CHAPTER 4

RESULTS

1. Polyphenol composition of Siamois®, Siamois 1 and Siamois 2

Some polyphenols were identified by comparison of retention times and absorption spectra with standards including, quercetrin (quercetin-3-o-rhamnoside), rutin (quercetin-3-o-rutinoside), quercetin, apigenin, resveratrol, eriodictyol, kaempferol, caffeic acid, gallic acid and benzoic acid. Figure 14 a illustrates the various chromatographic profiles of the mixture of standard polyphenols. From these profiles, the retention time (r_t) of each pure polyphenol standard in the standard mixture solution can be determined; **phenolic acid** [(**1**) gallic acid (3.04 min), (**2**) caffeic acid (10.03 min)]; **flavonoid glycoside**s [(**3**) rutin (16.81 min), (**4**) quercetrin (18.91)], (**5**) **benzoic acid** (23.20 min), **flavonoids**; [(**6-7**) eriodictyol (25.10 min), (**8-9**) resveratrol (27.37 min), (**10**) quercetin (30.94 min), (**11**) apigenin (34.05 min), and (**12**) kaempferol (36.00 min)]. Figure 14 b and c are typical HPLC chromatograms of Siamois® and Siamois 1 polyphenols. Fourteen and sixteen major peaks were characterized in Siamois® and Siamois 1, respectively. The typical HPLC chromatograms and r_t of the major peaks of Siamois 2 are shown in Figure 14 d. We would like to stress here that about 30 min after mixing, the solution was analyzed by HPLC and the peaks 6 to 14 of Siamois® and 6 to 16 of Siamois 1 disappeared. In order to verify whether these polyphenols can undergo oligomerization or polymerization during this experimental time, the mixture solution

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was eluted using open Sephadex® G-50 column and silica gel 40 at various times after mixing.

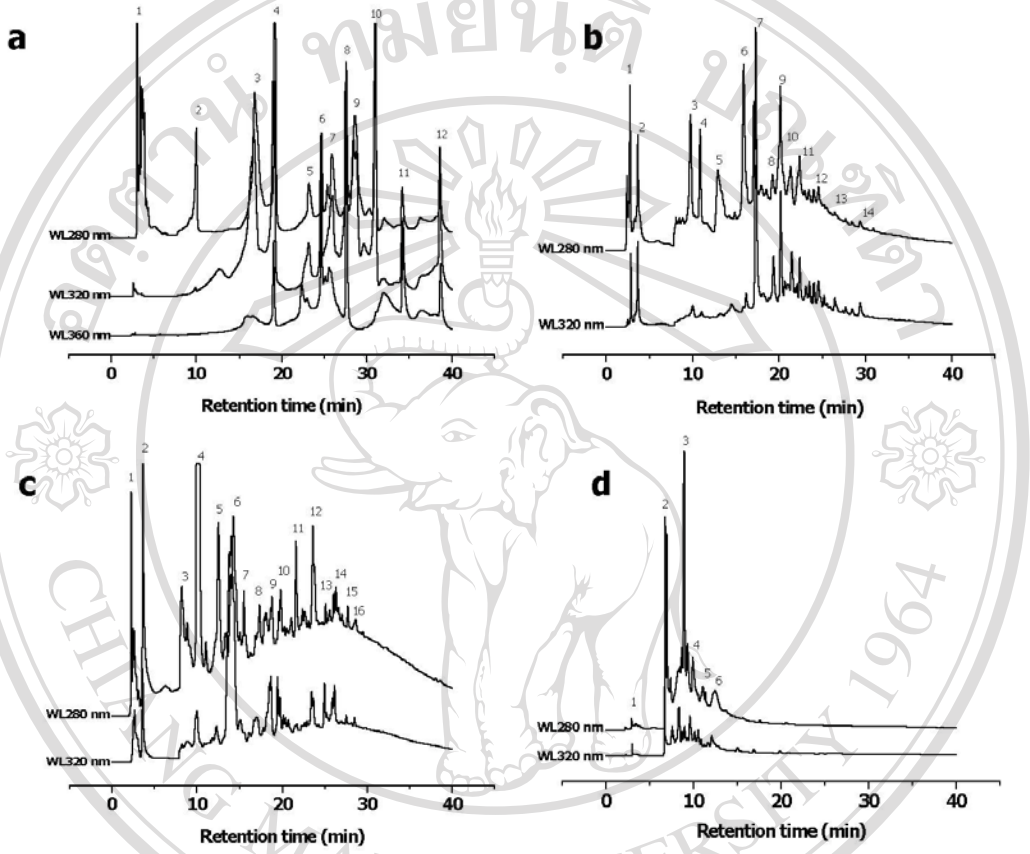

Figure 14. HPLC chromatograms of **(a)** mixture of standard polyphenol solution, **(b)** Siamois®, **(c)** Siamois 1 and **(d)** Siamois 2.

