CHAPTER II

Red wine polyphenols promote normal myocyte growth but exhibit anticancer and apoptosis-inducing activities against cancer multidrug-resistant cells

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Red wine polyphenols promote normal myocyte growth but exhibit anticancer and apoptosis-inducing activities against cancer multidrug-resistant cells

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Abstract

We sought to investigate the potential use of red wine polyphenols for cancer treatment. The Siamois® red wine was used as polyphenolic source (SRPE) through out the experiments. This study clearly showed for the first time that the red wine polyphenols exhibited different actions; promoted the normal myocyte but inhibited the cancer cell growth. SRPE (up to 0.5 mg/mL) significantly stimulated normal human myocyte cell growth while doxorubicin, an anticancer drug, completely inhibited it. On the other hand, SRPE significantly inhibited cancer cell growth of both drug-sensitive and corresponding multidrug resistant cells with IC_{50} value of 5.0 ± 0.2 mg/mL for MDA-MB-435, 3.28 ± 0.38 mg/mL for K562 and K562/*adr* 3.2 ± 0.2 mg/mL for GLC4 and GLC4/*adr* cells. The action of SRPE on cancer cells can be described as "assisted suicide" or induction of apoptosis. The SRPE exhibited similar efficacy to induction of apoptosis against all cancer cell lines used; about 50% total apoptosis was measured when cell were exposed to15 mg/mL at 24 h.

Our results suggested that SRPE is a non toxic compound and has it is available as a nutrition-based intervention in cancer treatment, following further clinical investigation.

Key words: Siamois® red wine polyphenols (Siamois®); Human myocyte; Multidrug resistance phenomenon; Apoptosis-inducing activities; Cell growth

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INTRODUCTION

Red wine is a rich source of biologically active phytochemicals known as polyphenols which beneficial against degenerative conditions such as cardiovascular disease and carcinogenesis (Manach *et al.*, 2005; Chung *et al.*, 2003). The cancer prevention effects of red wine

polyphenols are also the subjects studied by many research groups (Gurjeet *et al.*, 2006; Dolara *et al.*, 2005; Kris-Etherton *et al.*, 2002). Dolara P *et al.* (2005) reported that polyphenols from red wine inhibit the process of chemical colon carcinogenesis in rodents, modify colon microbial ecology, reduce colonic mucosa DNA oxidation and have complicated effects on gene regulation, possibly affecting the mucosal response to inflammatory and carcinogenic agents. It is not clear at present how the observed variations in gene regulation are specifically connected to protection from oxidative damage and/or inhibition of carcinogenesis.

According to the recent studies, polyphenol is considered as an effective general inhibitor of cancer cell growth, and induces apoptosis against various cancer cell lines including human colon carcinoma cells (Wenzel *et al.*, 2000) and human prostate cancer cell lines (Romero *et al.*, 2002; Hsieh *et al.*, 1999). The relationship of chemical structures of polyphenols and anticancer has been widely studied with *in vitro* and *in vivo* system, and polyphenol concentrations required for anticancer effects vary depending on the types of cancer cell lines (Kothan, 2004). The polyphenols were demonstrated to affect the function of multidrug-resistance transporters such as P-glycoprotein and MRP proteins (Jodoin *et al.*, 2002; Ferguson and De Flora, 2005). In fact this multidrug resistance phenomenon is the major cause of failure in cancer chemotherapy. We have recently reported that flavonoids that are abundantly found in red wine such as quercetin, apigenin, kaempferol and eriodictyol significantly inhibited cell growth with similar efficacy ($IC_{50} \approx 15 \pm 2$ μM) against human erythromyelogenous leukemic drug-sensitive K562 and drug-resistant K562/*adr* with overexpression of P-glycoprotein and human small cell lung carcinoma drug-sensitive GLC4 and drug-resistant GLC4/*adr* with overexpression MRP1 protein (Kothan, 2004). Moreover, these flavonoids efficiently acted as apoptosis-inducing agents.

Apoptosis, or programmed cell death, is an important physiologic process in the normal development (Hong *et al.*, 2003), and induction of apoptosis is a highly desirable mode as a therapeutic strategy for cancer control (Koo *et al.*, 2002; Thompson, 1995). The major challenge in treating cancer is that many tumor cells carry mutations in key apoptotic genes such as p53, Bcl family protein, or those affecting caspase signaling (Hollstein *et al.*, 1994). The Bcl-2 family determines the life-or-death of a cell by controlling the release of mitochondrial apoptogenic factors associated with death protease called caspases which are considered as a central player for the apoptotic process and cascade of proteolytic cleavage event (Oltvai *et al.*, 1993).

In this study, the investigation of SRPE's interaction with normal human myocytes compared with cancer cell lines was performed. Indeed, the SRPE stimulated myocyte cell growth, no significant toxicity was observed; contrast to doxorubicin, an anthracyclines that use in clinics. Obviously, the SRPE exerted anticancer activities with similar efficacy via induction of apoptosis against human carcinoma MDA-MB-435, human erythromyelogenous leukemic drug-sensitive

K562 and drug-resistant K562/*adr* with overexpression of P-glycoprotein and human small cell lung carcinoma drug-sensitive GLC4 and drug-resistant GLC4/*adr* with over-expression MRP1 protein. The results showed that the SRPE exhibited collateral sensitivity to multidrug resistant cells of both P-glycoprotein and MRP1 protein phenotypes.

MATERIALS AND METHODS

Siamois wine making

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 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. 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 sponta-neously, when yeast (3×10^7 cells/mL) was added. Fermentation processes were followed by daily measuring temperature, yeast density, the total sugar content (% Brix) and total alcohol content (% Alcol). Once fermentation was finished, decanting was performed and the newly wines were kept at 28 °C in the stainless steel tank for 3 months before their transfer into bottle. Conventional chemical analysis; total acidity, volatile acidity, alcohol content, free and total SO_2 and reducing sugar were carried out in wine.

