CHAPTER 3

MATERIALS AND METHODS

1. Chemicals and Reagents

Pirarubicin (THP) and doxorubicin (Dox) are kindly provided by Professor Arlette Garnier-Suillerot, Laboratoire de Physicochimie Biomoléculaire et Cellulaire, UPRES A 7034 CNRS, UFR Santé Médecine et Biologie Humaine, Bobigny, Université de Paris Nord. Stock solutions of THP and Dox were prepared in distilled water and the concentrations were spectrophotometrically determined by diluting stock solutions in water to approximately 0.1 mM by using a molar extinction coefficient at 480 nm of 11500 M^{-1} $cm^{-1}$and stored at -20°C. Quercetin, quercetrin, kaempferol and eriodictyol were from Extrasynthèse (Genay, France) and home-purified 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (WP283) has been described elsewhere (180). These compounds were stored as 100 mM solution in DMSO at -20°C. A stock solution of MTT was prepared by dissolving 5 mg mL^{-1} of MTT (12 mM) in HEPES-Na^{+} buffer then filtered through a 0.22 µm filter and stored at 4 °C.

- HEPES-Na+ buffer consists of 20 mM HEPES buffer containing 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.25 at 37 ^{o}C

- Phosphate buffered saline (PBS) consists of 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, supplemented with 2.7 mM KCl, and 137 mM NaCl, pH 7.4 at room temperature.

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- Antibodies: anti-human CD95/Fas/TNFRSF6 antibody MAB142, anti-IκBα, anti-p65 (C20), anti-p50 (NLS), anti-cRel (N), anti-RelB (C19), anti-Fra1(H50), anti-Nrf2 (C10) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p38, anti-p44/42, anti-cfos, anti-cjun, anti-junB, anti-junD were purchased from Active Motif (xxx); anti-Sirt1 was purchased from Biomol (xxx); anti-Stat3 was purchased from Upstate (xxx); anti-histone-H3 antibody was purchased from Abcam (xxx) and anti-α-tubulin was purchased from Sigma (Bornem, Belgium). The phospho-specific antibodies directed against p65 Ser536, p38 and p44/42 MAPK, cjun, Akt, MEK were purchased from Cell signalling (Beverly, CA).

- Other chemical agents include: Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Company (St Louis, MO, USA) and stored as 1 mg mL^{-1} solution in DMSO at -20 ^{o}C. Recombinant murine TNF, produced in *Escherichia coli* and purified to at least 99% homogeneity, had a specific biological activity of 8.58 x 10^7 IU mL^{-1} of protein as determined in a standard TNF cytolysis assay. Reference TNF (code 88/532) was obtained from the National Institute of Biological Standards and Control (Potters Bar, UK). Glucose, RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal calf serum, Trypan blue, dimethyl sulfoxide (DMSO), acetonitrile, acetone, n-butanol, hydrochloric acid, chloroform, annexin V-FITC, propidium iodide (PI), rhodamine B (RhoB), acridine orange (AO), monensin, saponin, concanamycin, sodium azide (NaN_3), Sephadex® G-50 bead, silica gel 40.

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2. Characterization of Siamois® polyphenols

2.1. Preparation of crude extracts

The Siamois® red wine used in this study was made of purple grapes (*Vitis vinifera Portugieser*) and vinified by Laboratory of Physical Chemistry, Molecular and Cellular Biology (PCMCB), Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiangmai University, Chiangmai, Thailand. Grapes were collected during the year 2000 harvest from a vintage located in Sam Phran District, Nakhon Pathom Province and Damnoen Saduak District, Ratchaburi Province, Thailand. The grape bunches were removed and grapes were crushed. The must was supplemented with potassium meta-bisulphite at 50 mg L^{-1} final concentration. 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 10^7 cells mL^{-1}) was added. Fermentation processes were followed daily by measuring the temperature, yeast density, total sugar content (24% Brix) and total alcohol content (12% alcohol). Once fermentation was finished, decantings were performed and the aging process was performed at 28˚C for 1 year before transfering 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.

Mamao (*A. thwaitesianum Müll.Arg.*) wood of 5 years age, were collected from Mueang District, Kalasin Province, Thailand. The compounds were extracted by using hydroethanolic model solution, 1 kg of 5 months air-dried wood 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 lyophilization or analysis.