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The oligomeric compounds such as anthocyanidins were isolated using opened-column chromatography. The result showed that the fractions possess absorption spectra corresponding to those of anthocyanidins, such as pelargonidin, cyanidin, peonidin (Figure 15) (192). Oligomeric anthocyanidin species can be dissociated to monomers under boiling acid conditions and this transformation is followed by a color change from yellow to red (193). The acid-butanol hydrolysis study of the fractions was carried out

and the solution change in color from yellow to red was observed as indicated in Figure 16. This result suggested that all fractions of Siamois® polyphenols obtained from Sephadex® G-50 column also comprised oligomeric anthocyanidins. In addition, the efficacy of various liquid phases used in solid/liquid extraction was investigated. The Siamois® polyphenols were also isolated and characterized with similar techniques. The solid/liquid extraction using 12% of ethanol, absolute ethanol, 70% of acetone and the mixture of acetronitrile and ethanol was performed prior to isolation by injection into the HPLC column. As demonstrated in Figure 17, HPLC chromatograms corresponding to those of pigments were found in all fractions and abundant flavonoids were found in absolute ethanol but also in 12% ethanol and a mixture of acetronitrile and ethanol phase in less quantity, but not in 70% acetone phase. The chromatograms of pigments and flavonoids found in Siamois® polyphenols are shown in the inset of the corresponding absorption spectra as illustrated in Figure 18 and these chromatograms were compared to those of standard compounds. There were not enough data to identify all pigments while flavonoids possessed chromatograms corresponding to those of resveratrol, quercetin, eriodictyol and apigenin. The total polyphenolic content in Siamois® determined from lyophilized powder of each fraction was about 45% of solid content (12 g L^{-1}). Altogether, Siamois® polyphenols comprise the major group of polyphenols, including anthocyanidins, flavonoids, phenolic acids, catechin, epicatechin, proanthocyanidins.

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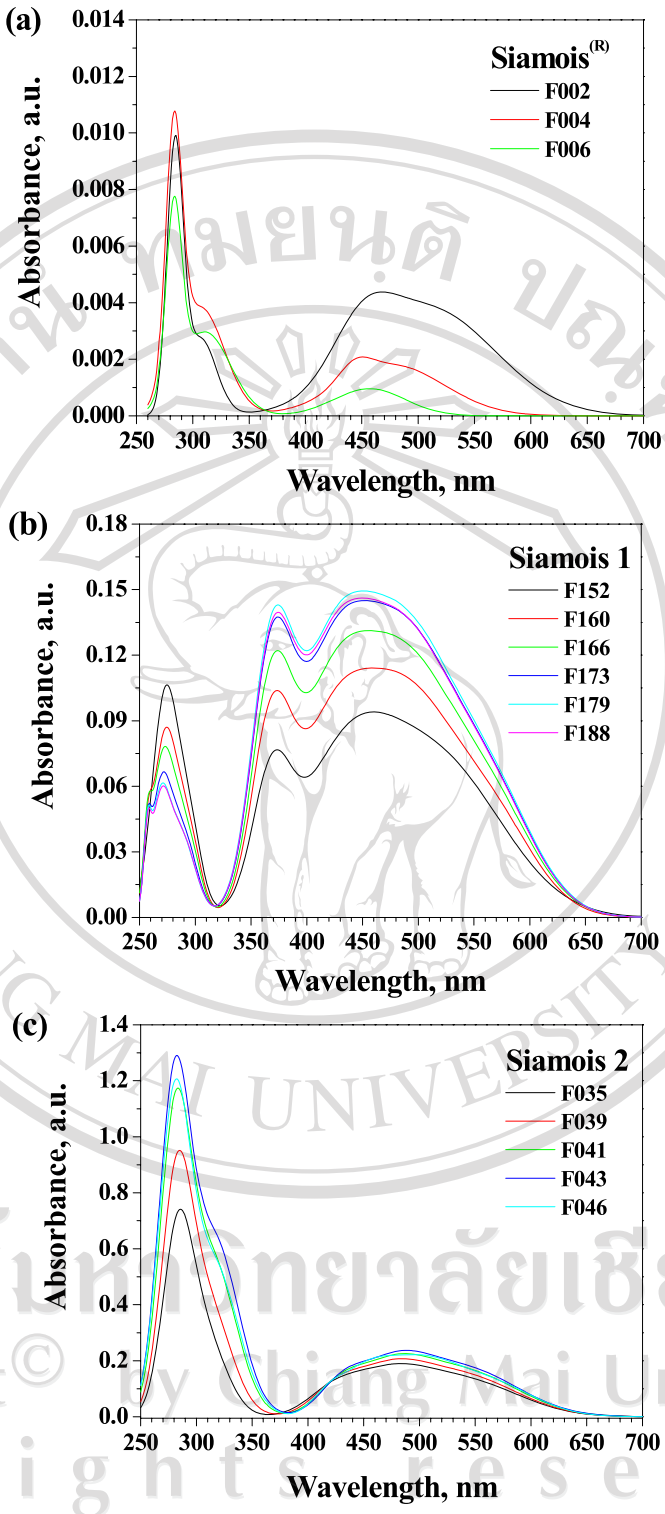

