

**APPENDIX**

**IN VITRO AND IN VIVO ASSESSMENT OF CANCER CELL  
RESPONSE TO FLAVONOIDS BY USING MOLECULAR  
IMAGING TECHNIQUES**

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## **In vitro and In vivo assessment of cancer cell response to flavonoids by using molecular imaging techniques**

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### **Summary**

This study clearly showed for the first time that the Siamois® polyphenols exhibited protective actions by promoting the normal myocyte but mediated cytotoxicity against cancer cells. Siamois® polyphenols significantly inhibited cancer cell growth of both drug-sensitive and their corresponding multidrug resistant cells; IC<sub>50</sub> value was 5.0 ± 0.2 mg/mL for MDA-MB 435 (estrogen-receptor negative), 3.28 ± 0.38 mg/mL for K562 and K562/adr and 3.2 ± 0.2 mg/mL for GLC4 and GLC4/adr cells. The action of Siamois® polyphenols on cancer cells can be described as "assisted suicide" or induction of apoptosis. Two-fold of IC<sub>50</sub> of these compounds were clearly found to induce apoptosis in breast tumor tissue which can be determined by 99mTc-Annexin V scintigraphy and histological staining. This study clearly shows that the apoptosis-inducing effects of Siamois® polyphenols on the MDA-MB-435 cell in vitro were effectively extrapolated to the in vivo situation.

**Keywords:** Anticancer activity, flavonoids, multidrug resistance (MDR), xenograft mice, molecular imaging

## Introduction

We have previously reported that Siamois® red wine was designed as an enrich source of polyphenols and vinified under the conditions of tropical climate by the Laboratory of Physical Chemistry, Molecular and cellular Biology (PCMBC), Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand [1]. The health benefits and anticancer activity of the Siamois® red wine are the research subjects of our group [2, 3].

Recent development in the area of the antioxidant and anticancer activities of the natural flavonoids such as quercetin, apigenin, genistein, myricetin, etc. has been demonstrated both *in vitro* and *in vivo* levels. Flavonoids efficiently scavenge and chelate the species which can undergo cellular oxidative stress or may serve as an intracellular electron donor for a trans-plasma membrane oxidoreductase, suggesting that the flavonoids exert beneficial effects under oxidative stress conditions [4, 5]. By using a normal cell such as H9c2 cardiomyoblast cell, quercetin could protect hydrogen peroxide from inducing H9c2 cardiomyoblast cells from undergoing apoptosis [4].

A huge of reports showed that flavonoids efficiently exhibited apoptosis-inducing activity, particularly in cancer cells and mitochondria were proposed to be the potential intracellular target [2, 3]. The ability of flavonoids to induce cellular apoptosis may depend on the extent and characteristics of the mitochondrial injury. Various forms of cellular stress result in mitochondrial alteration such as mitochondrial membrane depolarization and release of cytochrome c

from the intermembrane space to the cytosol [6, 7]. In the presence of ATP, the cytosolic cytochrome c interacts directly with apoptotic protease activating factor 1 and procaspase 9 to form an apoptosome, a macromolecular complex that cleaves procaspase 9 to active caspase 9, which, in turn, cleaves procaspase 3 to active caspase-3 [8-9].

The aims of the study are to establish parallel panels of *in vitro* cell lines (as a renewable source for biochemical and macular studies) and corresponding *in vivo* xenograft counterparts (for pharmacological studies) for investigating cancer cell response to flavonoids from SRPE by using molecular imaging techniques.

## Materials and Methods

### Cell culture and apoptotic induction assay

MDA-MB-435, erythromyelogenous leukemic drug-sensitive (K562) and -resistant, P-gp overexpressing, (K562/adr) and Small cell lung cancer drug-sensitive (GLC4) and -resistant, MRP1-overexpressing, (GLC4/adr) were used in this study. The rationale and validation of experimental set-up for measuring cytotoxicity and apoptosis-inducing activity of flavonoids has been extensively described and discussion [1-3].

