CHAPTER IV

Quercetin, extracts of mamoa wood and Siamois® red wine induce apoptosis in human breast cancer MDA-MB-435 cells xenografts *in vivo*

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Quercetin, extracts of mamoa wood and Siamois® red wine induce apoptosis in human breast cancer MDA-MB-435 cells xenografts *in vivo*

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Abstract

We sought to investigate the apoptosis-inducing activities of quercetin, extracts of mamoa wood, and Siamois® red wine against invasive estrogen-receptor negative MDA-MB-435 cells xenografted in athymic nude mice. This study clearly demonstrated that these compounds exhibited apoptosis-inducing activities in cell culture system. Quercetin (20 μg/mL), extracts of mamoa wood (100 μg/mL), and Siamois® red wine polyphenols (200 μg/mL) can induce apoptotic cell death by 40 ± 5%, 44 ± 14%, and 31 ± 13%, respectively. Two-fold of IC_{50} of these compounds were clearly found to induce apoptosis in breast tumor tissue which can be determined by ^{99m}Tc-Annexin V scintigraphy and histological staining. This is the first report that the apoptosis-inducing effects of quercetin, extracts of mamoa wood and Siamois® red wine 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.

Keywords: Quercetin; Extracts of mamoa wood; Siamois® red wine; ^{99m}Tc-Annexin V; MDA-MB-435 cell; Breast cancer

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INTRODUCTION

Flavonoids have been considered as phytoestrogens and drugs for various pathologies such as cancer, viral infection, inflammation, allergy, hypertension as well as atherosclerosis. Among bioflavonoids, quercetin frequently uses for testing the pharmacological properties such as induction of apoptosis resulting in an inhibition of the growth of various cancer cell types (Wang *et al.*, 1999; Yin *et al.*, 1999; Ellis *et al.*, 1991). The

mechanisms of the anticancer activity of quercetin are complicated, starting with metabolic changes and decreasing in IP3 concentration and down-regulation of oncogenes (*c-myc* and *Ki-ras*). The products of *c-myc* and *Ki-ras* oncogenes are required for induction of proliferation and of apoptosis (Choi *et al.*, 2001; Evan *et al.*, 1992). In fact quercetin is the most widely distributed flavonoid in vascular plants and was abundantly found in extracts of mamoa wood (*Antidesma thwaitesianum* Müll. Arg.) and Siamois® red wine.

Recently we reported that quercetin induced apoptosis in a concentration- and time-dependent manner was found in drug-sensitive K562, drug-resistant K562/*adr*, drug-sensitive GLC4 and drug-resistant GLC4/*adr* cells at 1h after exposure to 10 μM quercetin. At such a low quercetin concentration as 10 μM, an increase followed by a decrease in $|\Delta\Psi_m|$ value associated with an induction of apoptosis was detected at 1 h (Kothan *et al.*, 2004). It was also reported that quercetin (60 μM) induced apoptosis in HL-60 cells via decrease in $\Delta\Psi_m$ due to Cytochrome c release and induction of caspase-9 processing (Wang *et al.*, 1999; Murphy *et al.*, 1999). In fact quercetin provoked its cytotoxicity at mitochondria level, impairing mitochondrial energetic state followed by an induction of apoptosis and inhibition of cancer cell growth.

The potential beneficial use of quercetin in preventing ischemia/ reperfusion-induced myocardial damage by reactive oxygen species has been reported. By using a normal cell such as H9c2 cardiomyoblast cell, quercetin could protect hydrogen peroxide from inducing H9c2 cardiomyoblast cells from undergoing apoptosis (Park *et al.*, 2003). It was also reported that quercetin showed a higher value of antioxidant activity than Vitamin C, Vitamin E and β-carotene on a molar basis (Rice-Evans *et al.*, 1995) and probably due to the antioxidant action, it prevented the generation of reactive oxygen species by cyclosporine and thereby suppressed the cyclosporine-induced nephrotoxicity (Satyanarayana *et al.*, 2001). This is strong evidence suggesting that quercetin is safe and has potential for exploring their *in vivo* toxicity.

This study was intended to answer two questions. First, as the mitochondria were proposed as an intracellular target of bioflavonoids (Kothan, 2004), their cellular toxicity should be independent of its estrogen like activities. Second, would quercetin alone or with extracts enriched in a mixture of flavonoids at physiological concentrations (≤ 20 μM) mediate cytotoxicity against cancer, not to normal tissues? For these purposes, estrogen receptor-negative MDA-MB-435 breast carcinoma cells were xenografted in nude mice to determine whether quercetin, extracts of mamoa wood and Siamois® red wine might have therapeutic utility in the treatment of breast cancer. The xenografted nude mice models demonstrated a statistically significant correlation between the incidence of metastases and microvessel counts in invasive breast carcinoma as suggested by Weidner *et al.* (Weidner *et al.*, 1991).

