Research Articles

Effects of the three flavonoids; kaempferol, quercetin, and myricetin on Baby hamster kidney (BHK-21) cells and Human hepatocellular carcinoma cell (HepG2) cells proliferations and total Erk1/2 protein expression

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Abstract

Kaempferol, quercetin, and myricetin were regarded as the potential therapeutic flavonoids in several types of cancers. And the cancer cell proliferations that were directly induced by MAPK/Erk signaling pathway had been the important target of cancer treatment. Thus, this study was investigated the anti-proliferative effects of these three flavonoids together with total Erk1/2 protein expressions on Human hepatocellular carcinoma cell (HepG2) and Baby hamster kidney cells (BHK-2). The data showed the cytotoxic effects of 100 μM kaempferol, 5 μM myricetin and 50 μM quercetin on BHK-21 cells at 24h. After same incubation time, kaempferol (5 μM), myricetin (1 μM) and quercetin (1 μM) could significantly inhibit HepG2 cell proliferations. By western blot analysis, kaempferol and myricetin could not affect Total Erk1/2 proteins expression. But quercetin obviously suppressed total Erk1/2 protein expression in HepG2 cells at 24h. In conclusion, all three flavonoids had significant inhibitory effects on Hepatocellular carcinoma cells without cytotoxic effects on normal fibroblast. And inhibitory effects of quercetin in cancer cell proliferation related to total Erk1/2 protein reduction.

Keywords: kaempferol, quercetin, myricetin, HepG2, Erk

ประสิทธิภาพของสารกลุ่มฟลาโวนอยด์สามชนิด ได้แก่ kaempferol quercetin และ myricetin ที่มีต่อการเพิ่มจำนวนของเซลล์ (proliferation) และการแสดงออกของโปรตีน total Erk1/2 ในเซลล์ Baby hamster kidney (BHK-21) และ Human hepatocellular carcinoma cell (HepG2)

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บทคัดย่อ

kaempferol quercetin และ myricetin เป็นสารกลุ่ม flavonoids ที่พบว่ามีประสิทธิภาพในการรักษาโรคมะเร็งหลายชนิด และการเพิ่มจำนวนของเซลล์มะเร็ง (proliferation) ซึ่งถูกกระตุ้นโดยตรงผ่าน MAPK/Erk signaling pathway เป็นเป้าหมายสำคัญของการรักษา ดังนั้น การทดลองครั้งนี้ จึงต้องการทดสอบประสิทธิภาพของสารกลุ่มฟลาโวนอยด์สามชนิดดังกล่าว ที่มีต่อการเพิ่มจำนวนของเซลล์ พร้อมกับผลที่มีต่อการแสดงออกของโปรตีนชนิด total Erk1/2 ของเซลล์มะเร็งตับชนิด Human hepatocellular carcinoma cell (HepG2) และ Baby hamster kidney cells (BHK-21) จากการทดลองพบว่า 100 μM kaempferol 5 μM myricetin และ 50 μM quercetin มีความเป็นพิษต่อเซลล์ BHK-21 ที่เวลา 24 ชั่วโมง ส่วนผลการทดลองใน HepG2 พบว่า kaempferol (5 μM) myricetin (1 μM) และ quercetin (1 μM) สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งได้อย่างมีนัยสำคัญทางสถิติ และจากการทดสอบด้วยวิธี western blot analysis นั้น kaempferol และ myricetin ไม่มีผลต่อการแสดงออกของโปรตีนชนิด total Erk1/2 ขณะที่ quercetin สามารถยับยั้งการแสดงออกของชนิด total Erk1/2 ในเซลล์มะเร็งชนิด HepG2 ได้อย่างชัดเจนที่ 24 ชั่วโมง สรุปผลการทดลองดังกล่าวพบว่า สารกลุ่ม flavonoids ทั้ง 3 ชนิด สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งตับได้อย่างมีนัยสำคัญทางสถิติด้วยความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ปกติชนิด fibroblast และผลการยับยั้งของ quercetin เกี่ยวข้องกับการลดลงของโปรตีนชนิด total Erk1/2