Siamois®, Siamois 1 and Siamois 2 crude extracts :

- Siamois® is the dried powder obtained from Siamois® red wine

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- Siamois 1 is the dried powder obtained from the 12% ethanol extraction of Mamao wood

- Siamois 2 is the dried powder obtained from the 1:1 mixture solution (vol:vol) of Siamois® and Siamois 1

The various stock solutions of crude extracts were obtained by dissolution of the corresponding dried powder in pure DMSO.

2.2. Preparative liquid column chromatography and high performance liquid chromatography

Crude extracts were injected into a open column of Sephadex® G-50 beads Sigma-Aldrich (Switzerland) equibrated in double-distilled water containing 12% ethanol (99.9%) and pre-washed 3 times with 12% ethanol in water solution; 7 g silica gel 40 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 various fractions obtained from Sephadex® G-50 open column chromatography 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 elution condition was a gradient of solvent A (Water/HCl, pH 2.5) and solvent B (acetronitrile): initial 0% solvent B, linear from 0 to 15% (vol:vol) 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}.

2.3. Acid-butanol hydrolysis

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The fractions obtained from Sephadex® G-50 open column chromatography were dried by using rotating evaporation. 1 mg of the dried fractions were resuspended in 0.5 ml of DMSO and then, added with concentrated hydrochloric acid and butanol (HCl-BtOH). Thereafter, the solutions were boiled for 1 h and a change in solution color was observed.

3. Cells and culture conditions

3.1. Cancer cell lines

Adriamycin-sensitive erythroleukemia cells (K562), Adriamycin-resistant erythroleukemia cells (K562/Adr), Adriamycin-sensitive small lung cancer (GLC_4) and Adriamycin-resistant small lung cancer cells (GLC_4/Adr, over-expressing MRP1 protein) (Figure 10) were grown in RPMI-1640 medium supplemented with 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and 10% (vol:vol) fetal calf serum at 37 ^{o}C in a humidified incubator of 95% air. Cultures were initiated at a density of 10^5 cells mL^{-1} and grew exponentially to about 10^6 cells mL^{-1} in 3 days. For the assays and in order to have cells in the exponential growth phase, the cell seeding density in cultures was 5 x 10^5 cells mL^{-1} and used 24 hr later after reaching a density of about 8-10 x 10^5 cells mL^{-1}. Cell viability was assessed by Trypan blue exclusion. The number of cells was determined using a haemocytometer.

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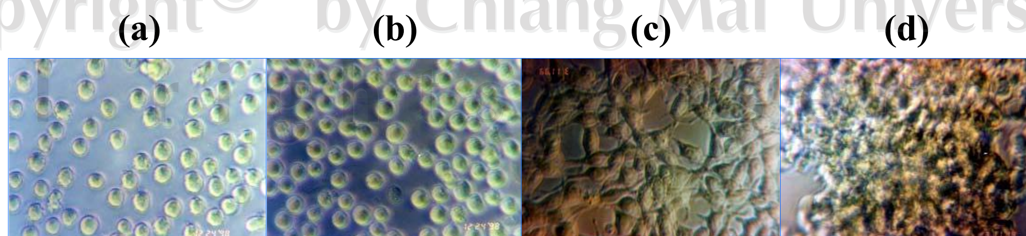

Figure 10. Cancer cell lines; **(a)** K562, **(b)** K562/Adr, **(c)** GLC_4 and **(d)** GLC_4/Adr cell.

Murine fibrosarcoma L929sA cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum, 5% fetal calf serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes, such that they were confluent at the time of the experiment.

3.2. Establishment of primary human myoblasts cultures

Under local anesthesia, a human biopsy sample was taken of the thigh *vastus lateralis* through a 1-cm incision. Local anesthetic agents appear to stimulate dormant myogenic cells. The sample was then immersed in complete RPMI 1640 (GIBCO BRL, USA) culture medium and stored at 4 ˚C. Cell isolation and culturing procedures were started within 10 h of biopsy.