### Animal experiments

Female athymic mice (3 weeks-old; NMRI-nu (nu/nu NUDE: France) were purchased from Janvier Laboratory (Le-Genest-st-Isle, France). They were housed in a pathogen-free isolation facility with rodent chow and water *ad libitum* and treated in accordance with institutional guidelines for animals. MDA-MB-435 ( $5 \times 10^6$  in 0.1 mL PBS sterile) were injected s.c. on the flanks of nude mice.

Tumor-bearing mice were used in the studies when tumor volume was approximately 1 cm<sup>3</sup>. The mice were divided into 5 groups. Each investigated animal group was composed of three mice. Four groups received s.c. injections of anti-CD95, quercetin, Siamois 1 and Siamois 2 dissolved in 12% ethanol, and the other one received injection of the vehicle only [1-3].

### **99mTc-hynic-Annexin V Scintigraphy**

Scintigraphy was immediately performed after intravenously injecting in the eye vein with 150-300  $\mu$ Ci 99mTc-hynic-Annexin V using a gamma camera (DST-XL, double head) equipped with a low energy, high resolution pinhole collimator. Data were recorded using 20% window centered on the 140 keV photopeak of 99mTc into a 256  $\times$  256 matrix of a dedicated computer system for digital display and analysis. All images were recorded with a preset time of 5 min (5 min/frame, 45 frames totally). A syringe with a known amount of radioactivity was scanned along with the mice to allow semiquantification of the results using region-of-interest (ROI) analysis. Mice were sacrificed after radiolabelling imaging. Tumor and liver organs were harvested and the activity of 99mTc-Annexin V of tumors were measured using gamma well counter (LKB Wallac, 1261 Multigramma) prior to fixation in 3.7% formaldehyde at 18-24 °C for the paraffin embedding. The paraffin-embedded sections (5 $\mu$ m) were histochemically determined apoptotic cells with the TumorTACS™ in situ apoptosis detection kit (R&D Systems). The brown staining of the DNA-biotinylated-diaminobenzidine (DAB)

was shown in the apoptotic cells by the kit.

### **Statistical analyses**

The results are presented as means  $\pm$  SD. Multiple statistical comparisons were performed using the T-tests analysis.

### **Results**

Lyophilized form of Siamois® red wine, the so-called “Siamois 1 and Siamois 2” has been standardized in polyphenolic content to 12 g/L was used through out the study. It consists of gallic acid, anthocyanins, flavonoids, phenolic acids, proanthocyanidin units. In fact quercetin is the most abundantly found in the Siamois® powder.

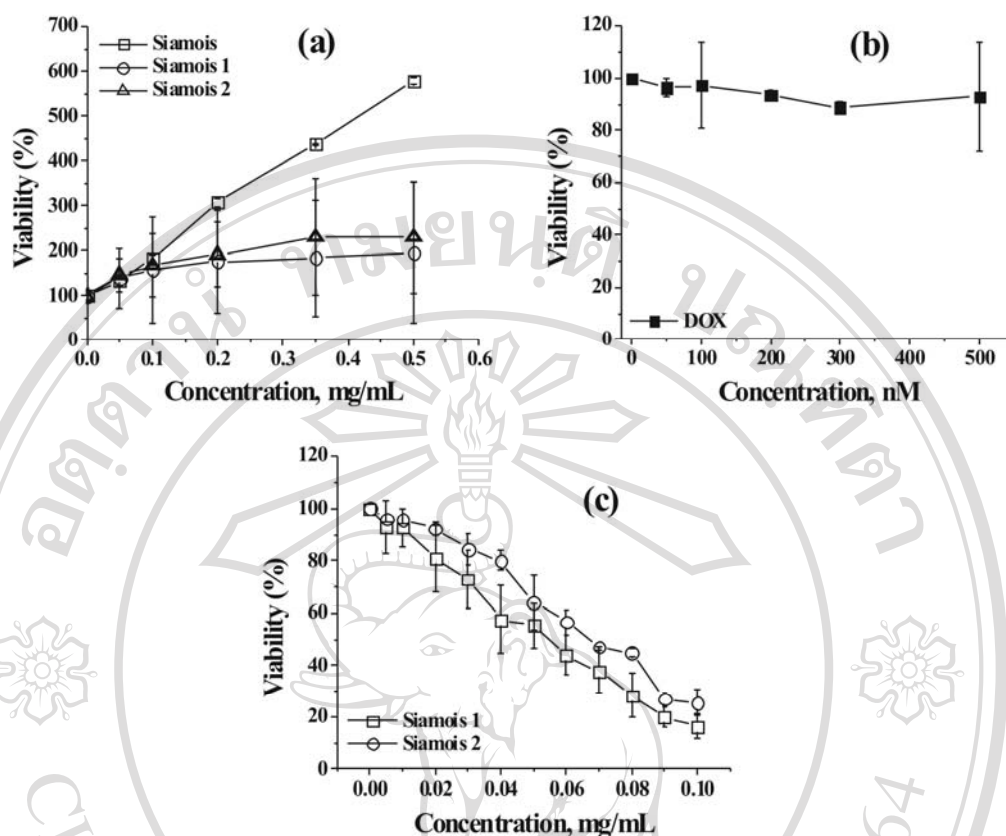
### **Cytotoxicity of Siamois® polyphenols against myoblast and cancer cells**