คำสำคัญ : kaempferol quercetin myricetin HepG2 Erk

Introduction

The three flavonoids: kaempferol, quercetin, and myricetin, which could be extracted from many herbal plants, were the potential polyphenolic compounds in human cancer treatment. Kaempferol could inhibit cell proliferations and induced apoptosis of several types of cancers such as colon cancer (Lee et al., 2014), cervical cancer (Tu et al., 2016), breast cancer cells (Kim et al., 2016) and cholangiocarcinoma (Quin et al., 2016) via the mitochondrial membrane disruption, the changing of intracellular ion (Tu et al., 2016), cellular antioxidant activities (Liao et al., 2016) and mitogen-activated protein kinase (MAPK) signaling (Lee et al., 2016). Quercetin could induce cancer cell apoptosis by activation of caspase cascade (Kim et al., 2013; Jakubowicz-Gil et al., 2013) and inhibit cancer cell proliferation directly through Raf and MAPK signaling (Khan et al., 2016). And myricetin, the constituents from many plants such as teas and fruits, also exhibit anticancer activities by various signaling pathways (Devi et al., 2015). Myricetin could decrease cancer cell proliferation by restriction some enzyme kinase activities in MAPK pathway (Kang et al., 2011; Semwal et al., 2016).

Thus, the aims of the study were to investigate the effects of three flavonoids: kaempferol, quercetin, and myricetin, on Human Baby hamster kidney (BHK-21) cells and Human hepatocellular carcinoma cell (HepG2) cell proliferations. And the HepG2 cells were studied the mechanism of three flavonoids on total Erk1/2 protein expressions. The liver cancer was the highest rate cancer in Asia and Africa. The incidences were about 88/100,000 in male and 35/100,000 in female population (McGlynn and London, 2011). In Thailand, the hepatocellular carcinoma (HCC) has very high incidence, especially in North-Eastern region. Liver and bile duct cancer was the major common cancer in male (Srivatanakul, 2001). The development and progression of this cancer type closely related to the activation with multiple steps of MAPK signaling pathway (Lamarca et al., 2016; Hu et al., 2016; Zhang and Zhou, 2016). The expression of extracellular signal-regulated kinases (ERKs), following by the activation of dual phosphorylation on tyrosine and threonine residues play one of the key roles in cancer cell proliferation (Samatar and Poulikakos, 2014) and should be the one of interesting target for liver cancer therapy.

Materials and Methods

Cell culture and treatment

Baby hamster kidney cell (BHK-21), the adherent fibroblast cell line and Human hepatocellular carcinoma cell line (HepG2) were culture in T-75 flasks with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 unit/ml penicillin G sodium, and 100 μg/ml streptomycin at 37°C until confluent. Then cells were sub-cultured into 96-well and 6-well culture plates with or without fetal bovine serum DMEM before reagent treatment. BHK-21 was used to determine the cytotoxicities of three flavonoids; kaempferol, quercetin, and myricetin (Sigma, USA) by calorimetric MTT and SRB assay. Various concentrations of kaempferol, quercetin, and myricetin (1, 5, 10, 50, 100, 250, 500 and 1000 μM) in 0.001% Dimethyl sulfoxide (DMSO) were used to evaluate the cytotoxicity of BHK-21 and inhibitory effects on HepG2 proliferation at 24h.

MTT calorimetric assay

The MTT assay was modified from Mosmann's method for using in high throughput screening of cell proliferation (Mosmann, 1983; Riss et al, 2013). After treatment by various concentrations of three flavonoid reagents, BHK-21 and HepG2 were incubated with 2 mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; MTT (Sigma) 3h at 37°C. Then the intracellular MTT-formazan was solubilized by dimethyl sulfoxide; DMSO (Sigma) 5 min at room temperature. The absorbance of each well in 96-well culture plates was determined at 570 nm wavelength by using Synergy™ HT Multi-Detection Microplate Reader (BioTek, USA).