To harvest human myoblasts, adipose tissue and fascia were first removed and the muscle was carefully minced with scissors in a biohazard cabinet using an aseptic technique. The muscle pieces were washed with RPMI 1640, incubated in 5 mL calcium and magnesium free Hank's balanced salt solution (HBSS) containing 20 $mg.mL^{-1}$ amphotericin B (ABBOTT France SA, France) for 20 minutes, washed again with RPMI 1640, incubated in 5 mL HBSS containing 10000 U mL^{-1} and 10000 μg mL^{-1} of penicillin and streptomycin, respectively (Biochrom AG, Berlin, Germany) and then washed once with RPMI 1640. The pieces were mechanically minced using a surgical blade and then digested using collagenase (20 mg mL^{-1}, Biochrom AG, Berlin, Germany) at 37 ˚C for 30 minutes. Ten milliliters of RPMI 1640 was then added to stop collagenase digestion. The sample was centrifuged, and the pellets were washed once with RPMI 1640, resuspended in 1 mL HBSS containing 0.25% trypsin-EDTA (GIBCO BRL, USA) and then incubated at 37 ˚C for 30 minutes. Trypsin digestion was stopped by the addition of fresh RPMI

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1640 (5 mL). The sample was then centrifuged and the pellets were washed twice with RPMI 1640. The cells were resuspended in RPMI 1640 supplemented with 20 % fetal calf serum (GIBCO BRL, USA) and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Biochrom AG, Berlin, Germany) and plated in a culture flask coated with 0.2% gelatin. Cells were grown in a CO_2-incubator at 37 °C at 5 % CO_2 and 95% humidify for approximately six weeks, or until the cultures reached approximately 70% confluence. The cells were harvested by trypsinization (2 mL 0.25% trypsin-EDTA) and passaged at a 1:10 dilution every six weeks. All experiments were performed using cells not exceeding five passages.

3.3. Detection of desmin and CD56 co-expression

To identify myoblasts, the cells were stained for co-expression of desmin and CD56 (181, 182). Cell culture flasks were periodically observed using an inverted light microsope and an inverted fluorescence microscope (Nikon model TE-2000E) equipped with a filter box model B-2E/C coupled to a Nikon digital camera, model DXM 1200F. When the cells reached subconfluency, they were harvested by trypsinization and 10^4 cells were resuspended in 500 μL of a phosphate-buffered solution in the presence of 10 μL CD56-PE (Immunotech, a Coulter Company), the myocyte-associated neural cell adhesion molecule (NCAM/CD56) (182). Cells were incubated in the dark at 25˚C for 30 minutes prior to the addition of paraformaldehyde (500 μL phosphate-buffered solution containing 1% (vol:vol) formaldehyde) and cells were then washed in PBS. The fixed cells were incubated for 30 min at 4°C with a FITC-conjugated mouse anti-desmin antibody (clone D33; Dako Corp) at 2.5 mg mL^{-1} in PBS containing 0.1% (vol:weight) saponin and 10% (vol:vol) fetal bovine serum (FBS). The immunolabeled cells were then washed, incubated 30 min at 4°C, and analyzed by flow cytometry. After 3 passages, cells

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at 70 % confluence demonstrated a well organized, mussel tissue like lining, and 51 ± 3% of the cell population co-expressed the two myoblast markers CD56 and desmin.

4. Fluorescence micrographs of living cells

Myoblast cells were cultured in RPMI 1640 supplemented with 20% FBS (Gibco Biocult Ltd.) and 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. Cell cultures were started with 10^5 cell mL^{-1} and allowed to grow to approximately 70% confluence. The culture medium was removed, and cells were rinsed once with HEPES-Na^+ buffer pH 7.25. A mixture of acridine orange, a specific marker of lysosomes and cytoskeleton proteins (183, 184), rhodamine B, a specific mitochondrial dye (29) and pirarubicin, a nuclear compartment marker (185) was then added to the cells (1 µM final concentration). The sample was incubated at 37˚C for 30 minutes prior to examination with an inverted fluorescence microscope (Nikon model TE-2000E) equipped with a filter box model B-2E/C coupled to a Nikon digital camera, model DXM 1200F.

5. Cytotoxicity assay

5.1. Cancer cells

Human cancer cells (5 x 10^4 cells mL^{-1}) were incubated in 1 mL RPMI 1640 supplemented with 10% fetal calf serum in 24 well plates with various concentrations of Siamois® polyphenols at 37 ^oC in a humidified atmosphere of 5% CO_2 for 72 h, then the number of cells was determined by flow cytometry (Beckman Coulter, Miami, FL., USA). The concentration of polyphenol required for 50% inhibition of proliferation of cells (IC_{50}) was determined by plotting the percentage of the cell growth inhibition (%IC)

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as a function of Siamois® polyphenol mixtures and pure flavonoidconcentration. The %IC was determined by using the following relationship:

%IC = [(number of control cell - number of treated cell) / (number of control cell - number of initial cell)] x 100

The resistance factor (RF) was defined as the IC50 of drug-resistant cells divided by the IC50 of drug-sensitive cells. The cellular morphology change was simultaneously observed using forward and side scattering (FS and SS) parameter obtained by flow cytometry. Data represent mean ± SD of 3 independent experiments.