Figure 1a shows that the concentration of 3 formulations of Siamois® polyphenols ranging from 0.05 mg/mL to 0.5 mg/mL stimulated a proliferation of normal myocytes to about 600 % of control. Contrary to the series of experiments treated using doxorubicin; the cell growth was completely inhibited but did not cause any cell death even at a high concentration of 500 nM (Figure 1b).

Siamois 1 exhibited about 30 to 100-fold anticancer activity against those five cancer cell lines compared with Siamois 2 (Figure 1c). IC50 values were 0.05  $\pm$  0.01 mg/mL for MDA-MB 435, 0.12  $\pm$  0.02 mg/mL for K562 and K562/adr 0.18  $\pm$  0.02 mg/mL for GLC4 and 0.10  $\pm$  0.2 mg/mL GLC4/adr cell.

### **Decrease in the ROSi and alteration of $\Delta\Psi_m$ of cancer cells**

Quercetin, kaempferol, apigenin and eriodictyol are found as active ingredients of “Siamois 1 and

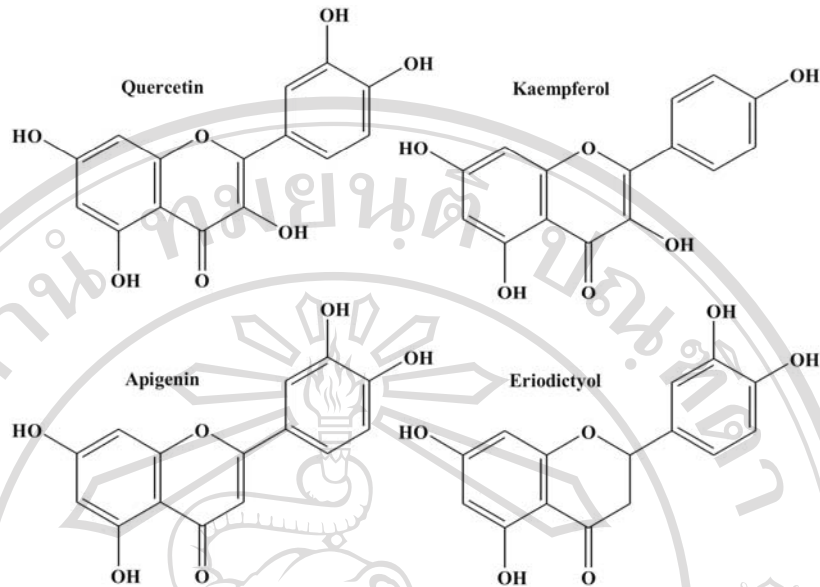


**Figure 1** Effects of (a) Siamois, Siamois 1 and Siamois 2, (b) doxorubicin on myocyte cell growth and (c) Siamois 1 and Siamois 2 on MDA-MB 435 cells. Results were averaged from triplicate cultures