SRB calorimetric assay

The sulforhodamine B (SRB) assay were efficient and highly cost-effective method for screening the drug-induced cytotoxicity and cell proliferation of adherent cell treated within a few days (Voigt, 2005; Vichai and Kirtikara, 2006). After 24h incubation, the cells in 96-well culture plates were fixed with 50 µl ice-cold 50% trichloroacetic acid; TCA (Sigma) in sterile distilled water and incubated at 4°C for 1h. After 5 times washing by distilled water, the cells were dried in room temperature and then were stained with 50 µl of 0.4% w/v sulphorhodamine B; SRB (Sigma) dissolved in 1% acetic acid solution for 30 min. The SRB solution was poured off and washed with 1% acetic acid 3-5 times to remove unbound dye and left the plates to air-dry. 10 mM un-buffered Tris-based solution (pH 10.5) (200 µl/well) was used to solubilize the SRB dye and placed on plate shaker for 5 min in room temperature. The absorbances of solution in 96-well plates were measured at 540 nm wavelength by Synergy™ HT Multi-Detection Microplate Reader.

Immunoblotting

After reagent treatment for 24h, the cells were lysed by cold phosphate buffer saline (PBS) containing 138 mM NaCl, 2.7 mM KCl, 8 mM $Na2HPO_4$, 1.46 mM $KH2PO_4$ before being lysed with cold extract buffer (50 mM Tris base; 10 mM EDTA; 1% (V/V) Triton X-100; 0.57 mM PMSF; 1.5 µM pepstatin A; 2 µM leupeptin). Protein concentrations were determined by Bio-Rad protein assay kit (Life Science). The equal amounts of protein of each sample were loaded into 12% separating SDS/PAGE gel and electrophorised for 2 h at 120 constant voltages. The separated protein bands were transferred onto nitrocellulose membrane. Protein band on membranes were blocked by 5% low fat milk in Tris buffer saline (20 mm Tris (pH 7.6) and 250 mm NaCl) and then incubated with primary monoclonal antibody 1.5 h (Anti-total Erk [dilution 1:500], Anti-beta-actin [dilution 1:5,000]) (Signaling) following by incubation with anti-rabbit IgG- conjugated horse radish peroxidase (dilution 1:2,000) (Signaling) for 1h. Immunoblots were developed with 3,3'-Diaminobenzidine; DAB Substrate Kit (Pierce). Beta-actin was used as internal control because of its high conservation as an endogenous housekeeping gene.

Statistical analysis

The percentages of cell proliferations were statistically presented as mean ± SD. All statistical analyses were performed by using GraphPad Prism 5 and SPSS version 19. The mean values of data among treatment group were compared by using one-way analysis of variance (ANOVA) and the t-test. All results were considered to be statistically significant at $p < 0.05$.

Results

Cytotoxicity of three flavonoids on fibroblast cells

BHK-21 was used to evaluate the cytotoxicity of kaempferol, quercetin, and myricetin by MTT and SRB assay at 24h (Figure 1). The 5-fluorouracil (20 μM) and PD-98059 (20 μM) were used as the positive control. All results were shown as percentage of cell proliferation compare with non-treatment control. The study revealed that 100 μM kaempferol had significant cytotoxic effects on BHK-21 cell compared with non-treatment group by both calorimetric assay (MTT: 60.90 ± 4.41 and SRB: 31.79 ± 10.19 %). By MTT assay, 5 μM myricetin (71.76 ± 5.11 %) and 100 μM quercetin (68.59 ± 11.95 %) also had significant cytotoxic effects on fibroblast cell proliferations. By SRB assay, the toxic concentration of myricetin and quercetin were 50 μM (21.44 ± 1.05% and 28.49 ± 10.52 %, accordingly).

Anti-proliferative effects on Human hepatocellular carcinoma cell line (HepG2)

The inhibitory effects of three flavonoids were shown in Figure 2. 0.001% Dimethyl sulfoxide (DMSO) did not have any cytotoxicity on HepG2 at 24h. By MTT assay, all three flavonoids; kaempferol (5 μM), and myricetin (1 μM) could significantly inhibit HepG2 cell proliferations (78.53 ± 4.28 % and 52.76 ± 1.88 %, accordingly). By SRB assay, the inhibitory concentrations were 100 μM in kaempferol (50.67 ± 2.56 %) and 10 μM in myricetin (7.57 ± 3.71 %). And quercetin significantly affected HepG2 cells at 1 μM by MTT (82.15 ± 2.65%) and 10 μM by SRB methods (80.11 ± 2.83 %).