5.2. Normal myoblasts

Myoblasts (10^4 cells) were seeded into 0.2% gelatine coated six well plates containing 4 mL RPMI 1640 supplemented with 20 % fetal calf serum and 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (BioMedia). The cells were incubated at 37 °C in 5 % CO_2 and 95% humidity for one week to allow the viable myocytes to attach to the gelatin. When the cell density in the wells reached 10^5 cell mL^{-1}, compounds ranging in concentration from 0 to 0.5 mg mL^{-1} were added. Cell viability was then determined using an MTT assay based on the reduction of MTT to purple-colored formazan by living cells. The percentage viability was determined at 72 h and was plotted versus the concentration of the compound tested.

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6. Determination of intracellular ATP, ADP and AMP levels

Human cancer cells (10^6 cells mL^{-1}) were incubated in the presence of various concentrations of Siamois® crude extracts at 37 °C in 5 % CO_2 and 95% humidity for 30 minutes. The cells were collected and washed once using HEPES-Na^+ pH 7.3. Cell extraction was performed at 4°C using 500 µL lysis solution containing 0.6 M $HClO_4$.

The samples were vigorously mixed for 5 minutes, centrifuged at 3000 x g for 10 minutes, and the perchloric residue was collected. The supernatant was precipitated by the addition of 2 M KOH (120 μL) and then the $KClO_4$ was removed following centrifugation at 3000g for 10 min. The sample was passed through a 0.45 μm filter before HPLC analysis (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 50 mM NaH_2PO_4 pH 7.0 at flow rate 1 mL min^{-1}.

7. Determination of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a non-invasive functional spectrofluorometric method, which can be used to determine and to monitor a spontaneous change in mitochondrial function in drug-sensitive and drug-resistant cells. In principle, rhodamine B was used as a molecular probe to determine the $\Delta\Psi_m$. Rhodamine B is a lipophilic cation which can passively diffuse into the cell and accumulate in the mitochondria depending on the mitochondrial membrane potential. Normally the accumulation of the rhodamine B in mitochondria is not sufficient to modulate the fluorescence intensity. Therefore, we used MTT which its reduced state forms formazan crystals by complex II of the electron transport chain as the quencher of rhodamine B in mitochondria leading to an amplification of the difference of fluorescence intensity. Finally, the $\Delta\Psi_m$ can be determined following the Nernst equation.

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The determination of $\Delta\Psi_m$ was carried out as described previously (29). Briefly, cells (2×10^6 cells) were incubated in 2 mL of HEPES–Na+ buffer with 40 nmol L^{-1} rhodamine B in 1 cm quartz cuvettes and vigorously stirred at 37 °C. The rhodamine B fluorescence intensity (F_0) was measured at an emission wavelength (λ_{em}) of 582 nm using an excitation wavelength (λ_{ex}) of 553 nm, and monitored as a function of time. An accumulation of rhodamine B in cells follows the Nernst distribution, but the plasma membrane potential does not contribute to rhodamine B uptake by the cells. The estimation of $\Delta\Psi_m$ was done using Nernst equation:

$$C^o{}_m/C^o{}_i = 10^{(-\Delta\Psi mF/2.303RT)}$$

where $C^o{}_m$ is the rhodamine B concentration in the mitochondrial matrix, $C^o{}_i$ is the rhodamine B concentration in the cytosol at steady state and F, R, and T are the Faraday constant, gas constant and Kelvin temperature respectively. $C^o{}_m$ was determined by adding 200 μM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma Singapore Science Park II, Singapore) to the solution yielding a progressive decrease in rhodamine B fluorescence. The kinetic uptake of rhodamine B was recorded by conventional spectrofluorometer (Figure 11). The slope of the tangent to the curve F = f(t) after the addition of MTT was defined as – dF/dt and the initial rate of decrease in rhodamine B fluorescence was equal to:

$$V_i = (dF/dt) \times (CT/F_0)$$

where F_0 represents the initial fluorescence intensity of rhodamine B. This method has its foundation in the quantification of the Nernst distribution of dye across the mitochondrial membrane; V_i is largely empirical in design, representing the mitochondrial dye concentration. V_i can be used to estimate the value of the $\Delta\Psi_m$:

$$\Delta\Psi_m = -61.51 \log V_i - 258.46$$

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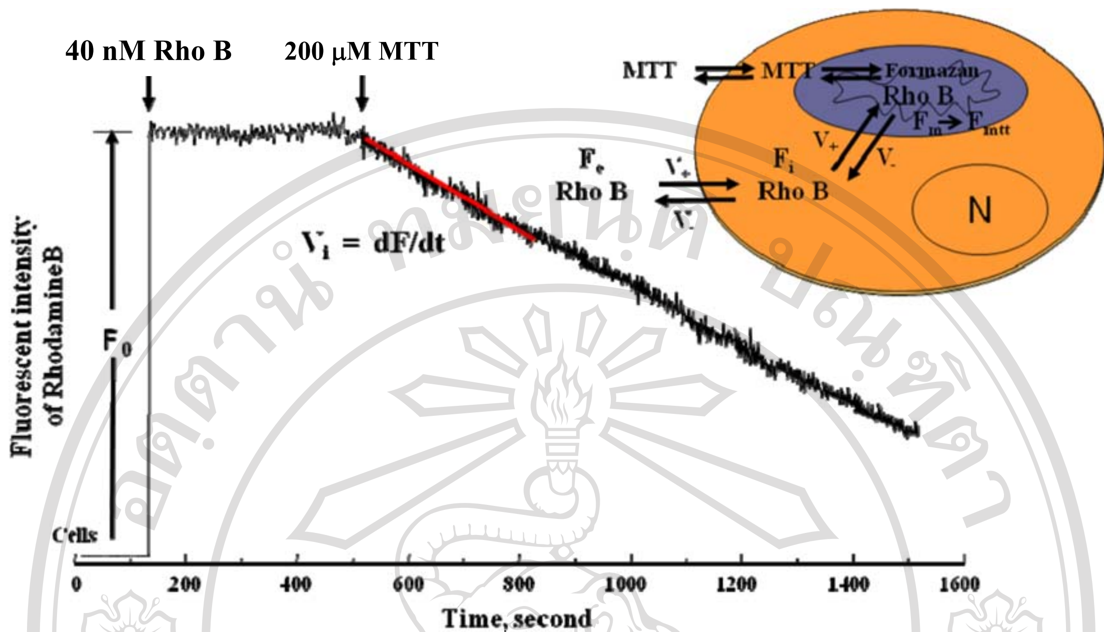

Figure 11. Typical kinetics of cellular rhodamine B uptake recorded from spectrofluorometer (λ_{ex} = 553 nm, λ_{em} = 582 nm).

8. Apoptosis assay

To determine the apoptotic cell, phosphatidyl serine (PS) was used as an early apoptotic marker. PS is a membrane phospholipid which is restrictively distributed in the inner leaflet of the plasma membrane by the control of two ATP-dependent enzymes, flippase and translocase. It was found that PS externalized to the outer leaflet of the plasma membrane in the early state of apoptosis. PS on the outer membrane can specifically bind to annexin V, a non-fluorescent protein which can be conjugated with the fluorescent probe, FITC allowing *in vitro* investigation of apoptotic cells by flow cytometry (Figure 12).

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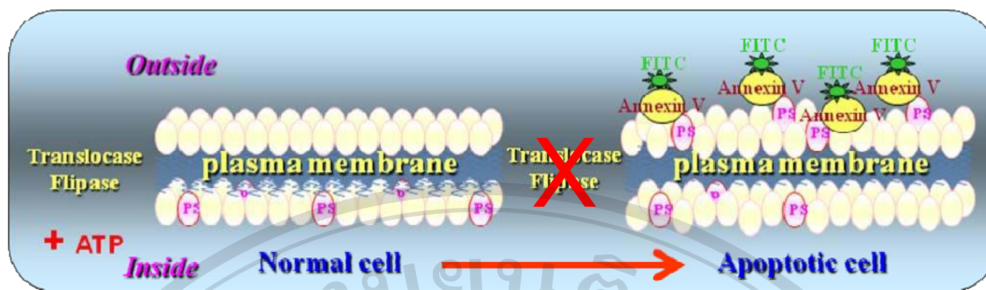

Figure 12. Annexin V-FITC binding with phosphatidyl serine (PS) on cell membrane.