Isolation of red wine polyphenols

Red wine was prepared by passage through a column of silica gel G-100, C18 Sigma-Aldrich (Switzerland) made up in double-distilled water containing 12% ethanol (99.9%) and pre-washed 3 times with 12% ethanol in water solution; 7 g silica gel was used to adsorb polyphenol from 150 mL red wine. The column was eluted with 150 mL ethanol followed by 150 mL of 70% acetone (Merck, Thailand). The three fractions were analyzed using high performance liquid chromatography (HPLC) Shimadzu (SPD-M20A photodiode array detector, LC-20AD parallel type double plunger pump unit). Analytical RP-HPLC chromatography was performed on an Innertsil-ODS-3, C_8, 5 μm particle size, 250 x 4.6 mm i.d. column (GL Sciences Inc.) protected with a guard column of the same material (20 mm x 2.1 mm, 3 μm packing (GL Sciences Inc.). The solvent system was a gradient of solvent A (Water/HCl, pH 2.5) and solvent B (acetronitrile): initial 0% B, linear from 0 to 15% in 30 min, 15 to 30 in 45 min, 30 to 100% in 65 min, isocratic with 100% for 20 min, followed by washing and re-equilibrating the column. Flow rate was 1 $mL.min^{-1}$.

All analyses were performed in duplicate, and calibration curves were established using the following external commercial standards of analytical grade: (i) gallic acid was purchased from Fluka (Switzerland) to quantify gallic acid at 270 nm, (ii) caffeic acid from Sigma-Aldrich (Switzerland) to quantify caffeic acid and t-caftaric acid at 320

nm, (iii) quercetin and isoquercetrin from Extrasynthèse (Genay, France) to quantify flavonols at 360 nm, and (iv) anthocyanidin, dimer compounds (B1 and B2) and gallotannin were from Sigma-Aldrich (Switzerland) to quantify red pigments at 520 nm.

Establishment of primary culture of human myocytes

Muscle biopsy, myocyte cell isolation and culture technique

A human biopsy sample of the thigh *vastus lateralis* was taken through a 1-cm incision under local anesthesia. Local anesthetic agents appear to stimulate dormant myogenic cells. The sample then immerged in completed RPMI 1640 (GIBCO BRL, USA) culture medium and kept in 4 ˚C. The procedure for cell isolation and culture was started within 10 h.

Adipose tissue and fascia were removed and the muscle was carefully minced with scissors in a biohazard cabinet using an aseptic technique. The muscle pieces were washed using RPMI 1640 culture medium and then incubated in 5 mL Hank's balanced salt solution, free of calcium and magnesium ion containing 20 mg/mL amphotericin B (ABBOTT France SA, France) for 20 minutes. The muscle pieces were washed using RPMI 1640 culture medium and followed incubated 5 mL Hank's balanced salt solution containing 10,000 U/mL and 10,000 mg/mL penicillin/ streptomycin (Biochrom AG, Berlin, Germany), and washed once using RPMI 1640 culture medium. The pieces were mechanically digested using surgical blade followed by collagenase (Biochrom AG, Berlin, Germany) digestion (20 mg/mL) at 37 ˚C for 30 minutes and 10 mL RPMI 1640 was added in order to stop the collagenase activity. The sample was centrifuged and the pellets were obtained and washed once using RPMI 1640 culture medium and resuspended in 1 mL Hank's balanced salt solution, free of calcium and magnesium ion containing 0.25% trypsin-EDTA (GIBCO BRL, USA) and further incubate at 37 ˚C for 30 minutes. Fresh RPMI 1640 medium (5 mL) was added in order to stop the trypsin activity. The sample was centrifuged and the pellets containing cells were washed twice using RPMI 1640. The cells were resuspended in RPMI 1640 supplemented with 20 % fetal calf serum (GIBCO BRL, USA), and 1 % penicillin/streptomycin (Biochrom AG, Berlin, Germany) and plated in culture flask coating with 0.2% gelatin and let incubate in an CO_2-incubator at 37 °C in 5 % CO_2 and 95% humidify. The culture systems were grown approximately six weeks yielded about 70% confluence. The cells were harvested by trypsinization (2 mL 0.25% trypsin-EDTA) and diluted 10 times every six weeks. All series of experiments were performed using the cells with the passage number was ≤ 5.

Quantification of myoblasts by flow cytometry

Cell culture flasks were periodically observed using an inverted light microscope and an inverted fluorescence microscope (Nikon model TE-2000E) using a filter box model B-2E/C coupled with Nikon, digital camera model DXM 1200F. When subconfluency was obtained, cells were harvested by trypsinization. Cells (10^4) were resuspended in 500 µL in phosphate-

buffered solution in the presence of 10 μL CD56-PE (Immunotech, a Coulter Company), the known myocyte-associated neural cell adhesion molecule (NCAM/CD56) (Stewart *et al.*, 2003) in dark at 25°C for 30 minutes prior to an addition of fixant solution (500 μL phosphate-buffered solution containing 1% (v/v) formaldehyde) before flow-cytometric analysis.

The cytotoxicity assay were performed as follow; myocytes (10^4 cells) were seeded into 0.2% gelatine coated plates (six wells) containing 4 mL RPMI 1640 supplemented with 20% fetal calf serum, and 1% penicillin/ streptomycin (BioMedia). The cells were further incubate in an CO_2-incubator at 37 °C in 5 % CO_2 and 95% humidify for one week allowing the viable myocytes to attache onto gelatin at the bottom site of wells. Each well consisted of 10^5 cells/mL. The variation of final concentrations of compounds ranging from 0 to 0.5 mg/mL was added into the cells. The viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live, but not dead, cells. The concentration of polyphenols required to inhibit cell growth by 50% when measured at 72 h (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the compound concentration

Fluorescence micrographs

Myocyte cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum (Gibco Biocult Ltd.) and 1% penicillin/streptomycin. For the assays, a culture was initiated at 10^5 cells per mL and allowed to grow to approximately 70% confluence. The culture medium was removed and rinsed once adding HEPES-Na^+ buffer pH 7.25. The mixture solution of acridine orange, a specific marker of lysosomes and cytoskeleton protein (Pourahmad *et al.*, 2001), rhodamine B, a specific mito-chondrial dye (Reungpatthanaphong *et al.*, 2003) and pirarubicin, a nuclear compartment marker (Laochariyakul *et al.*, 2003) 1 μM (final concentration) was added into the cells and it was allowed to further incubate at 37°C for 30 minutes prior to placement on the sample holder of inverted fluorescence microscope (Nikon model TE-2000E) using a filter box model B-2E/C coupled with Nikon, digital camera model DXM 1200F.