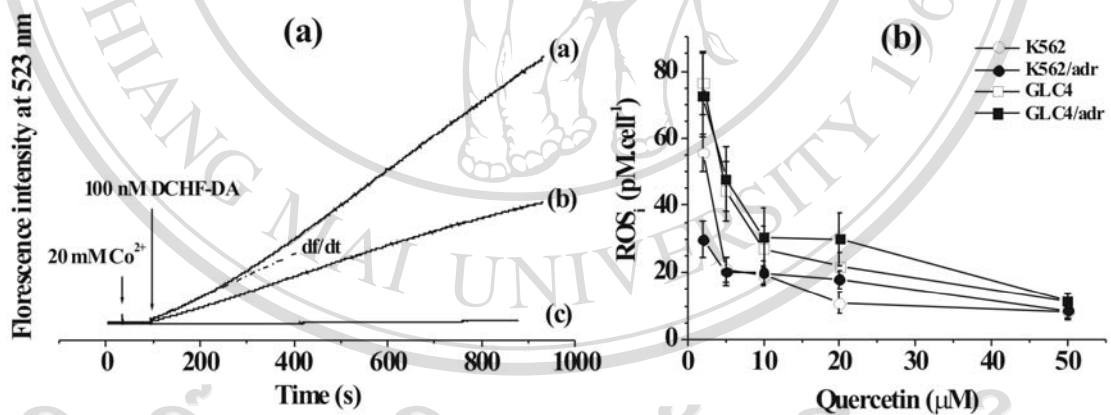
Siamois 2". The 4 molecules are very similar in chemical structures (Figure 2) and have similar Log P values ( $\sim 1.5 \pm 0.4$ ). All compound excepted eriodictyol mediated cytotoxicity by impairing the mitochondrial energetic state of cancer cells resulting in an induction of apoptosis. Figure 3a shows that the 4 compounds diffused across the plasma membrane into cytosol of cells and immediately deplete the ROSi content as time- and concentration-dependent manner. The similar concentration of the 4 molecules required to deplete the ROSi content by 50% was about  $8 \pm 2 \mu\text{M}$  in all cell lines studied (Figure 3b).

#### Determination of apoptosis against cancer cells

It is clear that all compounds used induced apoptotic cell death even after a very short exposure time (30 min). The early apoptotic cells (%) increased with the time to reach a pseudo plateau at about 24 h after incubation when  $10 \mu\text{M}$  of each flavonoid was used. We observed that quercetin for example; an increase in the early apoptotic cells (%) occurred at the first hour ( $42 \pm 2\%$  in K562 and K562/adr cells) and then no further change until 24h. For longer times, the late apoptotic cells (%) and necrotic



**Figure 2** Chemical structure of quercetin, kaempferol, apigenin and eriodictyol



**Figure 3** (a) Kinetics of DCHF-DA oxidation: cells ( $2 \times 10^5$ ) were suspended in 2 mL of HEPES-Na<sup>+</sup> buffer for 10 min before addition of (1) 20 mM CoCl<sub>2</sub> and 100 nM DCHF-DA, (2) of 20 mM CoCl<sub>2</sub>, 100 nM DCHF-DA and 10 μM quercetin and (3) of 20 mM CoCl<sub>2</sub>, 100 nM DCHF-DA and 10 μM quercetin without cell. The fluorescence intensity at 523 nm (excited 502 nm) were recorded as a function of time and the initial rate of an increase in DCF fluorescence intensity ( $V_i$ ) was determined by the tangent to the curve of  $F = f(t)$  during a first 50s after addition of DCHF-DA. The determined ROS<sub>i</sub> in M.cell-1 was calculated using the expression  $[ROS_i] = a.V_i$ , where “a” is the ratio of ROS<sub>i</sub>/ $V_i$ .; (b) Variation of ROS<sub>i</sub> obtained from the series of experiments (a) as a function of flavonoid concentration used in (○) K562, (●) K562/adr, (□) GLC4 and (■) GLC4/adr cell. Data are the mean  $\pm$  SD of three independent experiments.