Exponentially growing cells were seeded in flask-T25 at an initial density of 10^5 cells in 5 mL medium. After 24 h, Siamois®polyphenol mixtures and pure flavonoids were added and cells were further incubated at 37 °C as a function of time. Cells were also incubated with 15 mg mL^{-1} of Siamois® as a function of wine age (0, 8, 12 months) for 24 h. Different concentrations of anti-human CD95/Fas/TNFRSF6 antibody MAB142 (R&D Systems Inc.) ranging from 2.5 to 15 μg mL^{-1} were used as positive controls of apoptosis.

To measure the level of apoptosis, 10^6 cells were centrifuged for 5 min at 1000 × g at room temperature (18-24 ^{0}C), resuspended and washed once with 5 mL phosphate-buffered saline prior to staining with annexin V (apoptosis detection kit, R&D Systems). Flow cytometric analysis was performed using a Coulter Epics XL-MCL (Coultronics France SA), and 10,000 cells per sample were evaluated. Biparametric histograms were used to visualize cells distribution as a function of signal intensity with respect to annexin V-FITC and propidium iodide (Figure 13). 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 both PI- and annexin V-FITC-positive.

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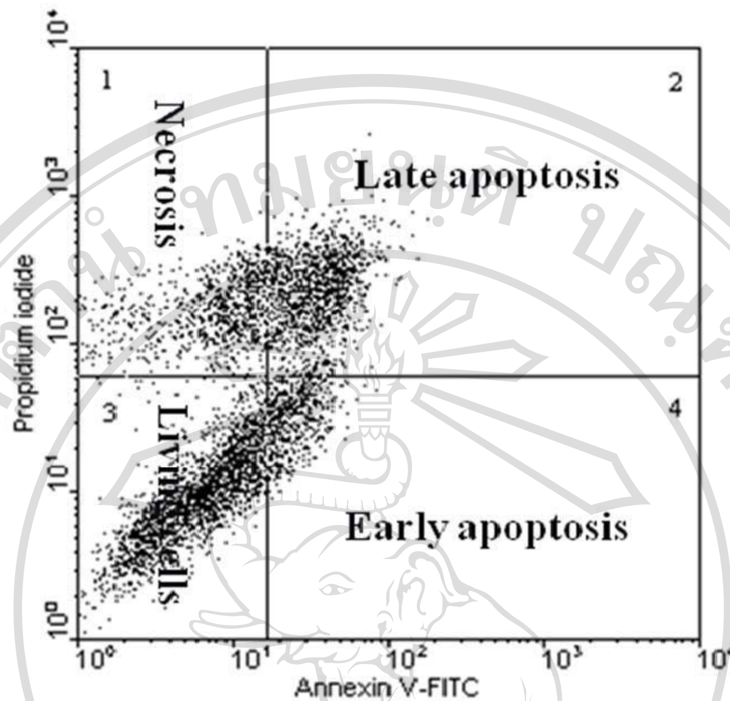

Figure 13. Biparametric histogram of annexin V-FITC versus PI of MDA-MB435 cells.

9. Reporter gene analysis

Reporter gene analysis

The full-size IL6 promoter reporter gene constructed p1168hu.IL6P-Luc+ and the recombinant plasmids p(IL6κB)$_3$50hu.IL6P-luc+ were described previously (186, 187). Stable transfection of L929sA cells was performed by the calcium phosphate precipitation procedure according to standard protocols (186). Luciferase and galactosidase reporter assays were carried out according to the manufacturer's instructions (Promega) and have been described previously (186). Normalization of luciferase activity was performed by measurement of β-galactosidase levels in a chemiluminescent reporter assay Galacto-Light kit (Tropix, Bedford, MA). Light emission was measured in a luminescence microplate reader (Packard Instrument Co.).

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Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample by normalization to the co-expressed β-galactosidase levels. β-Galactosidase protein levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (Tropix).

10. RNA isolation and real-time polymerase chain reaction analysis

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method using the Trizol reagent (Invitrogen, Merelbeke, Belgium). Reverse transcription was performed on 500 ng of total RNA in a 30 µl total volume. For normalization, cDNA concentrations in each sample were determined prior to quantitative real-time PCR (Q-RT-PCR). The Q-RT-PCR was performed on 5 µl of each condition using Invitrogen Sybr green platinum Supermix-UDG on a iCycler apparatus (Bio-Rad, Eke, Belgium). All amplifications were performed in duplicate or triplicate, and data were analysed using Genex software (Bio-Rad, Eke, Belgium). Data were expressed as mRNA expression normalized with that of cells incubated in control medium with 0.1% DMSO. QPCR primers are summarized in Table 4.