Total Erk1/2 protein expression in BHK-21 and HepG2 cells

After treatment with various reagents at 24h, BHK-21 cells and HepG2 cells were determine the Total Erk1/2 protein expression by Western Blotting Technique (Figure 3-4). Kaempferol and myricetin could inhibit BHK-21 cell proliferations without any effects on total Erk1/2 protein expression. And quercetin obviously suppressed total Erk1/2 protein expression at 5 μM (Figure 3b). The various concentrations of kaempferol and myricetin could not effect on total Erk1/2 proteins expression in HepG2 cells. But quercetin (1, 5, 10 and 50 μM) distinctly inhibited expression of total Erk1/2 protein (Figure 4b). The phosphorylated Erk protein expressions at 24h were also evaluated, but the protein band did not express at this incubation time (data not shown).

Discussion

The three flavonoids: kaempferol (5 μM), myricetin (1 μM) and quercetin (1 μM) could significantly inhibit HepG2 cell proliferation without any effects on this normal fibroblast. Thus, three flavonoids tend to be the valuable therapeutic agents for human hepatocellular carcinoma treatment. And mechanisms of these flavonoids were investigated because the Erk1 and Erk2 ubiquitously expressed in normal cells and were usually induced into over-active state. Then the overexpression of Erk1/2 further activated the Ras/Raf/MEK/ERK cascade during cancer proliferations (Lamarca et al., 2016). From this study, 5 μM Kaempferol and 1 μM myricetin significantly inhibit cancer cell proliferation but these flavonoids could not inhibit total Erk1/2 protein expression at 24h. As previous study, kaempferol could slightly decrease the total levels of Erk1/2 protein expression (Lee et al.,