Cell lines, cell culture and cytotoxicity assay

The K562, human erythromyelo-genous leukemia cell line and its DOX-resistant, P-glycoprotein-overexpressing K562/*adr* subline (Mankhetkorn *et al.*, 1996; Tarasiuk *et al.*, 1993), and the GLC4, human small cell lung carcinoma cell line and its DOX-resistant, MRP1-overexpressing GLC4/*adr* subline (Reungpatthanaphong *et al.*, 2003) were routinely cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For the assays, cell cultures were initiated 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.

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% ethelenediaminetetra acetic 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.

The cytotoxicity assay was performed as follows. Cells (5×10^4 cells/mL) were incubated in the presence of various drug concentrations. The viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live, but not dead, cells. The concentration of drug required to inhibit cell growth by 50% when measured at 72 h (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the drug concentration. The resistance factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of the corresponding sensitive cells (Mankhetkorn *et al.*, 1996). Under our experimental conditions, the IC_{50} values were 10 ± 2 nM for K562 and GLC4 cells. The RF values were 40 and 7 for K562/*adr* and GLC4/*adr* cells, respectively.

Induction of apoptosis

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 MAB142 (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.* (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 ^{0}C), resuspended and washed once with 5 mL phosphate-buffered saline prior to be 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 on 5,000 events per sample. Biparametric histograms were used to visualise cells distributed as a function of their signal intensity with respect to Annexin V-FITC and PI.

RESULTS

Lyophilized form of Siamois red wine® (SRPE) which have been standardized in polyphenolic content to 12 g/L was used through out the study. The main wine polyphenols included anthocyanins, flavonols, phenolic acids, catechin, epicatechin, proanthocyanidin

units. All compound accounted for 45% of polyphenolic powder in weight.

SRPE promoted myocyte growth

The adult human skeleton muscle-derived cells have characterized with respect to cell identity and state of differentiation before their use. The co-expression of desmin and CD56 were used to identify myoblasts. After propagation through a 3rd passage, the cells with 70% confluence demonstrated a well organization lining like in the muscle tissue (Figure 1a) and 51 ± 3% cell population expressed the myoblast marker, CD56 (Figure 1b). The presence of myoblasts within propagated the cell culture was confirmed by the increased appearance of desmin-positive multi-nucleated myotubes (data not shown).

SRPE concentration ranging from 0.05 mg/mL to 0.5 mg/mL stimulated myocyte cell growth to about 600% of control (Figure 2a). Contrary to the series of experiments treated using doxorubicin; the cell growth was completely inhibited but did not cause any cell death even at high concentration such 500 nM (Figure 2b).

Figure 2a also showed that using other source of polyphenol extract such as from *A. thwaitesianum* Müll.Arg., Thai medicinal plant (MPE) and Siamois®, the mixture of SRPE and MPE (1:1 ratio) significantly stimulated myocyte cell growth but lesser degree than that of SRPE.

The morphology and cellular energetic state of myocytes after treatment was demonstrated using co-straining of acridine orange, rhodamine B and pirarubicin detected by optical imaging technique (Figure 2c). Figure 2d revealed more orange-red circles (lysosomes) and yellow circles (mitochondria) in cells treated using SRPE compared with untreated cells.

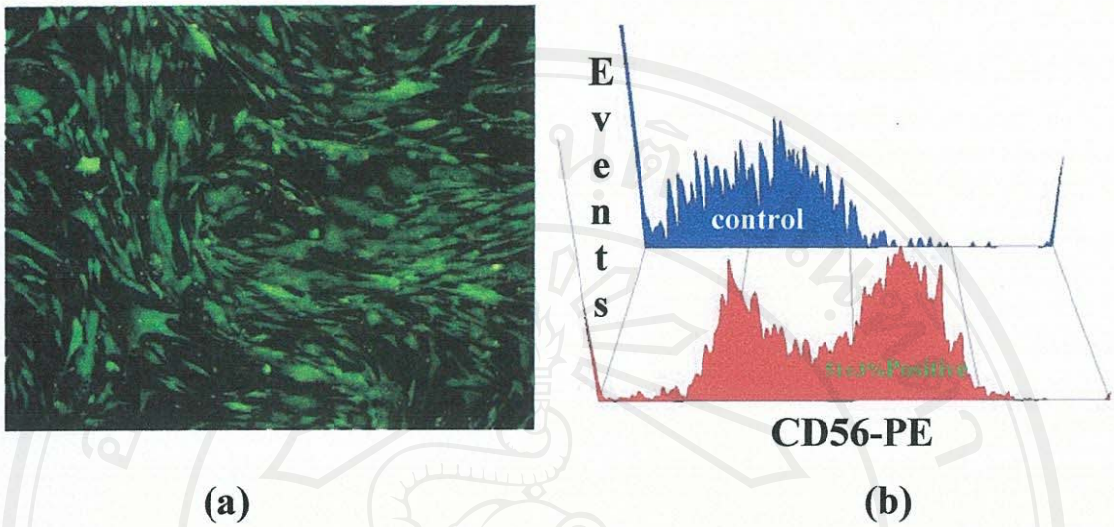

Figure 1. Myoblast Propagation after 3rd passage in culture. (a) Fluorescence micrograph of 3rd passage Myoblasts (200X). Cells were cultured in 1st passage in 0.2% gelatin-coated flasks as described for propagation of human skeleton muscle-derived cells in Materials and Methods. Then cells were seeded at low density onto the culture flask, propagated to confluent density in 2nd passage, then 3rd passage was performed and cells were maintained for 2 weeks. The differentiated cells were fixed while attached to the culture flask. The cytoskeleton, lysosomal and nuclei compartment were stained using the mixture of acridine orange (AO), rhodamine B and pirarubicin of 1 μM prior to placement on the sample holder of inverted fluorescence microscope. Note the appearance of multinucleate myotubes. (b) Fluorescence (Myoblast-bound CD56-PE); 3rd passage cells obtained from (a) at 10,000 cells/mL were incubated in the presence of 10 μL CD56-PE for 30 min then fixed using 1% formaldehyde and read by flow cytometer; 51 ± 3% of cells expressed the myoblast marker. Results were averaged from triplicate cultures.