Table 4. Primer sequences used in real-time Q-PCR. FW, forward; REV, reverse.

Primer	Sequence
IL-6 FW	GACAGCCACTCACCTCTTCA
IL-6 REV	AGTGCCTCTTTGCTGCTTTC
IL-8 FW	GCTCTCTTGGCAGCCTTCCTGA
IL-8 REV	ACAATAATTTCTGTGTTGGCGC
A1/BFL1 FW	GATTTCATATTTGTTGCGGAGTTC
A1/BFL1 REV	TTTCTGGTCAACAGTATTGCTTCAG
MCP1 FW	ACTCTCGCCTCCAGCATG
MCP1 REV	TTGATTGCATCTGGCTGAGC
A20 FW	CCTTGCTTTGAGTCAGGCTGT
A20 REV	TAAGGAGAAGCACGAAACATCGA
CYCLIN D FW	CGCCCCACCCCTCCAG
CYCLIN D REV	CCGCCCAGACCCTCAGACT

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Table 4. Primer sequences used in real-time Q-PCR. FW, forward; REV, reverse (continued).

VEGF FW	GCCTCCCTCAGGGTTTCG
VEGF REV	GCGGCAGCGTGGTTTC
MDR1 FW	CTGCTTGATGGCAAAGAAATAAAG
MDR1 REV	GGCTGTTGTCTCCATAGGCAAT

11. Western blot analysis

For the western blot analysis of total cell lysate, cells were washed with ice-cold PBS before lysis in SDS sample buffer (62.5 mM Tris-HCL, 2% (weight:vol) SDS, 10% (vol:vol) glycerol, 50 mM DTT and 0.01% (weight:vol) bromophenol blue). To shear DNA and reduce sample viscosity, lysates were sonicated for 1 min in a water bath sonicater and then heated to 95°C for 5 min, after which they were immediately cooled on ice and centrifuged for 5 min. For the western blot analysis of nuclear extract, the nuclear proteins were suspended in SDS sample buffer at the same concentration. The protein samples were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Blots were probed using the appropriate antibodies and the immunoreactive protein was detected using enhanced chemiluminescence reagents on a Kodak Imager system. Quantification of the chemiluminescence was done using the Quantity One software (BioRad, Nazareth Belgium).

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12. Electrophoretic mobility shift assay (EMSA)

After treatment, cells were washed with ice-cold PBS and pelleted in 1 ml PBS by centrifugation for 10 min at 2600 r.p.m. (4°C). Preparation of nuclear extracts has been described previously (187). For EMSA, equal amounts of protein were incubated for 25 min with an NFκB-specific ^{32}P-labeled oligonucleotide and binding mix as described

previously (163). For supershift assay, antibodies were preincubated to the sample of interest for 10 minutes prior to incubation with radiolabelled probe (188). Labeling of the oligonucleotides was performed with [α-^{32}P]-dCTP by using Klenow enzyme (Boehringer Mannheim). For EMSA competition assays, 100-fold excess of unlabelled NFκB oligonucleotide was added to the binding mix. The NFκB oligonucleotide comprises the sequence: 5'-AGCTATGT**GGGTTTTCCC**ATGAGC-3', in which the single IL6 promoter-derived NFκB motif is bold and underlined. Samples were loaded on a 6% polyacrylamide gel run in 0.5 X TBE buffer (pH 8) and complexes formed were analysed using Phosphor Imager Technology.

13. Measurement of caspase-3 activity

After appropriate induction, cells were washed with ice-cold PBS and the cytosolic cell lysate was prepared as described previously (189). Measurement of caspase-3 activity was carried out by the incubation of cytosolic cell lysate with fluorogenic substrates, Ac-DEVD-AMC. The release of fluorescent AMC was monitored for 1 h at 37°C at 2-min time intervals in a fluorescence microplate reader (Packard Instrument Co.) using a filter with an excitation wavelength at 360 nm and a filter with an emission wavelength at 460 nm (190, 191). Data are expressed as the increase in fluorescence intensity as a function of time (Δfluorescence min^{-1}) normalized with that of cells incubated in control medium with 0.1% DMSO.

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