To examine the potential anti-breast cancer activities of quercetin, extracts of mamoa wood and Siamois® red wine, firstly, the apoptosis and proliferation was measured in estrogen-receptor negative MDA-MB-435 cell line. Lastly, the *in vivo* effects of the compounds were assessed on

MDA-MB-435 xenografted in athymic nude mice. The results clearly demonstrated that quercetin, extracts of mamoa wood and Siamois® red wine have potent apoptotic-inducing activities both *in vitro* and *in vivo*. Given their availability as simple dietary supplements and with further clinical investigation, these could be used as a nutrition-based intervention in breast cancer treatment.

MATERIALS AND METHODS

Siamois® red wine vinification

The red wine used in this experiment was made of purple grapes vinified by Laboratory of physical chemistry, molecular and cellular biology (PCMCB), faculty of Science, Burapha University, Bangsaen, Chonburi, Thailand. Grapes were collected during the year 2000 harvest from a vintage located in Amphor Sampran, Nakhonprathom Province and Amphor Damneonsadoek, Rachaburi Province, Thailand. The grape bunches were de-stemmed and crushed. The must were supplemented with potassium meta-bisulphite at the final concentration 50 $mg.L^{-1}$. The alcoholic fermentation was performed at 28 °C, a local room temperature in Thailand in a sterile 500 liter stainless steel tank. The fermentation process started spontaneously, when yeast (3 x 10^7cells/mL) was added. Fermentation processes were followed daily by measuring the temperature, yeast density, total sugar content (% Brix) and total alcohol content (% Alcol). Once fermentation was finished, decantings were performed and the aging process was performed at 28 °C for 1 year before transferring into a bottle. Conventional chemical analysis for total acidity, volatile acidity, alcohol content, free and total SO_2 and reducing sugar were carried out in wine according to OIV method. The wine was lyophilized and kept under N_2-saturated atmosphere at -20 °C.

Preparation of extracts of mamoa wood

Mamoa wood (*A. thwaitesianum* Müll.Arg.), 5 years old was collected from Amphor Muang, Kalasin province, Thailand. Compounds were extracted by using a hydroethanolic model solution. One kg of air-dried wood during 5 months was extracted after soaking for 2 weeks in 2.5 liter of 12 % ethanol. The mixture solution was shaken daily then filtered using Whatman no. 4 (Merck) prior to analysis or lyophilization.

The solutions of quercetin (Extrasynthèse, France), extracts of mamoa wood and Siamois® red wine were freshly prepared and injected into HPLC for quality control before use.

Cell culture and apoptotic induction assay

MDA-MB-435 is an estrogen receptor-negative cell line isolated from the pleural effusion of a patient with breast carcinomas (Cailleau *et al.*, 1974). The cells were routinely cultured in RPMI 1640 medium with 0.3 g/L L-glutamine and supplemented with 10% foetal calf serum, 2 mM pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin (all supplements purchased from Life Technology, Inc.) at 37 °C in humidified air, and 5% CO_2 and subcultured twice a week.

Prior to experiments the cells were trypsinized (0.05% trypsin, 0.02% ethylenediaminetetraacetic acid, EDTA) and resuspended in the medium described above at a density of 5×10^5 cells/mL to have cells in the exponential growth phase; the cells

were used 24 h later when the culture had grown to about 8×10^5 cells/mL. Cell viability was assessed by trypan blue exclusion. The number of cells was determined with a haemo-cytometer.

For induction of the apoptosis assay, exponentially growing cell were seeded in flask-T25 at initial density at 1×10^5 cells with 5 mL medium. After 24 h, varied concentrations of compounds ranging from 0 to 200 µg/mL were added and cells were further incubated at 37 °C for various times: 0.5, 1, 3, 6, 18, and 24 h. The concentration of anti-human CD95/Fas/TNFRSF6 antibody MAB-142 (R&D Systems Inc.) ranging from 2.5 to 15 µg/mL were used as a positive control to induce apoptosis, this concentration was ten fold higher than that reported by Yu *et al.* (Yu *et al.*, 1999).

Cytofluorometric staining of the cells

Cells (1×10^6) were taken for detection of apoptosis and centrifuged for 5 min, 1000×g at room temperature (18-24 °C), resuspended and washed once with 5 mL phosphate-buffered saline prior to being stained with Annexin V (apoptosis detection kit (R&D Systems)). Flow cytometry analysis was performed in a Coulter Epics XL-MCL (Coultronics France SA) and cells were evaluated at 5,000 events per sample. Bi-parametric histograms were used to visualize cells distributed as a function of their signal intensity with respect to Annexin V-FITC and PI.