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Figure 15. Absorption smoothened spectra of the fractions obtained from Sephadex® G-50 open column chromatography of **(a)** Siamois®, **(b)** Siamois 1 and **(c)** Siamois 2.

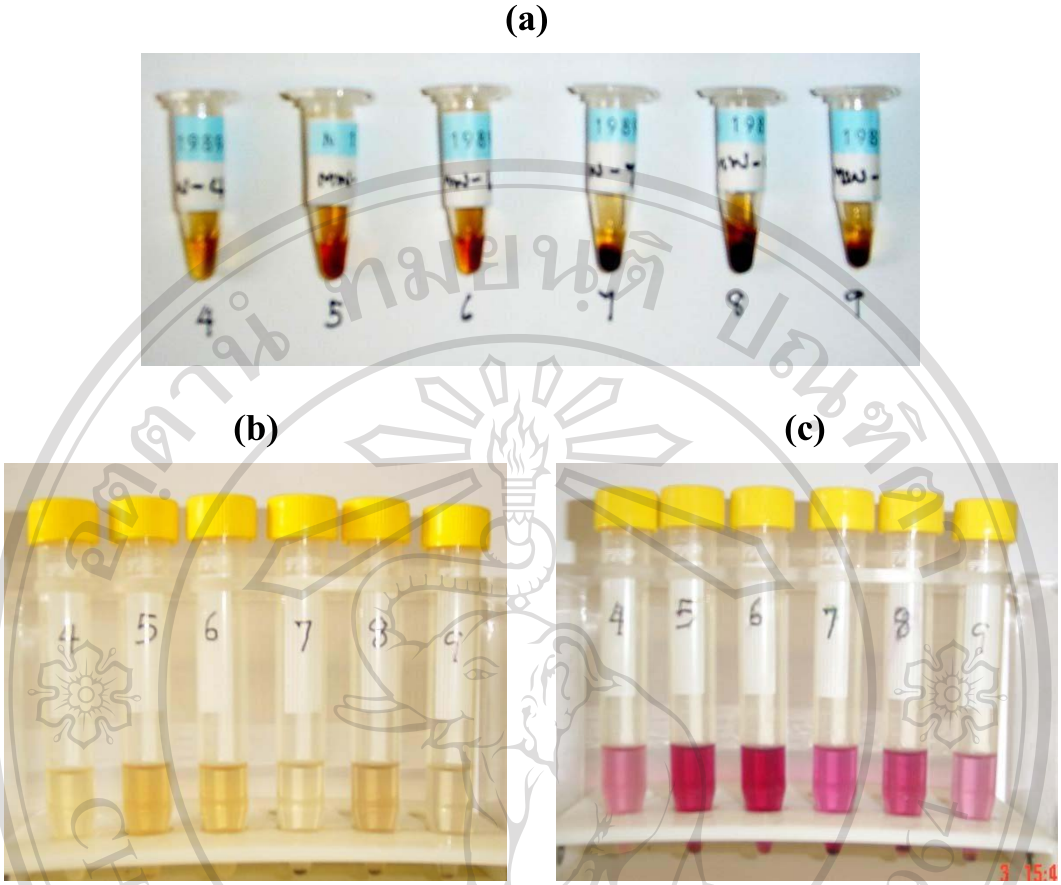

Figure 16. Acid-butanol hydrolysis of Siamois® polyphenols. **(a)** Fractions obtained from Sephadex® G-50 open column chromatography were **(b)** added with hydrochloric acid and butanol (HCl-BtOH) and then **(c)** boiled at 95 °C for 1 h.