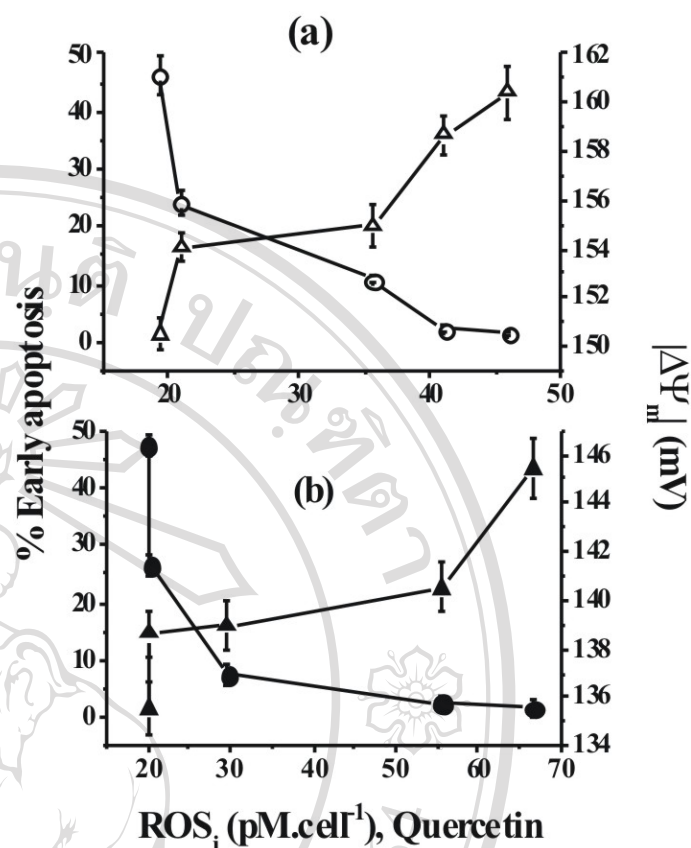
cells (%) slightly increased consistency with a slowly decreasing in early apoptotic cells (%) to  $32 \pm 2\%$  at 72h. However, the early apoptotic cells (%) increased with flavonoid concentration. The efficacies of apoptotic cell induction by these flavonoids from high to low activity is quercetin > kaempferol > apigenin > eriodictyol.

Figure 4a and b demonstrate a good correlation of apoptotic cell death (%), change in  $\Delta\Psi_m$  determined at 3 h after exposing to flavonoids and the ROS<sub>i</sub> content. It is clear that decreasing the ROS<sub>i</sub> content due the presence of flavonoids was leading to an increased in apoptosis while decreasing the  $\Delta\Psi_m$ .