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 at the Faculty of Medicine, the University of Paris13 (the work was carried on due to the approval of the Ethics Committee of the Paris13 University) MDA-MB-435 (5×10^6 cells in 0.1 mL PBS sterile) were injected s.c. on the flanks of nude mice. Tumor growth curves were obtained using vernier caliper twice a week and the estimate tumor weight formula, $TW\ (cm^3\ or\ g)=a^2.\frac{b}{2}$, where a and b are the short and the long axis of the tumor, respectively (Gonzales-Paz *et al.*, 2001). Tumor-bearing mice were used in the studies when tumor volume was approximately 1 cm^3. 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, crude extract of mamoa wood and Siamois® red wine dissolved in 12% ethanol, and the other one received injection of the vehicle only.

Preparation of ^{99m}Tc-Hynic-rh-Annexin V

The NAS 2020 kit for preparation of Technitium (Tc-99m)-Hynic-rh-Annexin V, as ^{99m}Tc-Annexin V containing stannous tricine complex (Lot#220-01-002) and Hynic-rh-Annexin V (Lot#60-027 JVL032202) was provided by Dr. Jean-Luc Vanderheyden (Division of Nuclear Medicine, Department of Radiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655). ^{99m}Tc-Annexin V (3.768 mCi; 140.43 MBq) complexes were carefully

prepared as per the instructions for radiolabeling. Briefly, 0.3 mL of 3.768 mCi sodium pertechnitate in a sterile, non-pyrogenic 0.9% sodium buffer saline were added to a thawed Hynic-rh-Annexin V vial (100 μg protein in 0.2 mL, 4 mg Tricine and is pH 6.8). To bind ^{99m}Tc to the Hynic-Annexin V conjugate, a reduced tin (stannous ion) and tricine solution was added to ^{99m}Tc pertechnetate with an aliquot of Hynic-Annexin V under anoxic conditions. This mixture was swirled and let to further incubate at room temperature for 20 min. The radiochemical purity of ^{99m}Tc-Annexin V was determined by instant thin-layer chromatography (ITLC) on ITLC-SG strips (Pall, Inc.) with ACD (citrate/dextrose solution, pH 5, from Sigma) as the mobile phase and found to be 90-94%. The radiolabeled material prepared above had calculated specific activities ranging from 1.26 to 1.32 MBq/μg proteins.

Radionuclide imaging

Planar gamma-camera images with ^{99m}Tc-Annexin V were acquired at 3 hours after a single dose of 10 μg/kg anti-CD95, 22 μg/kg quercetin, 200 μg/kg extracts of mamoa wood and 200 μg/kg Siamois® red wine or vehicle, or after 2 weeks of 2 treatments per week with the compounds or vehicle. Mice (18 weeks-old; 30-37.6 g) were sedated with 2.5% avertine (200 μL/100 g; Aldrich, Milwaukee WI) by intraperitonially injection. Scintigraphy was immediately performed after intravenously injecting in the eye vein with 150-300 μCi ^{99m}Tc-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 ^{99m}Tc into a 256 × 256 matrix of a dedicated computer system for digital display and analyasis. 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 ^{99m}Tc-Annexin V of tumors were measured using gamma well counter (LKB Wallac, 1261 Multigamma) prior to fixation in 3.7% formaldehyde at 18-24 °C for the paraffin embeding. The paraffin-embedded sections (5 μm) 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.

Tissue preparation and immuno-histochemical analysis

Immediately after surgical resection, primary tumor specimens were weighed and cut into small pieces. Fragments were fixed with 4% formalin, processed in paraffin in the usual way, and 5-μm sections were stained with H&E. Endothelial cells were specifically stained with GSL-1 lectin (Vector Laboratories, Burlingame, CA) as described previously (Bagheri-Yarmand *et al.*, 1999). For each GSL-1-labeled section, five fields containing exclusively viable tumoral cells, as indicated by the hematoxylin stain, were selected randomly for analysis. The percentage area of endothelial cells was then

calculated as the ratio of the labeled area: the total viewed area x 100.

Statistical analyses

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

RESULTS

The tumor vascular development was determined by immuno-histochemical staining of the endothelial cells presence in the tumor tissue. The typical results of histological studies showed a homogeneous distribution of endothelial cells in tumor tissue obtained from xenografted mice after 2 weeks (Figure 1a). The tumor growth pattern in athymic nude mice was studied by measuring the long and short diameters of tumors twice a week. The estimated tumor weights were found to be in agreement with those obtained from the reel tumor weight (from dissection at the end of series of experiments) as indicated in Figure 1b. These results suggested that the xenografted MDA-MB-435 tumors in athymic nude mice might contain vascular system, show good tumorigenic, and were useful for short-term analysis of differentiated homogenous cell populations.