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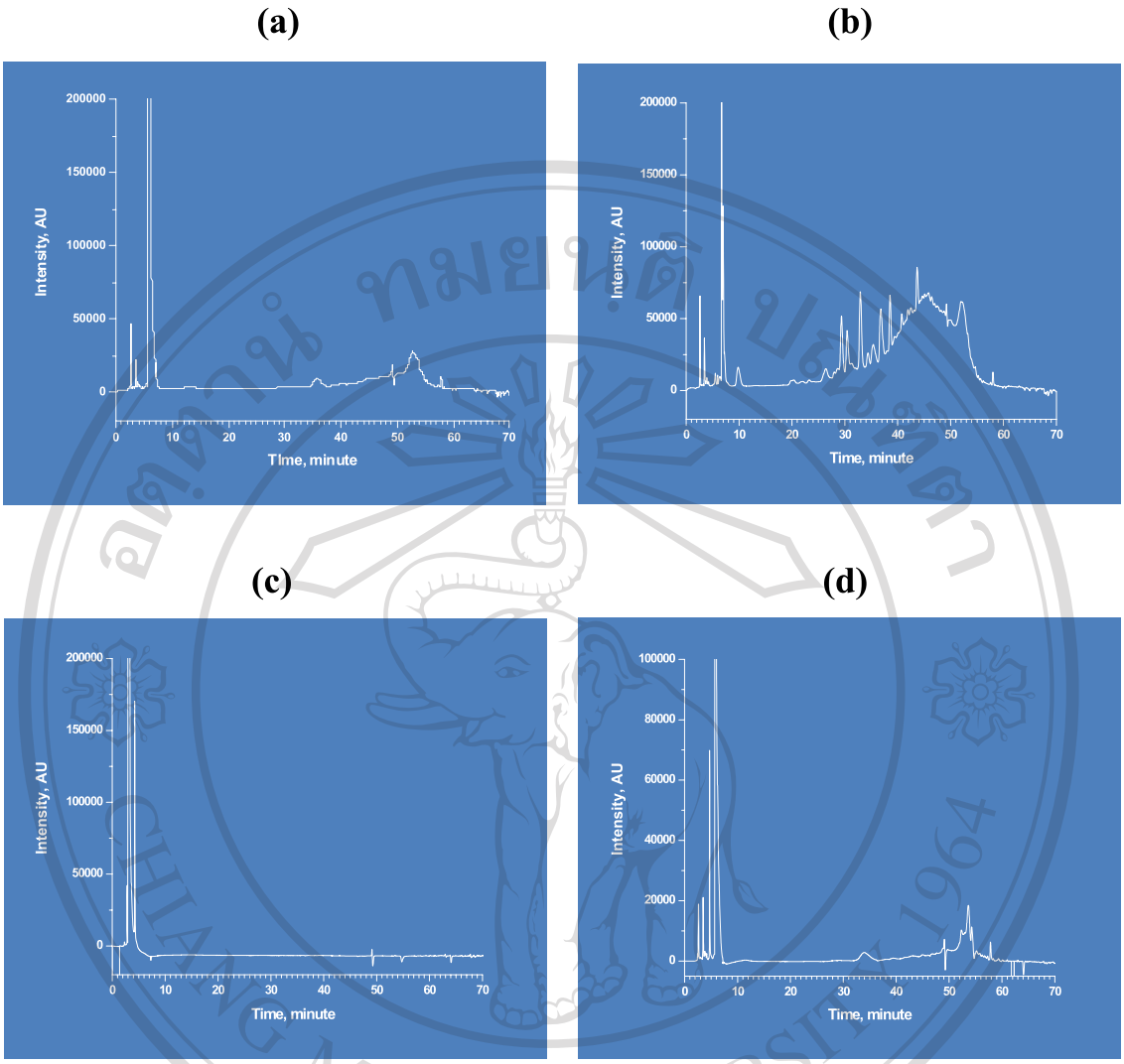

Figure 17. HPLC chromatograms of Siamois® crude extracts (absorption wavelength = 280 nm) obtained from solid/liquid extraction using **(a)** 12% EtOH in water, **(b)** 95% EtOH, **(c)** 70% acetone in watern and **(d)** 12% EtOH in acetronitrile.