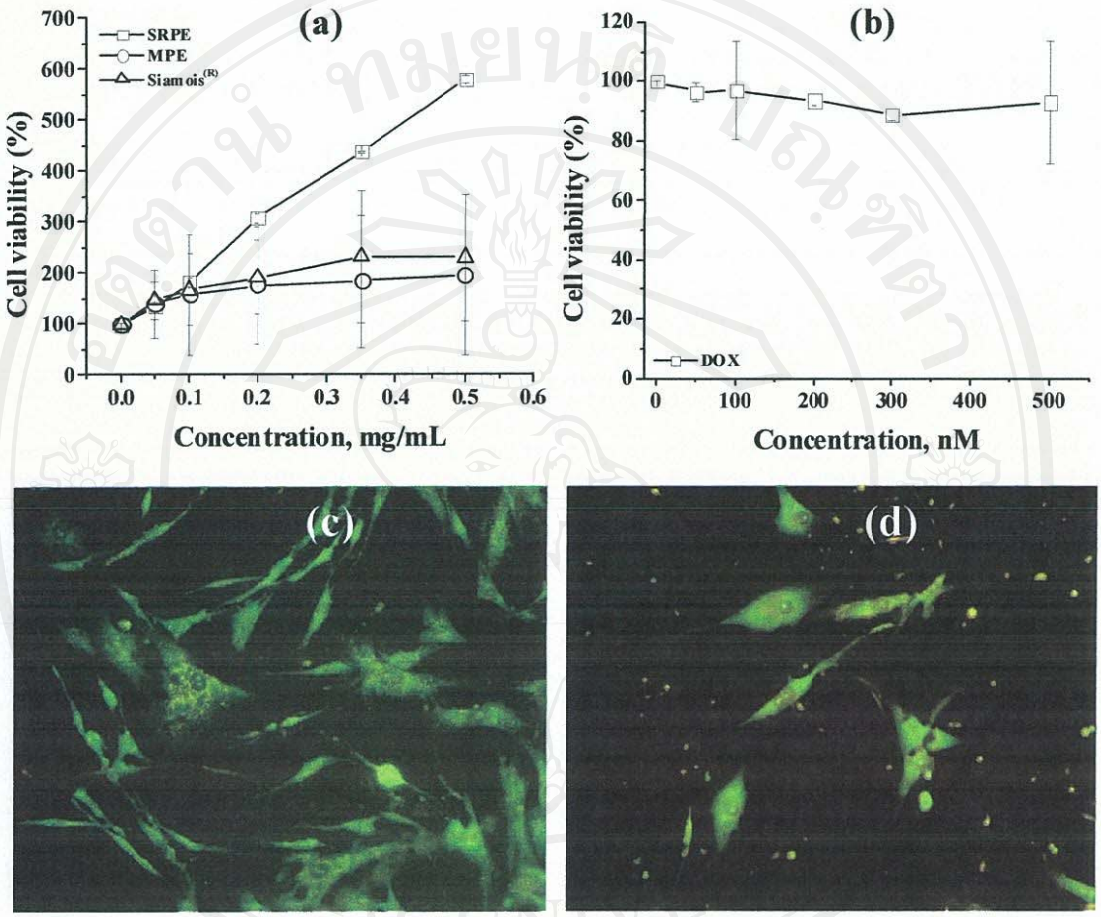

Figure 2. Effects of (a) SRPE, MPE and Siamois® and (b) doxorubicin (Dox) on myocyte cell growth. Cells (10^4 cells/mL) were seeded into 0.2% gelatine coated plates (six wells) containing 2 mL RPMI 1640 supplemented with 20% fetal calf serum and 1% penicillin/streptomycin. The cells were further incubate in an CO_2-incubator at 37 °C in 5 % CO_2 and 95% humidify for one week allowing the viable myocytes to attache onto the wells. Each well consisted of 10^5 cells/mL. The indicated concentrations of compounds were added into the cells. The viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live, but not dead, cells. Results were averaged from triplicate cultures. Fluorescence micrograph of myoblast cells (200X) treated (c) 500 μg/mL Siamois® and (d) 500 nM doxorubicin showing lysosomes (orange or red fluorescence in circles) and cytoplasm (green fluorescence).