MDA-MB-435 (5×10^6 cells in 0.1 mL PBS sterile) were injected s.c. on the flanks of female athymic nude mice (3 weeks-old). After two weeks, a mouse was sacrificed and immediately after surgical resection, primary tumor specimens were cut into small pieces. Fragments were fixed with 4% formalin, processed in paraffin in the usual way, and 5-μm sections were stained with H&E. Endothelial cells were specifically stained with GSL-1 lectin (a, arrow). Tumor growth curves were obtained using vernier caliper twice a week and the estimated tumor weight formula, $TW\,(cm^3 \text{ or } g) = a^2 \cdot \frac{b}{2}$, where a and b are the short and the long axis of the tumor, respectively. The reel tumor weights were obtained by weighing the tumor mass from dissection at the end of the series of experiments. The tumor weights from the two methods used were not significantly different by using T-test analysis ($p<0.25$).

Before investigating the effects of quercetin, extracts of mamoa wood and Siamois® red wine on apoptosis and tumor growth *in vivo*, their efficacy of induction apoptosis was studied *in vitro* at cellular level. Quercetin (20 μg/mL), extracts of mamoa wood (100 μg/mL), and Siamois® red wine polyphenols (200 μg/mL) can induce apoptotic cell death by 40 ± 5%, 44 ± 14%, and 31 ± 13%, respectively (Figure 2).

The cancer cell response to quercetin, extracts of mamoa wood and Siamois® red wine *in vivo* level was studied by measuring the apoptotic cells using ^{99m}Tc-Annexin V scintigraphy. ^{99m}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 (Kartachova *et al.*, 2004; Kown *et al.*, 2002).

The representatives of ^{99m}Tc-Annexin V scintigraphs of xeno-grafted mice 6 h after a single dose of 10 μg/kg anti-CD95 and 3 h after a single dose of 200 μg/kg extracts of mamoa are shown in Figure 3. It was found that there was a significant enhancement of ^{99m}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 treated or untreated mice. The activity of ^{99m}Tc-Annexin V in tumors of *ex vivo* studies was shown in Figure 4. The lowest activity of ^{99m}Tc-Annexin V was detected in the tumor region of the control group (untreated mice) compared with those treated with quercetin (200% of control), anti-CD95 (190% of control), extracts of mamoa wood (330% of control) or the Siamois® red wine (240% of control). These results suggested that all compounds used exhibited apoptosis-inducing activities in breast tumors *in vivo*. Among the compounds used, extracts of mamoa wood was the most efficient compound that induced cancer cells to undergo apoptosis.

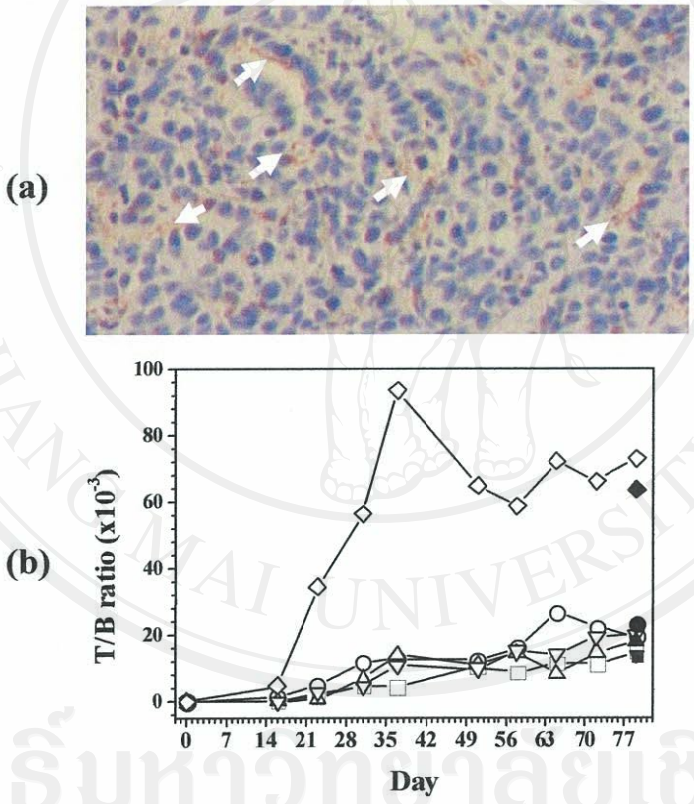

Figure 1. (a) Micrograph of tumor section, endothelial cells were shown in brown-stained (arrow) and (b) tumor growth curve; (open symbols) estimated and (bold symbols) the reel tumor weight.