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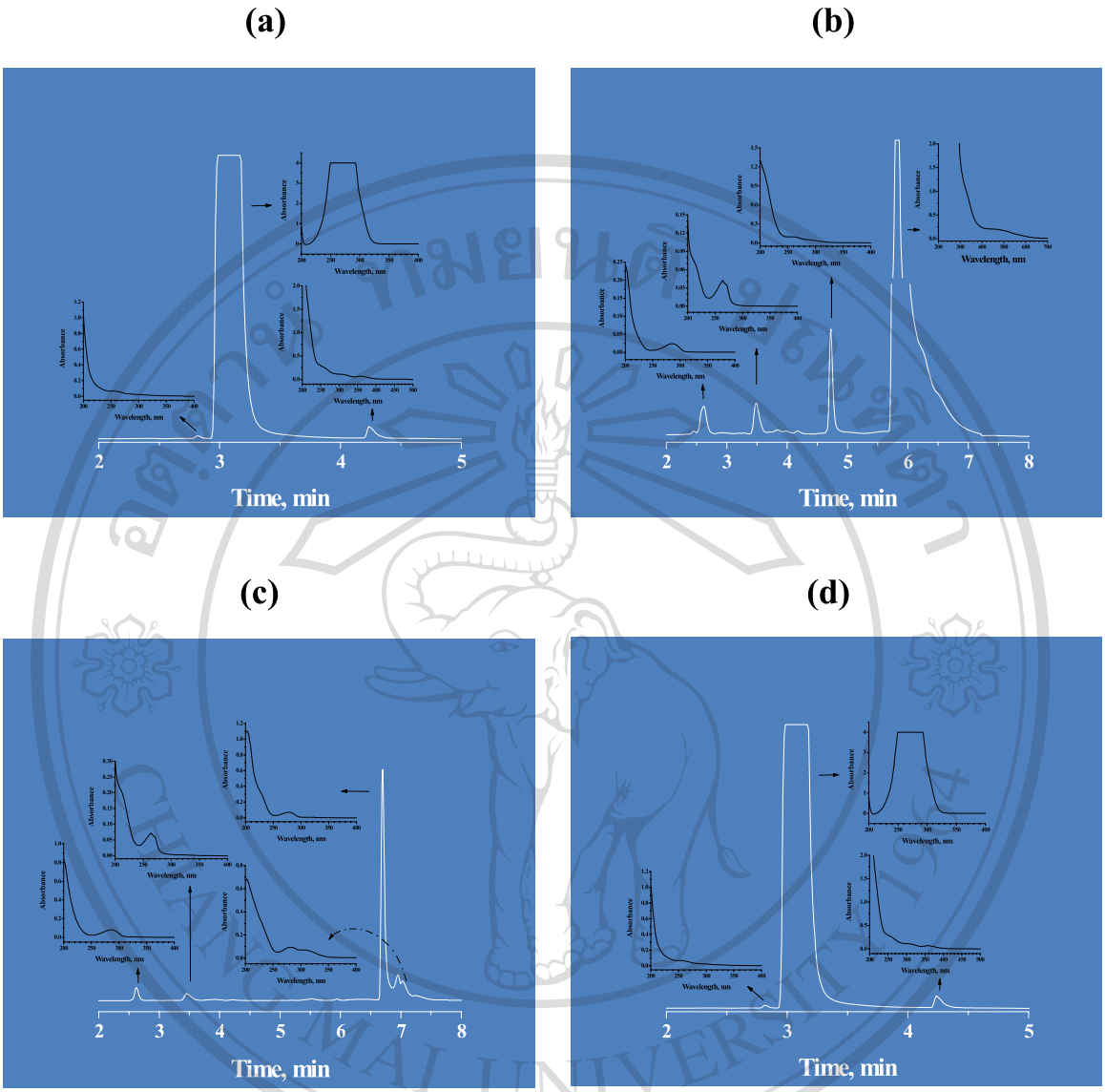

Figure 18. HPLC chromatograms of **(a-c)** pigments and **(d)** flavonoids present in Siamois® crude extract. HPLC peaks of flavonoids and pigments were analyzed by spectrophotometry and insets show the corresponding absorption spectra.