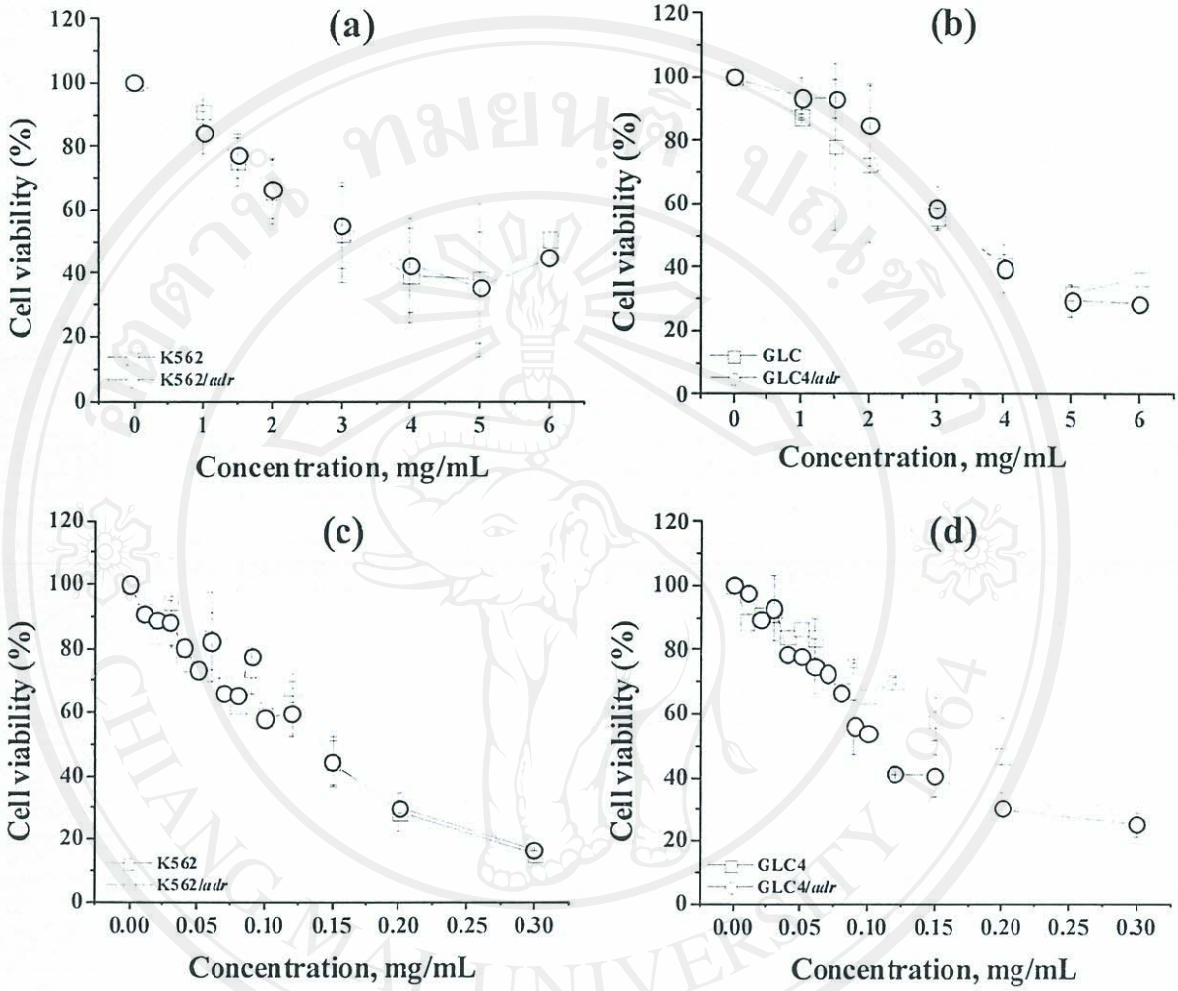

Figure 3. Effects of SRPE on (a) K562 and K562/*adr*, and (b) GLC4 and GLC4/*adr* cell growth and MPE on (c) K562 and K562/*adr* and (d) GLC4 and GLC4/*adr* cell growth.

Cells (5×10^4 cells/mL) were incubated in the presence of indicated drug concentrations. At 72 h after incubation, the viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live, but not dead, cells. The concentration of drug required to inhibit cell growth by 50% when measured at 72 h (IC_{50}) was determined. Results were averaged from triplicate cultures. Error bars identify the range of values.

Anticancer activity

SRPE significantly exhibited anticancer action against K562, K562/*adr*, GLC4, GLC4/*adr*, and MDA-MB-435 cell as indicated in Figure 3 (a, b) and Figure 4. The SRPE concentration

required to inhibit by 50% of cell growth (IC_{50} value) was 3.28 ± 0.38 mg/mL for K562 and K562/*adr*, 3.2 ± 0.2 mg/mL for GLC4 and GLC4/*adr*, and 5.0 ± 0.2 mg/mL for MDA-MB-435 cell.

MPE exhibited about 30-100- fold anticancer activity against those five cancer cell lines compared with SRPE (Figure 3c and 3d). IC_{50} values were 0.12 ± 0.02 mg/mL for K562 and K562/*adr*, 0.18 ± 0.02 mg/mL for GLC4, 0.10 ± 0.02 mg/mL for GLC4/*adr* and 0.05 ± 0.01 mg/mL for MDA-MB-435 cell (Figure 4),

Figure 4 indicates that the anticancer activities was considerable improved, when the MDA-MB-435 cell was treated using Siamois®; IC_{50} value was equal to 0.070 ± 0.003 mg/mL.

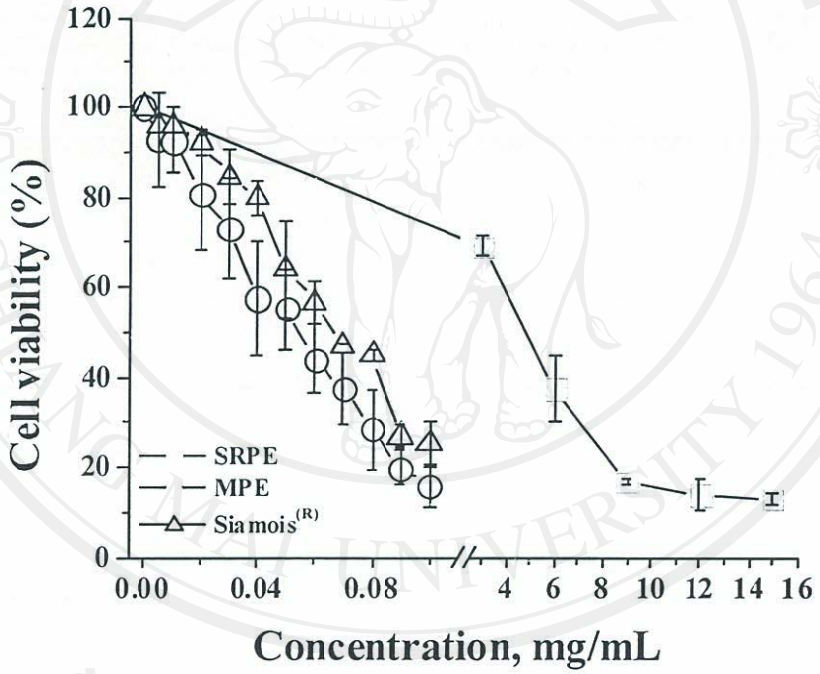

Figure 4. Effects of SRPE, MPE and Siamois® on MDA-MB-435 cell growth.

Cells (5×10^4 cells/mL) were incubated in the presence of indicated drug concentrations. At 72 h after incubation, the viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live, but not dead, cells. The concentration of drug required to inhibit cell growth by 50% when measured at 72 h (IC_{50}) was determined. Results were averaged from triplicate cultures. Error bars identify the range of values.

