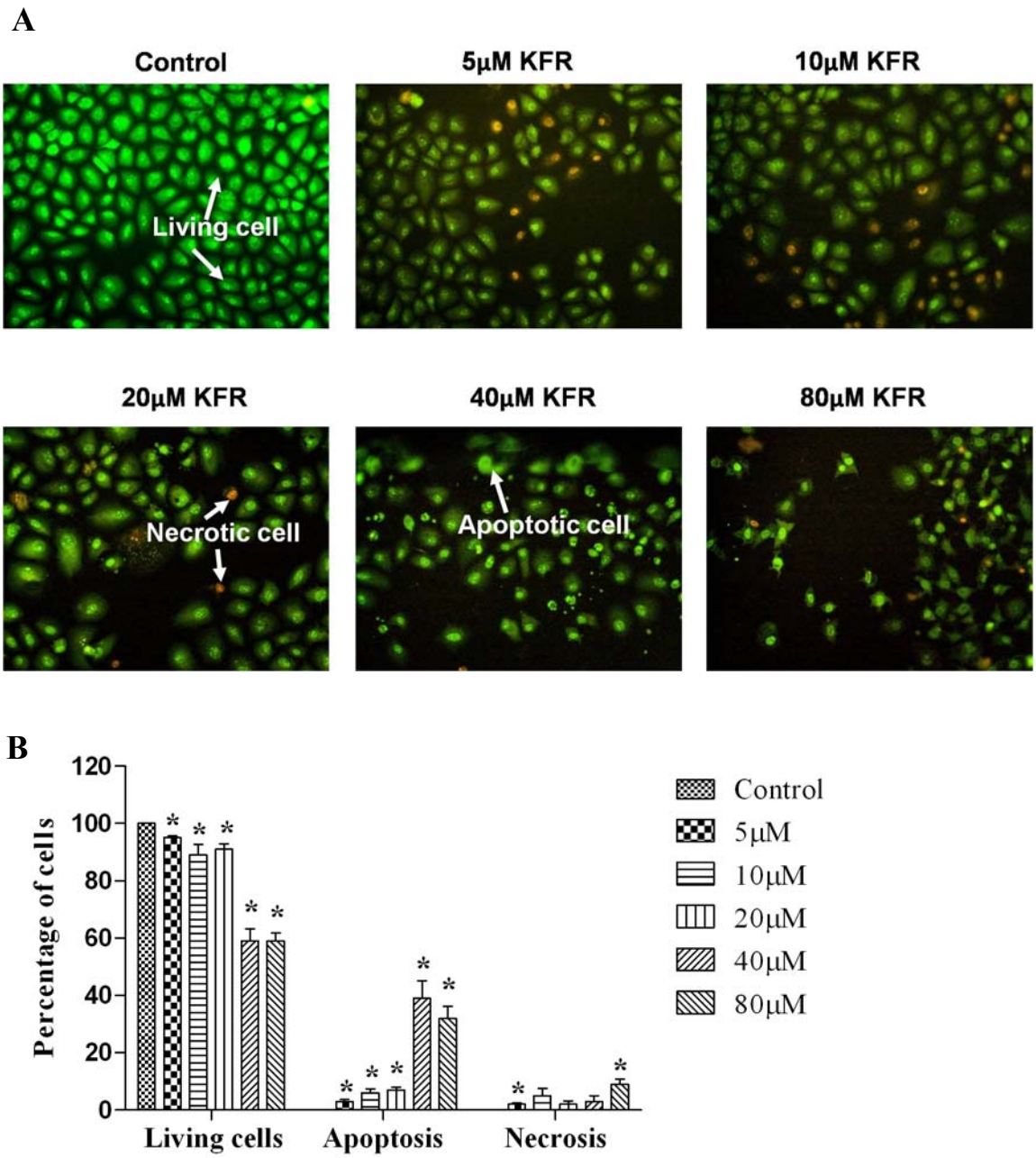

Figure 2 Effect of KFR on CCA cell death (both apoptosis and necrosis). KKU-100 Cells were treated with KFR at various concentrations (0, 5, 10, 20, 40 and 80 μM) for 48 h. (A) cells were stained with AO (acridine orange) and EB (ethidium bromide) and observed by fluorescent microscopy (at 20X magnifications). Live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus (Ribble et al., 2005). (B) The numbers of living, apoptotic, and necrotic cells were counted. The data are expressed as percent of apoptotic or necrotic cells over total number of cells in the same area. The data are the mean±SEM averaged from three independent experiments (triplicate in each experiment). *$p<0.05$ compared with control.