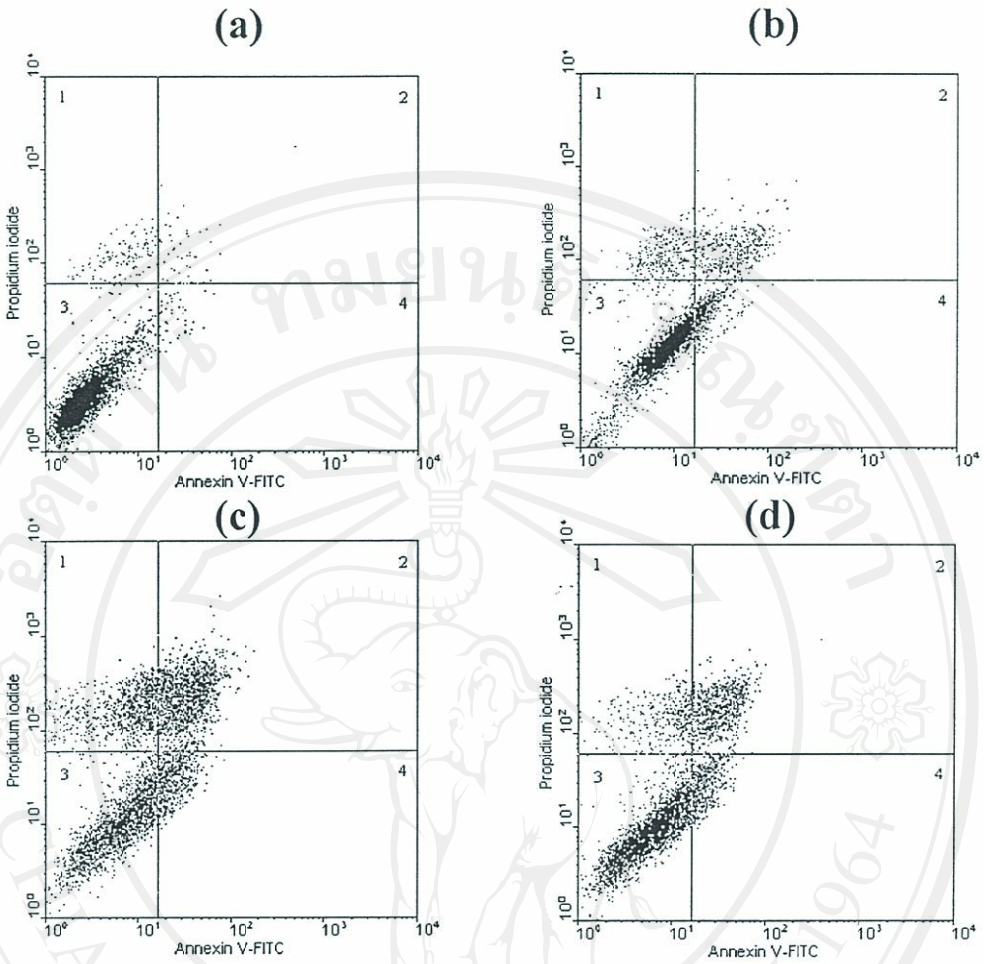

Figure 2. Apoptosis-inducing activities of quercetin, extracts of mamoa wood and Siamois® red wine on MDA-MB-435 in cell culture system. Representative biparametric histrogram of an Annexin V-FITC versus PI; Cells were exposed to (a) untreated, (b) quercetin (20 μg/mL), (c) extracts of mamoa wood (100 μg/mL), and (d) Siamois® red wine (200 μg/mL) 6h before staining using Annexin V-FITC and PI. Flow cytometry analysis was performed in a Coulter Epics XL-MCL (Coultronics France SA) and cells were evaluated on 5,000 events per sample.

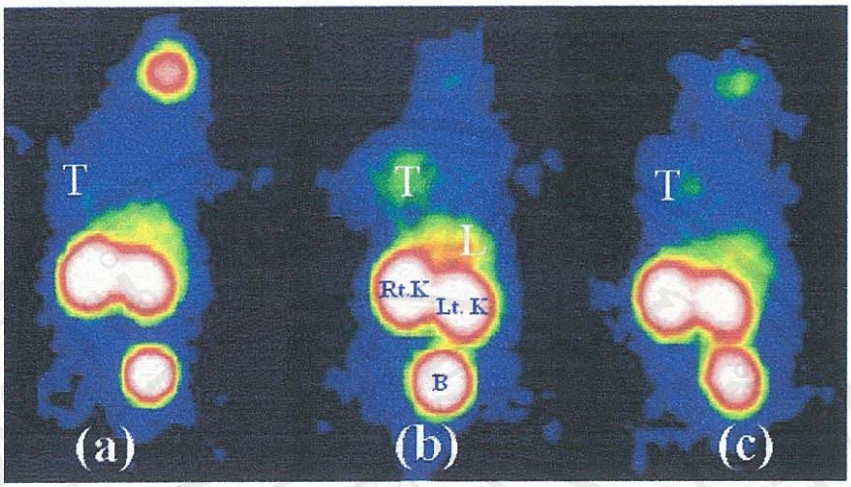

Figure 3. Gamma scintigraphic images showing the enhanced accumulation of ^{99m}Tc-Annexin V in the MDA-MB-435 breast tumor xenografted in athymic nude mice, (a) untreated and (b) after 3 h of single treatment using 10 μg/kg anti-CD95, and (c) after 6 h of single treatment using 200 μg/kg extracts of mamoa wood. Scintigraphy was immediately performed after intravenously injecting 150-300 μCi ^{99m}Tc-hynic-Annexin V in the eye vein. Data were recorded using 20% window centered on the 140 keV photopeak of ^{99m}Tc into a 256 × 256 matrix of a dedicated computer system for digital display and analysis.