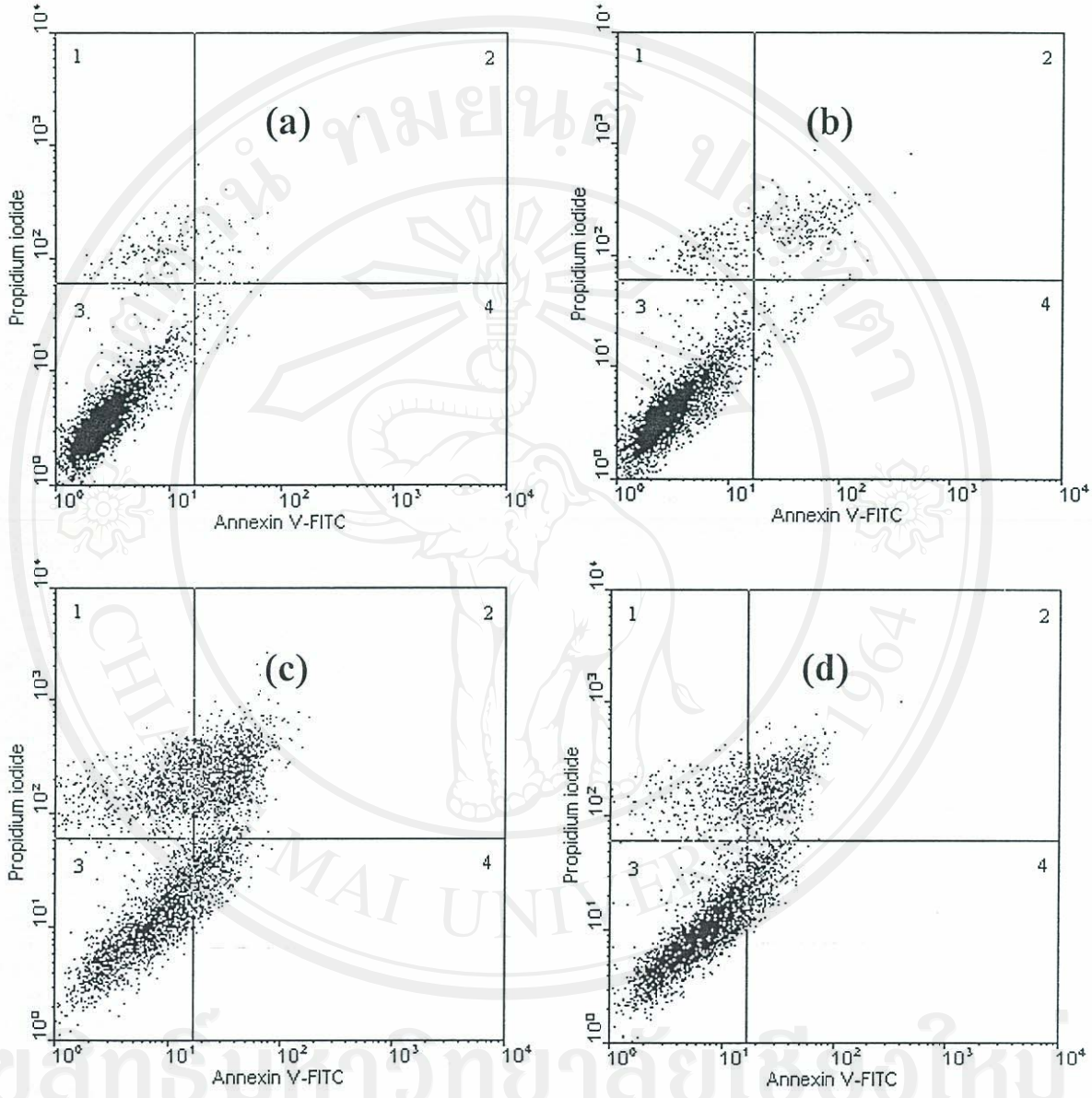

Figure 5. Representative biparametric histogram of an Annexin V-FITC versus PI of MDA-MB-435 cells untreated (a) and treated using (b) 5 μg/mL anti-CD95, (c) 60 μg/mL MPE and (d) 60 μg/mL Siamois® at 6h before staining as described in materials and methods.

Apoptosis-inducing activity

The typical histogram of Annexin V-FITC associated with the phosphatidylserine (PS) on apoptotic cells when MDA-MB-435 cells were incubated with anti-CD95/Fas/TNFRSF6 antibody MAB142, SRPE or MPE is shown in Figure 5. The total apoptotic

cells were the number of cells found in quadrant 2 and 4. The population of cells found in quadrant 4 corresponded to the early apoptotic cells by which the cells were PI negative and Annexin V-FITC positive. The population of cells found in quadrant 2 corresponded to the late apoptotic cells by which the cells were PI and Annexin V-FITC positive. Without treatment, basal apoptotic level of MDA-MB-435 cell line was 3.2 ± 1.8%. SRPE and MPE (up to 200 μg/mL) induce apoptosis in MDA-MB-435 cells as time and concentration dependent manner (Figure 6). Figure 6a demonstrates that the apoptotic cell death (%) can be detected within 30 min for all compounds used and then no modification of this percentage until 24 h. The total apoptotic cells (%) were determined at 6 h as indicated in Figure 6b.

The series of experiments were performed with the same conditions using K562, K562/*adr*, GLC4 and GLC4/*adr* cell. SRPE also exhibited similar apoptosis-inducing activities in the four cell lines as indicated in Figure 7 and these seem to be independent on the aging time of the wine (ranging from 0-12 months); 15 mg/mL at 24 h about 50% total apoptosis were measured.

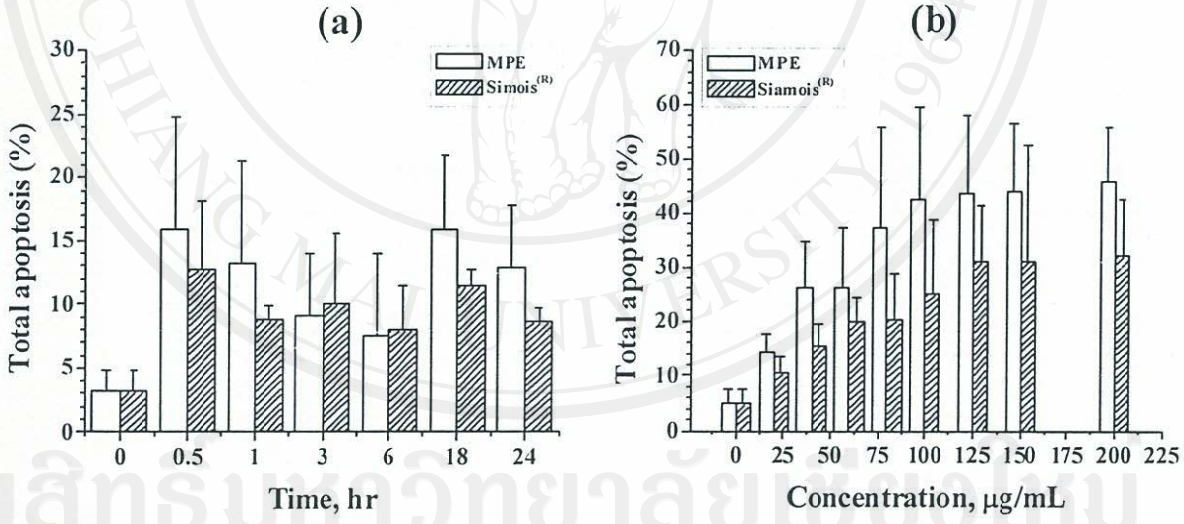

Figure 6. Apoptosis-inducing activities of MPE and Siamois® against MDA-MB-435 cells. The total apoptosis as a function of times (a) and concentrations (b).