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2. Visualization of potential intracellular targets of Siamois®, Siamois 1 and Siamois 2 crude extracts in normal cells

The crude extracts of Siamois®, Siamois 1 and Siamois 2 are an enriched source of flavonoids that could be potential pharmaceutical materials for developing new

generation of anti-cancer drugs. In order to ascertain their cytotoxicity, normal myoblasts between the 3rd and 5th passage were used. After propagation, the cells at 70 % confluence showed a typical morphology observedin the muscle. The functional study of intracellular organelles, including lysosomes and mitochondria of myoblasts were clearly visualized by using acridine orange and rhodamine B as specific probes combined with fluorescence microscopy. Figure 19 a shows the fluorescence micrograph of living myoblasts stained with acridine orange, rhodamine B and pirarubicin. The bright green fluorescence light is due to the interaction of acridine orange with cytoskeleton proteins. The reddish orange fluorescence light is due to the accumulation of acridine orange in lysosomes. The bright yellow fluorescence light is due to the rhodamine B accumulation in the energetic mitochondria. Finally, the dim red fluorescence light is due to pirarubicin accumulation in the nuclear compartment. The reddish orange fluorescence light completely disappeared in the presence of 3 μM monensin resulting from the successive elimination of any pH gradient in the lysosomes. Similarly, the bright yellow fluorescence light vanished in the presence of 7 μM FCCP and 10 μM oligomycin.

The micrographs of myoblasts after treatment using 500 μg mL^{-1} of Siamois® or 5 μg mL^{-1} Siamois 1 or Siamois 2 for 72 h were illustrated in Figure 19 b and c, respectively. The micrographs revealed cells with various morphology and they preserved proliferative and differentiated properties. All crude extracts studied and doxorubicin caused a decrease in reddish orange and bright yellow fluorescence intensity and density of organelles compared with untreated cells. However, Siamois 1 and Siamois 2 (each at 500 μg mL^{-1}) did not cause any deleterious effects on the viability of cells, contrary they promoted the growth of cells. Doxorubicin (500 nM) caused a decrease in reddish orange

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and bright yellow fluorescence intensity and density of organelles compared with untreated cells, as indicated in Figure 19 d.

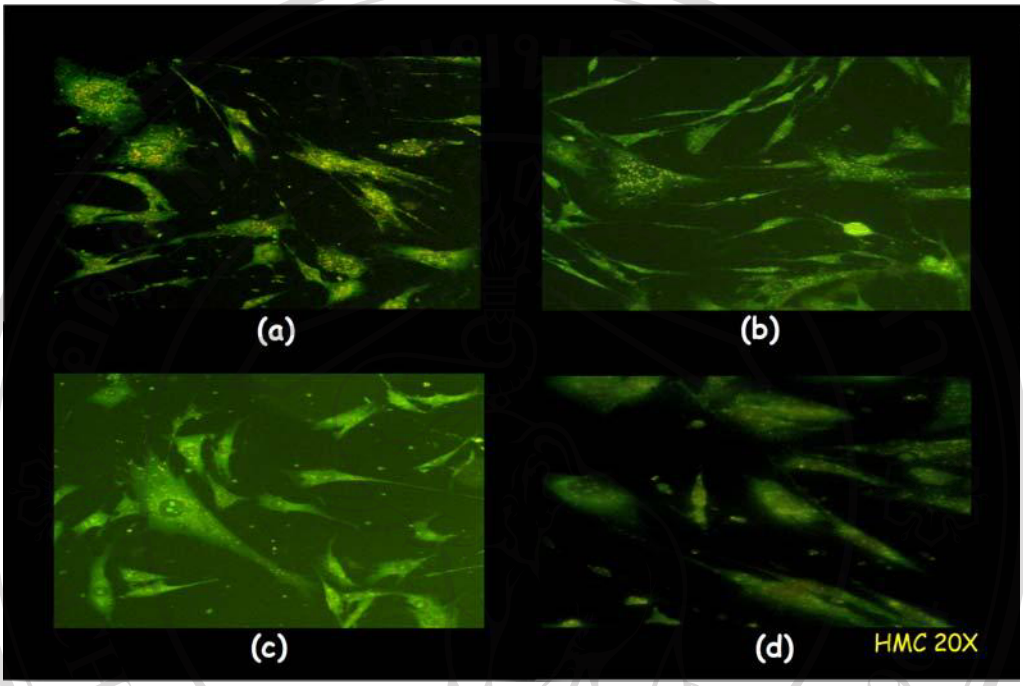

Figure 19. Fluorescence micrographs of myoblasts; **(a)** untreated myoblasts, or **(b)** myoblasts treated with 500 μg mL^{-1} Siamois 1, or **(c)** Siamois 2, or **(d)** 500 nM doxorubicin.