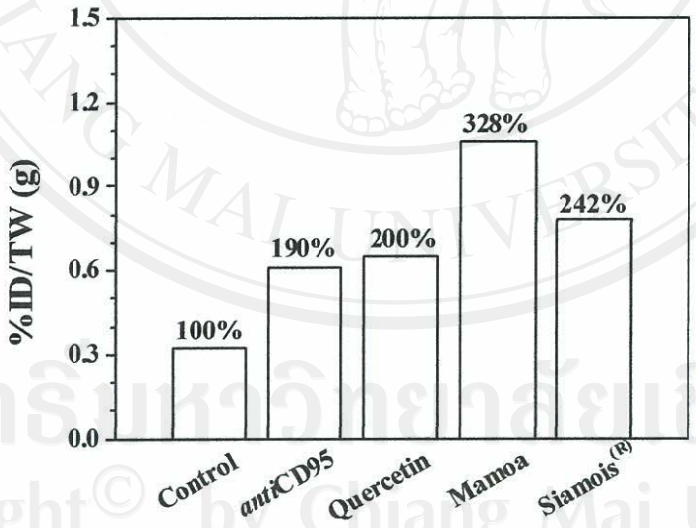

Figure 4. *Ex vivo* results of ^{99m}Tc-Annexin V of tumor activity. The series of experiments were similar to those described in Figure 3. At the end of experiments mice were sacrificed and the ^{99m}Tc-Annexin V of tumors and blood were counted. The tumor activity (%ID/TW) was reported as a percentage of the total dose injected by tumor weight. %ID/TW was the tumor counts divided by tumor weight and the total ^{99m}Tc-Annexin V activity injected. The results were a representative of three mice for each series of experiment.