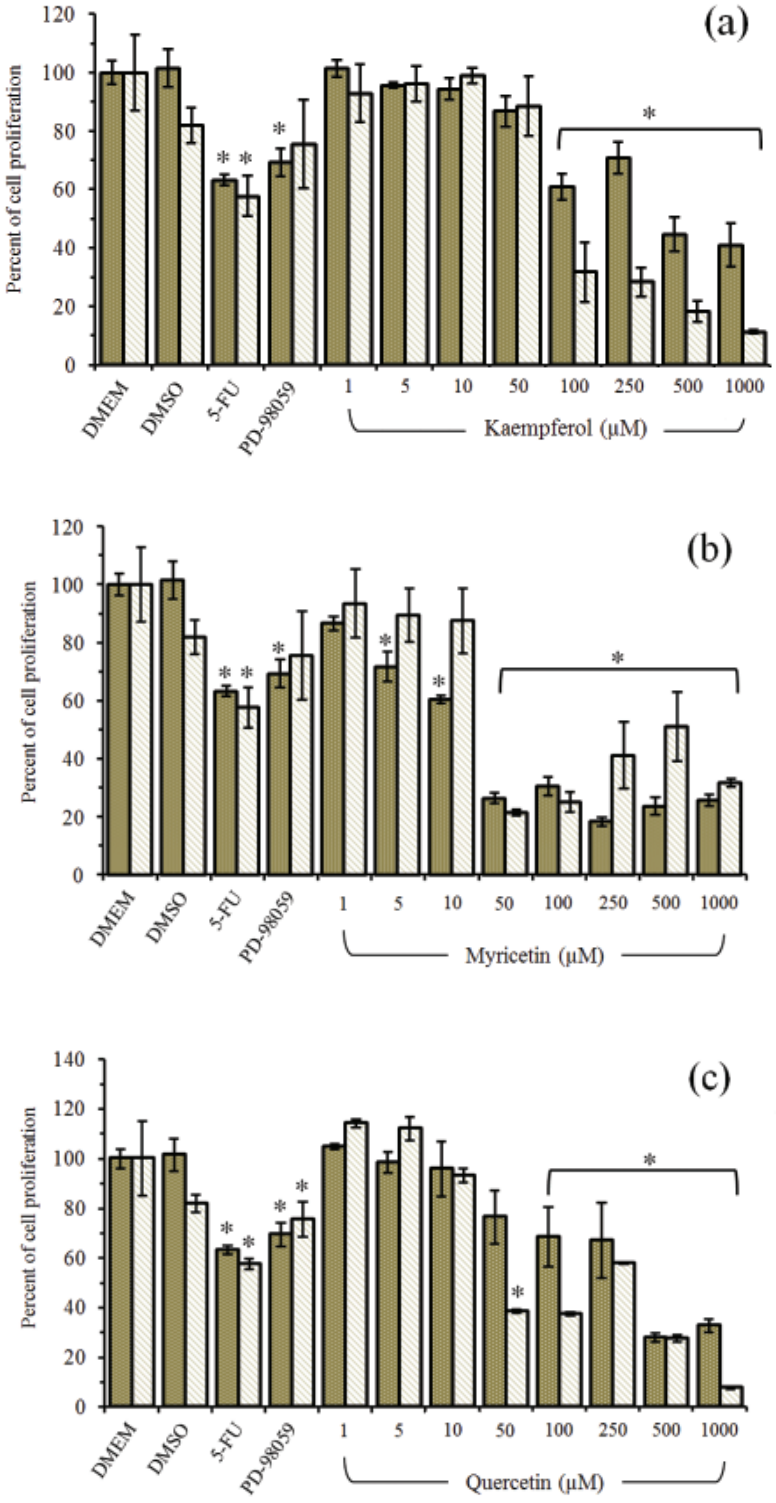

Figure 1 Cytotoxic effects of kaempferol (a), myricetin (b) and quercetin (c) on Baby hamster kidney cells (BHK-21) by MTT (▭) and SRB (▨) assay. Percentage of cell proliferation compared with non-treatment group (control) were represented as mean ± SD (n = 6). *P < 0.001 when compared with non-treatment control.

Note: Figure 1 and 2, DMEM: free serum Dulbecco's modified Eagle's medium, DMSO: 0.001% dimethyl sulfoxide, 5-FU: 20 μM 5-fluorouracil, PD-98059: 20 μM.