#### Determination of apoptosis in living xenografted mice

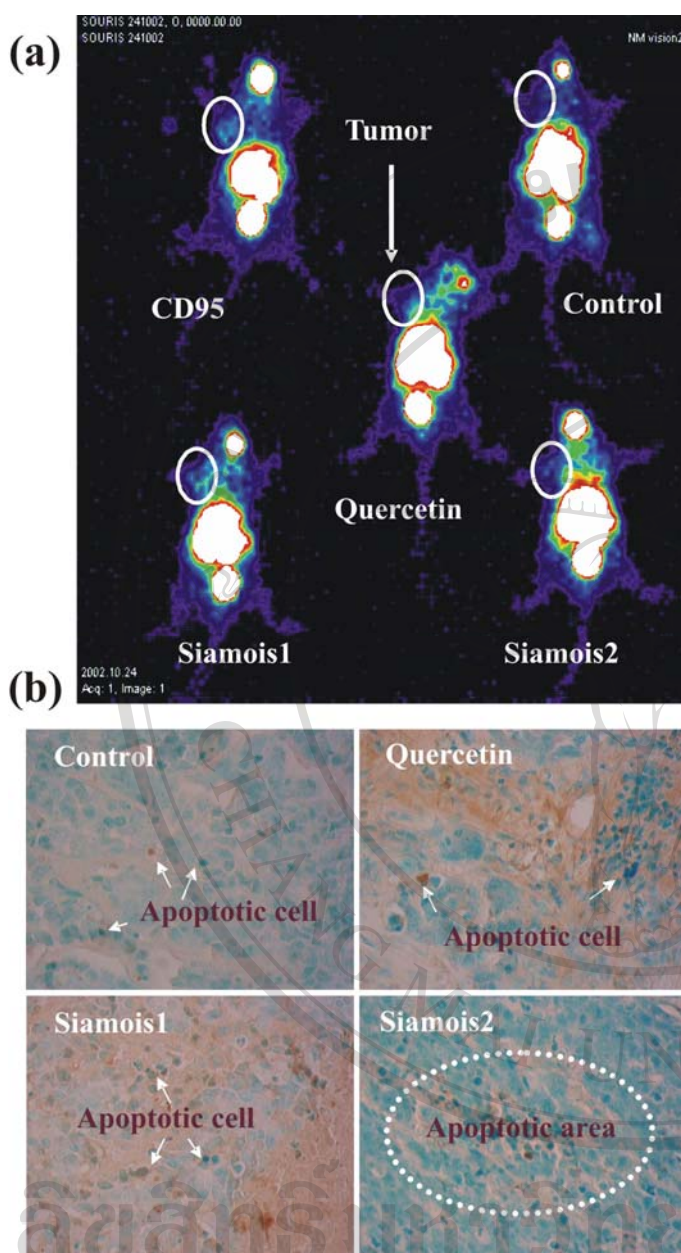
The cancer cells response to quercetin, Siamois 1 and Siamois 2 in vivo level was studied by measuring the apoptotic cells using <sup>99m</sup>Tc-Annexin V scintigraphy. <sup>99m</sup>Tc-Annexin V, a specific molecular probe, was bound to the externalized phosphatidylserine (PS) that had undergone flip-flop to the outer leaflet membrane of the tumor cell resulting from apoptotic pathways.

The representatives of <sup>99m</sup>Tc-Annexin V scintigraphes of xenografted mice 6 h after a single dose of 0.426 mg/kg anti-CD95 and 24 h after a single dose of 3.0 mg/kg quercetin, 17.4 mg/kg Siamois 1 and 14.3 mg/kg Siamois 2, are shown in Figure 5a. It was found that there was a significant enhancement of <sup>99m</sup>Tc-Annexin V in the tumor region of treated mice compared with untreated mice. Uptake in other organs, such as liver, heart, stomach and kidneys, did not differ significantly between the



**Figure 4** Variation of Absolute values of  $\Delta\Psi_m$  ( $\triangle$ ,  $\blacktriangle$ ) and percentage of early apoptotic cell ( $\circ$ ,  $\bullet$ ) as a function of ROS<sub>i</sub> in (a) K562 and (b) K562/adr cell. Data are the mean  $\pm$  SD of three independent experiments.

the treated or untreated mice. The micrographs of sectioned tumors showed consistent results with those obtained from scintigraphic technique. In particular, large areas of cancer apoptotic cell death were regularly found in the cancer tissue obtained from mice treated with Siamois 1 and Siamois 2 (Figure 5b). These confirmed that quercetin, anti-CD95, Siamois 1 and Siamois 2 induced apoptosis of breast cancer MDA-MB-435 tumor in nude mice. These results suggested that all compounds used



**Figure 5** (a) Gamma scintigraphic images showing the enhanced accumulation of  $^{99m}\text{Tc}$ -Annexin V in the MDA-MB 435 breast tumor xenografted in athymic nude mice and (b) ex vivo histological tumor tissue. The apoptotic cells were determined by TUNEL assay (white arrow).

exhibited apoptosis-inducing activities in breast tumors in vivo.

### Conclusions

This study clearly demonstrates that the panels of in vitro cell lines were a crucial source for studying biochemical and molecular basis of interactions, particular the intracellular target of flavonoids. The apoptosis-inducing activity of the compounds was clearly determined in xenografted mice by using  $^{99m}\text{Tc}$ -Annexin V scintigraphy and histological staining. This is the first report that the apoptosis-inducing effects of quercetin, Siamois 1 and Siamois 2 on the MDA-MB-435 cell in vitro were effectively extrapolated to the in vivo situation. These compounds might be considered as a simple dietary supplement and with further clinical investigation for their use as a nutrition-based intervention in breast cancer treatment.

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