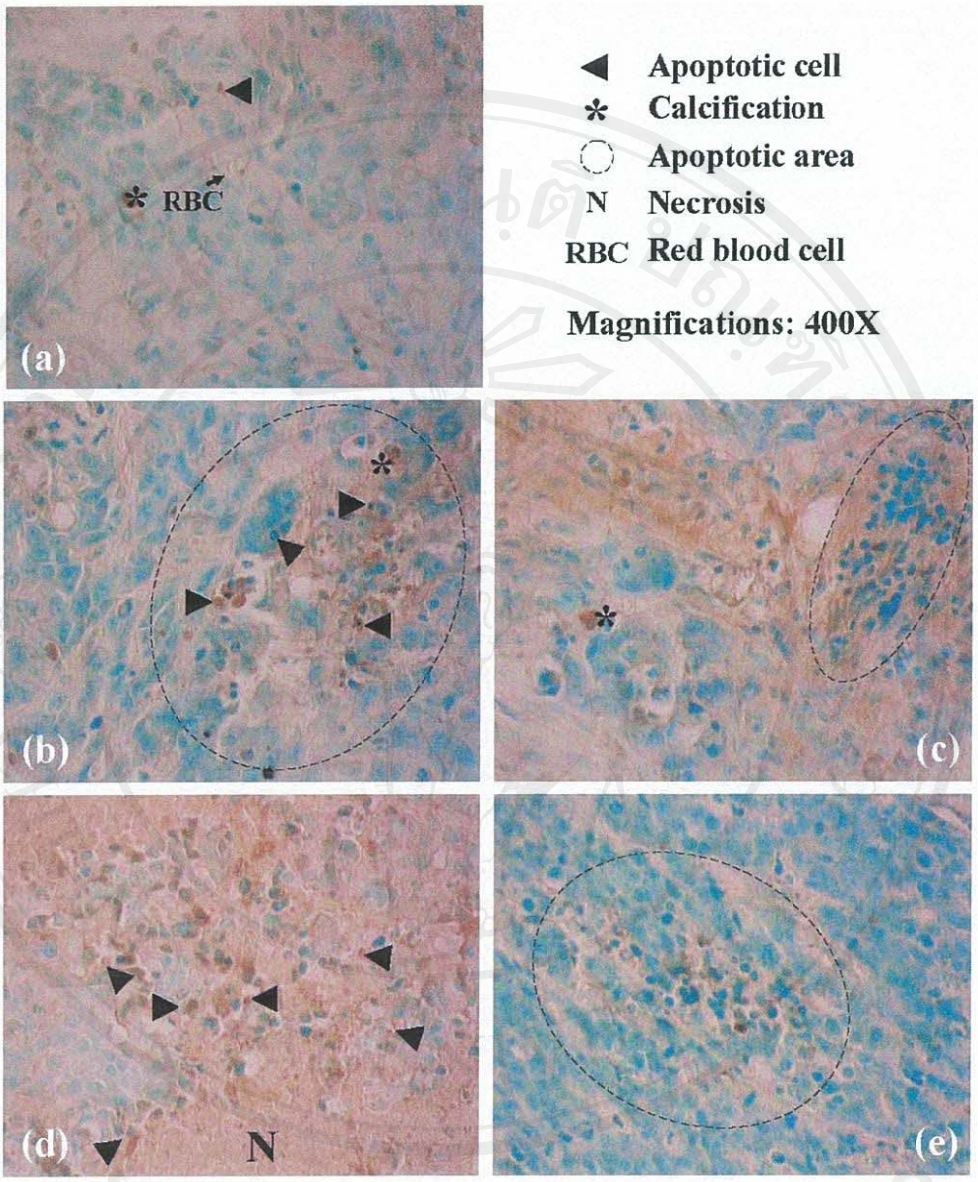

Figure 5. *Ex vivo* histological tumor tissue; (a) untreated mice and 2 weeks of 2 treatments a week of (b) 10 μg/kg anti-CD95, (c) 22 μg/kg quercetin, (d) 200 μg/kg extracts of mamoa wood, and (e) 200 μg/kg Siamois® red wine. The apoptotic cells were determined by TUNEL assay (black arrow). Haematoxylin/eosin stain demonstrated the presence of mitotically active neoplastic cells in MDA-MB-435 tumors. Necrotic foci were observed in the tumor (N). TUNEL assays demonstrated a significant rise of the apoptotic cells in 2 weeks of 2 treatments a week treated of MDA-MB-435 xenografted mice (outlined by dotted line) as compared to untreated tumors (a, arrow).