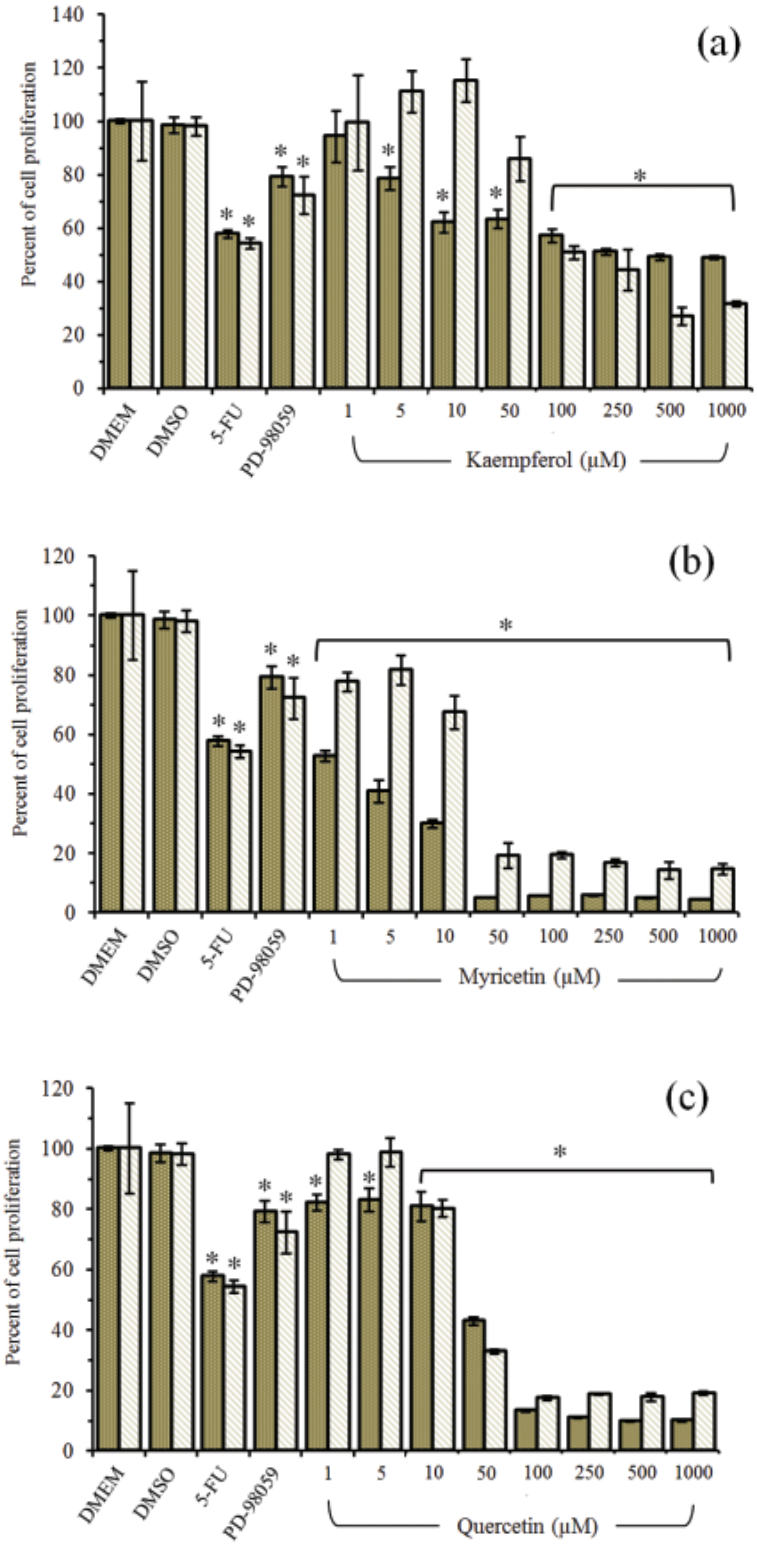

Figure 2 Antiproliferative effects of kaempferol (a), myricetin (b) and quercetin (c) on Human hepatocellular carcinoma cells (HepG2) by MTT (▒) and SRB (▨) assay. Percentage of cell proliferation compared with non-treatment group (control) were represented as mean ± SD (n = 6). $^{*}P < 0.001$ when compared with non-treatment control.

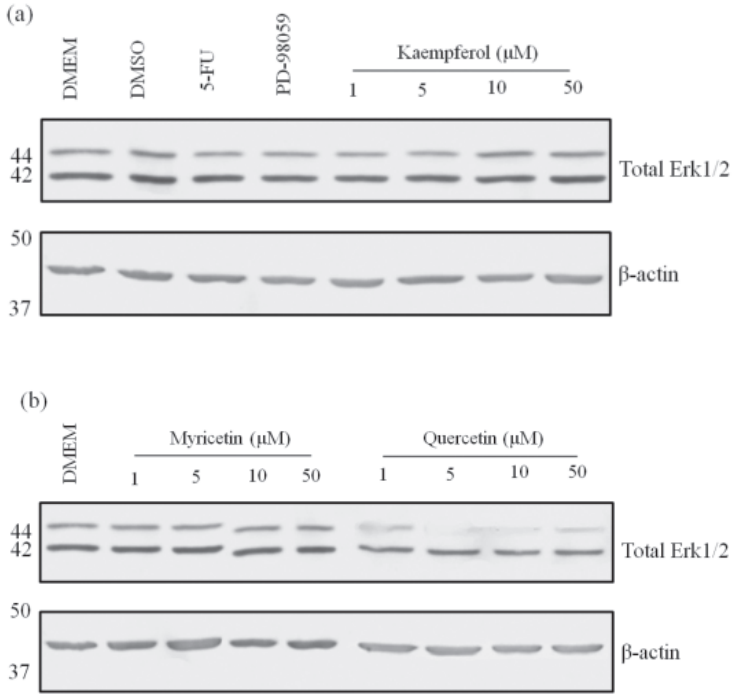

Figure 3 Effects of kaempferol (a), myricetin and quercetin (b) on total of Erk protein expression in BHK-21 cells.

Note: Figure 3 and 4, DMEM: free serum Dulbecco's modified Eagle's medium, DMSO: 0.001% dimethyl sulfoxide, 5-FU: 20 μM 5-fluorouracil, PD-98059; 20 μM PD-98059.