Cells were exposed to MPE and Siamois® with varied concentration and times as indicted in the figure 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. Each value is represented as the mean ± S.D. of three independent experiments. (All series of treatments $p < 0.05$)

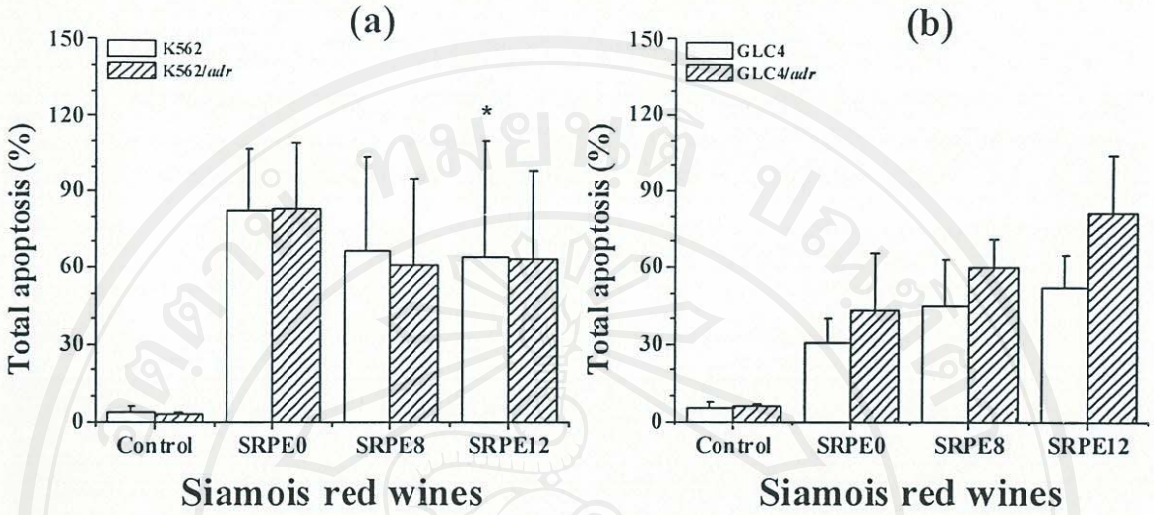

Figure 7. Apoptosis-inducing activities of SRPE against (a) K562 and K562/*adr* and (b) GLC4 and GLC4/*adr* cell.

Cells were exposed to 15 mg/mL SRPE for 24 h 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. Each value is represented as the mean ± S.D. of three independent experiments. ($p < 0.05$ except *)

DISCUSSION

The study clearly showed the different distinctive actions of red wine polyphenols in normal and cancer cells. And this is the first time that it is clearly demonstrated that SRPE stimulated normal myocyte cell growth, the contrary is that SRPE efficiently inhibited cancer cell growth and induction of apoptosis against cancer cells. The SRPE used was analyzed and the main polyphenols were anthocyanins, flavonols, phenolic acids, catechin epicatechin, and proanthocyanidin units. All compound accounted for 45% of polyphenolic powder in weight.

Normal human myocytes

The effects of SRPE on the myocyte cell proliferation were performed in comparison with doxorubicin. Doxorubicin is one of the most active anthracycline derivatives that use in cancer treatment at clinical. Indeed, human skeleton myocytes are very useful and suitable as normal model for cytotoxic test of polyphenols because they are big size and consist of large space of cytoplasm that allow to monitoring the intracellular organelle function by using co-straining of specific dye rhodamine B, a mitochondrial probe (Reungpatthanaphong *et al.*, 2003) and acridine orange, a lysosomotrophic probe (Pourahmad *et al.*, 2001) and measured by inverted fluorescence

microscope. This study clearly showed that SRPE promoted while doxorubicin (up to 500 nM) completely inhibited myocyte cell growth. The optical imaging technique revealed that SRPE provoked an enhancement of mitochondrial accumulation of rhodamine B, indicating that SRPE induced repolarization of the mitochondria, the sources of cellular ATP production. These are consistent with numerous reports that suggested the red wine polyphenols are non toxic compounds; as a matter of fact it looks as if the SRPE have very few side effects (Formica and Regelson, 1995; Hollman and Katan, 1997). We have previously reported that SRPE and MPE did not cause any liver tissue damage but clearly shown in tumor tissue in athymic nude mice xenografted using MDA-MB-435 cell (Dechsupa *et al.*, 2004).

Cancer cell lines

Contrary to normal cells, SRPE inhibited cell growth against 5 cancer cell lines with similar efficacy including MDA-MB-435, K562, K562/*adr*, GLC4 and GLC4/*adr* cell. It should be noted that these polyphenolic extracts similarly exerted their cytotoxic activities in drug-resistant sublines such as K562/*adr* with overexpression of P-gp and GLC4/*adr* with over-expression of MRP1 protein to their corresponding drug-sensitive cell lines. These suggested that SRPE stimulated collateral sensitivity of MDR cells. The results also showed that SRPE efficiently provoked an induction of apoptosis of cancer cells used in our study. However, the aging time of red wine in the conditions of our experiments did not affect such kind of activities. These results suggested that the action of these polyphenols on cancer cells can be described as "assisted suicide". In addition we have previously reported that the antiproliferative and apoptosis-inducing effects of quercetin, MPE and SRPE on the MDA-MB-435 cells *in vitro* were effectively extrapolated to the *in vivo* situation (Dechsupa *et al.*, 2005).