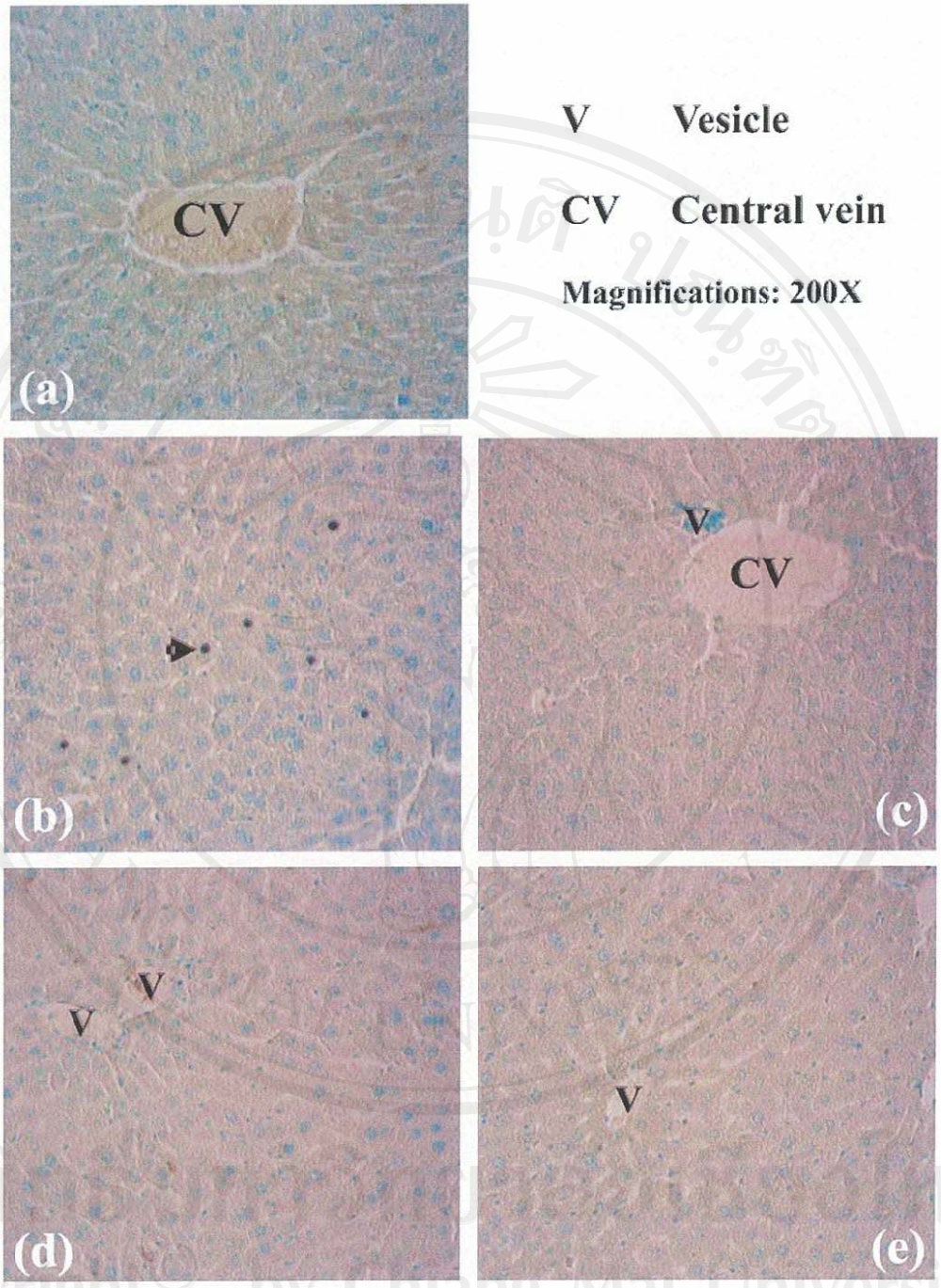

Figure 6. *Ex vivo* histological liver; (a) untreated mice and 2 weeks of 2 treatments a week of (b) 10 μg/kg anti-CD95, (c) 22 μg/kg quercetin, (d) 200 μg/kg extracts of mamoa wood, and (e) 200 μg/kg Siamois® red wine. Haematoxylin/eosin stain demonstrated the presence of hepatocyte cells, central vein (CV) and vesicle (V). The apoptotic cells were determined by TUNEL assay (black arrow). Apoptotic foci were observed in the liver tissue treated, using 10 μg/kg anti-CD95 (black arrow).

The micrographs of sectioned tumors showed consistent results with those obtained from scintgraphic technique (Figure 5). In particular, large areas of cancer apoptotic cell death were regularly found in the cancer tissue obtained from mice treated with extracts of mamoa wood (Figure 5b) and Siamois® red wine (Figure 5e). These confirmed that quercetin, anti-CD95, extracts of mamoa wood and Siamois® red wine induced apoptosis of breast cancer MDA-MB-435 tumor in nude mice.

The micrographs of sectioned liver (Figure 6) showed that apoptotic cells were rarely found on the tissue obtained from mice treated with polyphenols but regularly found on those treated with anti-CD95. These indicated that all compounds, except for anti-CD95, a well-known potent hepatocyte apoptosis-inducing agent (Yu *et al.*, 1999) at concentrations used did not damage liver tissue.

DISCUSSION AND CONCLUSION

In this study, the apoptosis-inducing activity of quercetin, extracts of mamoa wood and Siamois® red wine was assessed against estrogen receptor-negative MDA-MB-435 xenografted tumors in athymic nude mice. This model based on tumor cell injection into m.f.p. followed by metastasis to the lung mimics the clinical situation, in which tumors become estrogen receptor-negative and develop resistance to the anti-estrogen tamoxifen after some duration of treatment (Weidner *et al.*, 1992; Gasparini *et al.*, 1995; Tanigawa *et al.*, 1996; Czubako *et al.*, 1996; Price *et al.*, 1990). This study clearly demonstrated that, although the use of estrogen-receptor negative MDA-MB-435 cells were models, these compounds exhibited apoptotic activities of both in cell culture systems and in xenografted nude mice. This makes evident that these compounds mediated an action independent of their estrogen-like properties and answers of the first question. Since then we have previously reported that quercetin mediated apoptotic action at mitochondrial level (Kothan *et al.*, 2004). This confirms that mitochondria are potential intracellular targets for the selection of apoptotic agents, particularly flavonoids. It should be noted that the dosages of compound injection, except anti-CD95, were two-fold of their IC_{50} (IC_{50} is quantities of compound that are required to inhibit 50% of MDA-MB-435 cell growth and are equal to 4.58 ± 0.73 μg/mL (≈15.2 μM), 58 ± 9 μg/mL, and 70 ± 3.4 μg/mL for quercetin, extracts of mamoa and Siamois® red wine, respectively (Dechsupa *et al.*, 2005). This is the first time that a direct relationship between of the activities *in vitro* and *in vivo* of quercetin, extracts of mamoa wood, and Siamois® red wine level have been reported.

The scintigraphic images revealed that all compounds used induced apoptosis of a breast tumor and the ^{99m}Tc-hynic-Annexin V accumulation was found to be highest in treated groups using extracts of mamoa wood. Similar fixation was found for treated groups using quercetin, Siamois® red wine and anti-CD95. These results were consistent in the results of *ex vivo* and histochemical studies. In fact, the quantity of compounds used in this study was similar to the physiological concentration (≤ 20 μM) (Shen *et al.*, 2002), while the apoptotic cells were found in cancer sections. Moreover, for the range of concentration used, all

compounds except anti-CD95 do not damage to liver tissue.

This study demonstrated that the antiproliferative and apoptosis-inducing effects of quercetin, extracts of mamoa wood and Siamois® red wine polyphenols on the MDA-MB-435 cells *in vitro* were effectively extrapolated to the *in vivo* situation. These results also have shown their availability as simple dietary supplements and as a nutrition-based intervention in breast cancer treatment, following further clinical investigation.

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