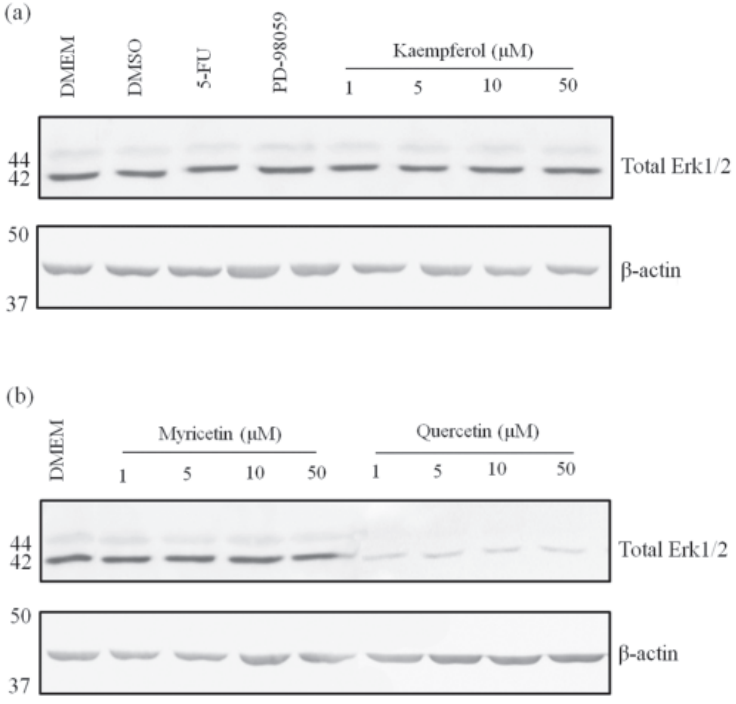

Figure 4 Effects of kaempferol (a), myricetin and quercetin (b) on total of Erk protein expression in HepG2 cell.

2014), but significantly reduce the expression of COX-2, phosphorylated Akt and phosphorylated Erk (Song et al., 2015). Kaempferol also induced HepG2 cell death via activation of the endoplasmic reticulum (ER) stress- the C/EBP homologous protein (CHOP) pathway (Guo et al., 2016; Kim et al., 2016; Tu et al., 2016). Ochiai et al showed that 100 μM kaempferol did not effect on both HepG2 viability and total Erk protein expression (Ochiai et al., 2016). Kaempferol may have many alternative pathways to inhibit the progression of cancer cells.

Myricetin are recently known as the natural inhibitor of MEK1 kinase activity and could induce cancer cell apoptosis through the reduction of pErk1/2 protein (Lee et al., 2007; Shih et al., 2009; Jiao and Zhang, 2016). The data were shown that the inhibitory effects of myricetin did not relate to total Erk1/2 protein expression. However, myricetin could also inhibit HepG2 cells via multiple pathway such as G2/M phase arrest (Zhang et al., 2011), mitochondria apoptotic pathway, Akt/p70s6k1/Bad signaling (Zhang et al., 2013) and phosphatidylinositol 3-kinase (PI3K) signaling pathway (Phillips et al., 2011).

Quercetin exhibited anti-carcinogenic properties in several type of cancer (Anand et al., 2016). From the study results, the effective concentrations of quercetin on HepG2 cell proliferation were very low when compare with the previous in vitro study (3-50 μM) (Lamson, and Brignall, 2000). And quercetin may have the anti-proliferative effects on human liver cancer via total Erk 1/2 protein inhibition. Because of the reduction of Erk protein in hepatocyte could slow down the cancer cell proliferation (Lefloch et al., 2009; Buscà et al, 2016). In MAPK pathway, this flavonoid could also inhibit p- Erk1/2 expression in liver cancer (Ren et al., 2017). Furthermore, the inhibitory effects of quercetin on cancer cell proliferation alternatively related to many tumor-induced factors such as tumor necrosis factor alpha (TNF-α), nitric oxide (NO), interferon (IFN)-γ production, COX-2 and NF-κB protein (Geng et al., 2013; Leyva-López et al., 2016) etc. The results can be seen that the total Erk1/2 or even phosphorylated Erk inhibition were not the only one major aspect on cancer cell progression. Thus, the chemotherapy that has multi-signaling targets must increase the potential of liver cancer treatment.

In conclusion, kaempferol, myricetin, quercetin could significant inhibit Human hepatocellular carcinoma cell (HepG2) cell proliferation without the cytotoxicity on normal cells at 24h. Kaempferol and myricetin inhibitory effects did not related to total Erk1/2 protein. And quercetin had anti-cancer proliferation effects via reduction of total Erk1/2 protein expression at 24h.

Acknowledgement

This research project was supported by grant from Mahidol University, Thailand.

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