Acknowledgements

This work was supported in part by Faculty of Medicine Research Grant, Khon Kaen University, Thailand. Wutthipong Duangarsong was supported by the Liver Fluke and Cholangiocarcinoma Research Center Khon Kaen University.

References

Aneknan P, Kukongviriyapan V, Prawan A, Kongpetch S, Sripa B, Senggunprai L. Luteolin arrests cell cycling, induces apoptosis and inhibits the JAK/STAT3 pathway in human cholangiocarcinoma cells. Asian Pac J Cancer Prev 2014; 15: 5071-6.

De Leo M, Braca A, Sanogo R, Cardile V, DeTommasi N, Russo A. Antiproliferative activity of Pteleopsis suberosa leaf extract and its flavonoid components in human prostate carcinoma cells. Planta Med 2006; 72: 604-10.

Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 1993; 20: 21-9.

Kang JW, Kim JH, Song K, Kim SH, Yoon JH, Kim KS. Kaempferol and quercetin, components of Ginkgo biloba extract (EGb 761), induce caspase-3-dependent apoptosis in oral cavity cancer cells. Phytother Res 2010; 24 Suppl 1: S77-82.

Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. Lancet 2005; 366: 1303-14.

Lazaridis KN, Gores GJ. Cholangiocarcinoma. Gastroenterology 2005; 128: 1655-67.

Leung HW, Lin CJ, Hour MJ, Yang WH, Wang MY, Lee HZ. Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. Food Chem Toxicol 2007; 45: 2005-13.

Marfe G, Tafani M, Indelicato M, Sinibaldi-Salimei P, Reali V, Pucci B, et al. Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. J Cell Biochem 2009; 106: 643-50.

Mutoh M, Takahashi M, Fukuda K, Matsushima-Hibiya Y, Mutoh H, Sugimura T, et al. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. Carcinogenesis 2000; 21: 959-63.

Patel T. Cholangiocarcinoma. Nat Clin Pract Gastroenterol Hepatol 2006; 3: 33-42.

Plochmann K, Korte G, Koutsilieri E, Richling E, Riederer P, Rethwilm A, et al. Structure-activity relationships of flavonoid-induced cytotoxicity on human leukemia cells. Arch Biochem Biophys 2007; 460: 1-9.

Prakobwong S, Gupta SC, Kim JH, Sung B, Pinlaor P, Hiraku Y, et al. Curcumin suppresses proliferation and induces apoptosis in human biliary cancer cells through modulation of multiple cell signaling pathways. Carcinogenesis 2011; 32: 1372-80.

Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 2005; 5: 12.

Senggunprai L, Kukongviriyapan V, Prawan A, Kukongviriyapan U. Quercetin and EGCG exhibit chemopreventive effects in cholangiocarcinoma cells via suppression of JAK/STAT signaling pathway. Phytother Res 2014; 28: 841-8.

Somerset SM, Johannot L. Dietary flavonoid sources in Australian adults. Nutr Cancer 2008; 60: 442-9.

Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, et al. Liver fluke induces cholangiocarcinoma. PLoS Med 2007; 4: e201.

Sriplung H, Sontipong S, Martin N, Wiangnon S, Vootiprux V, Cheirsilpa A, et al. Cancer incidence in Thailand, 1995-1997. Asian Pac J Cancer Prev 2005; 6: 276-81.

Suphim B, Prawan A, Kukongviriyapan U, Kongpetch S, Buranrat B, Kukongviriyapan V. Redox modulation and human bile duct cancer inhibition by curcumin. Food Chem Toxicol 2010; 48: 2265-72.

Tusskorn O, Prawan A, Senggunprai L, Kukongviriyapan U, Kukongviriyapan V. Phenethyl isothiocyanate induces apoptosis of cholangiocarcinoma cells through interruption of glutathione and mitochondrial pathway. Naunyn Schmiedebergs Arch Pharmacol 2013; 386: 1009-16.

Yongvanit P, Pinlaor S, Bartsch H. Oxidative and nitrative DNA damage: key events in opisthorchiasis-induced carcinogenesis. Parasitol Int 2012; 61: 130-5.

Yoshida T, Konishi M, Horinaka M, Yasuda T, Goda AE, Taniguchi H, et al. Kaempferol sensitizes colon cancer cells to TRAIL-induced apoptosis. Biochem Biophys Res Commun 2008; 375: 129-33.

Zhang Q, Zhao XH, Wang ZJ. Flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma cell line (OE33) by causing G2/M arrest and inducing apoptosis. Food Chem Toxicol 2008; 46: 2042-53.