Further information that we learned from this study is that not only SRPE but MPE and Siamois®, another source of polyphenol extracts also exhibited similar activities to SRPE. MPE and Siamois® stimulated myocyte cell growth (lesser efficient) but inhibited cancer cell growth and exhibited an apoptosis-inducing activities, particularly Siamois®, with more efficient than those of SRPE. This study provides evidence that *A. thwaitesianum* Müll.Arg. wood is appropriate to be used as wine cooperage or barrel for red wine aging, in order to developing the curative aspect of red wine.

Overall of our results showed that SRPE and MPE have their availability as a nutrition-based intervention in cancer treatment, following further clinical investigation.

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REFERENCES

Cailleau R, Young R, Olive M and Reeves WJ (1974). Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst*, 53: 661-674.

Chung FL, Schwartz J, Herzog CR and Yang YM (2003). Tea and cancer

prevention: studies in animals and humans. *J Nutr*, 133: 3268S–3274S.

Dechsupa S, Kothan S, Meesungnoen J, Leger G, Martineau A, Reungpatthaphong P, Vergote J, Beranger S, Moretti JL and Mankhetkorn S. "Scintigraphie de l'apoptose des tumours chez des souris athymiques a l'aide d'Annexine V-99mTc: Effets d'extraits de mamoa et vin rouge Siamois", Poster 71, 42eme Colloque de medicine nucléaire de lanque française, Québec, Canada. 30 Sept. -7 Oct. 2004.

Dechsupa S, Kothan S, Vergote J, Leger G, Martineau A, Beranger S, Kosanlavit, R, Moretti JL and Mankhetkorn S. "^{99m}Tc-hynic-Annexin V scintigraphy for monitoring apoptosis of breast cancer MDA-MB-435 cells induced by mamoa extract alone and mixture of mamoa extract and Siamois red wine powder in cancerous nude mice", The 4th annual general meeting of Asian regional cooperative council for nuclear medicine (ACRRNM), Bangkok, Thailand, 15-17 Aug. 2005, p.102.

Dolara P, Luceri C, De Filippo C, Femia AP, Giovannelli L, Caderni G, Cecchini, C, Silvi S, Orpianesi C and Cresci A (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mut Res*, 591: 237-246.

Ferguson LR and De Flora S (2005). Multiple drug resistance, anti-mutagenesis and anticarcinogenesis. *Mut Res*, 591: 24-33.

Formica JV and Regelson W (1995). Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxic*, 33(12): 1061-1080.

Gurjeet K, Marinella R, Francis R and Usha RP (2006). Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol. *Thromb Res*, In Press, Corrected Proof, Available online 28 February 2006.

Hollman PCH and Katan MB (1997) Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed & Pharmacother*, 51: 305-310.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R and Harris CC (1994) Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res*, 22: 3551–3555.

Hong MY, Chapkin RS, Davidson LA, Turner ND, Morris JS, Carroll RJ and Lupton JR (2003). Fish oil enhances targeted apoptosis during colon tumor initiation in part by downregulating Bcl-2. *Nutr Cancer*, 46: 44–51.

Hsieh TC and Wu JM (1999) Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res*, 249: 109–115.

Jodoin J, Demeule M and Beliveau R (2002). Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. *Biochim Biophys Acta*, 1542: 149–159.

Kothan S (2004). *Induction de l'apoptose et de la réversion de la résistance multiple drogues engendrées par les flavonoides et mise en évidence de la cible intracellulaire de ces molécules dans les cellules cancéreuses*. Ph.D. dissertation,

Université de Paris Nord, France and Burapha University, Thailand, 2004.

Koo HN, Jeong HJ, Hong SH, Choi JH, An NH and Kim HM (2002). High molecular weight water-soluble chitosan protects against apoptosis induced by serum starvation in human astrocytes. *J Nutr Biochem*, 13: 245–249.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE and Etherton TD (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med*, 113: 71-88.

Laochariyakul P, Ponglikit-mongkol M and Mankhetkorn S (2003). Functional study of intracellular P-gp- and MRP1-mediated pumping of free cytosolic pirarubicin into acidic organelles in intrinsic resistant SiHa cells. *Can J Physiol Pharmacol*, 81: 790–799.

Manach C, Mazur A and Scalbert A (2005). Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol*, 16: 77–84.

Mankhetkorn S, Teodori E, Scapecchi S and Garnier-Suillerot A (1996). Study of P-glycoprotein functionality in living resistant K562 cells after photolabeling with a verapamil analogue. *Biochem Phar*, 52(2): 213-217.

Oltvai ZN, Milliman CL and Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, 74: 609–619.

Pourahmad J, Ross S and O'Brien PJ (2001). Lysosomal involvement in hepatocyte cytotoxicity induced by Cu^{2+} but not Cd^{2+}. *Free Rad Biol & Med*, 30: 89–97.

Reungpatthanaphong P, Dechsupa S, Meesungnoen J, Loetchutinat C and Mankhetkorn S (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells. J Biochem Biophys Methods 57(1): 1-16.

Romero AP, Ferruelo A, Luján M and Berenguer A (2002). Polyphenols in red wine inhibit the proliferation and induce apoptosis of LNCaP cells. *BJU Int*, 89: 950–954.

Stewart JD, Masi TL, Cumming AE, Molnar GM, Wentworth BM, Sampath K, Mc-pherson JM and Yaeger PC (2003). Characterization of proliferating human skeletal muscle-derived cells in vitro: Differential modulation of myoblast markers by TGF-β2. *J Cell Physiol*, 196: 70-78.

Tarasiuk J, Foucrier J and Garnier-Suillerot A (1993). Cell cycle dependent uptake and release of anthracycline by drug-resistant and drug-sensitive human leukaemic K562 cells. *Biochem Phar*, 45(9): 1801-1808.

Thompson CB (1995). Apoptosis in the pathogenesis and treatment of disease. *Science*, 267: 1456–1462.

Wenzel U, Kuntz S, Brendel MD and Daniel H (2000). Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res*, 60: 3823–3831.

Yu W, Israel K, Liao QY, Aldaz CM, Sanders BG and Kline K (1999). Vitamin E succinate (VES) induces Fas sensitivity in human breast cancer cells: role for Mr 43,000 Fas in VES-triggered apoptosis. *Cancer Res*, 59: 953-961.