3. Cytotoxicity of Siamois®, Siamois 1 and Siamois 2 crude extracts against normal cells

The Siamois®, Siamois 1 and Siamois 2 concentration ranging from 0.05 mg mL^{-1} to 0.5 mg mL^{-1} efficiently promoted the growth of cells as shown in Figure 20 a. In contrast to doxorubicin treated cells, where the cell growth was completely inhibited although not causing death even at the highest concentration tested (500 nM), as shown in Figure 20 b.

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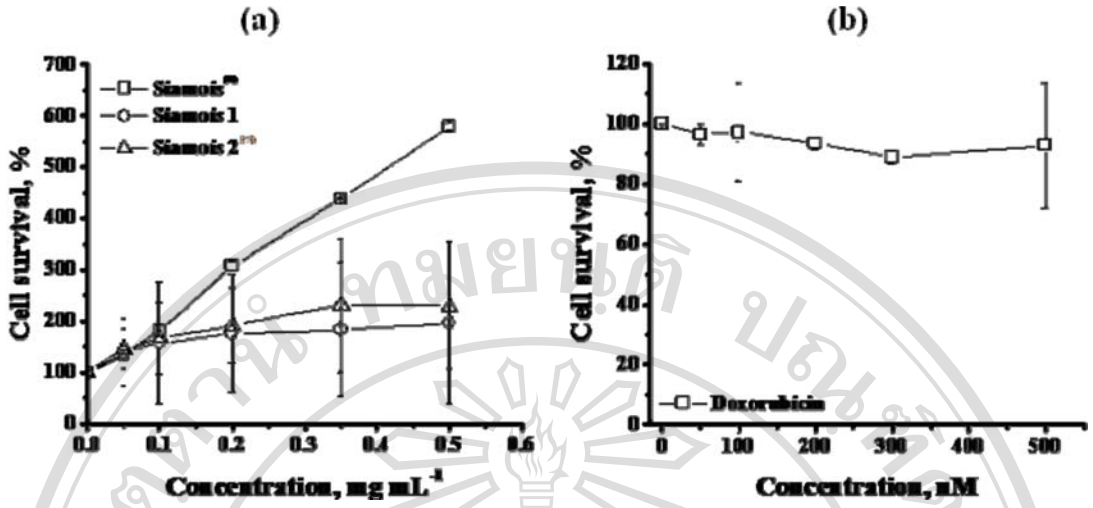

Figure 20. Effects of **(a)** Siamois®, Siamois 1 and Siamois 2 and **(b)** doxorubicin on myoblast cell growth.

4. Anti-cancer activity of Siamois® and Siamois 1 crude extracts

Anti-cancer activity of Siamois® polyphenols was evaluated by using MTT assay and the result demonstrated that Siamois® and Siamois 1 apparently exhibited anti-cancer activity against K562, K562/Adr, GLC4, and GLC4/Adr cells, as indicated in Figure 21. As indicated in Table 5, Siamois® concentrations required to inhibit by 50% the cell growth (IC_{50}) were 2650 ± 210, 2870 ± 110, 3290 ± 180, and 3230 ± 90 μg mL^{-1} for K562, K562/Adr, GLC4, and GLC4/Adr cells, respectively. Siamois 1 exhibited the most antiproliferative activity (40-160 folds) in the four cancer cell lines compared to Siamois®. The IC_{50} values were 60 ± 20, 30 ± 30, 60 ± 30 and 20 ± 10 μg mL^{-1} for K562, K562/Adr, GLC4 and GLC4/Adr cells, respectively. The Siamois 2 revealed more efficacy (40-160 folds) to inhibit cell growth in four cancer cell lines than Siamois® but slightly less than Siamois 1. The IC_{50} values were 70 ± 20, 50 ± 10, 110 ± 10 and 80 ± 20 μg mL^{-1} for K562, K562/Adr, GLC4, and GLC4/Adr cells, respectively.

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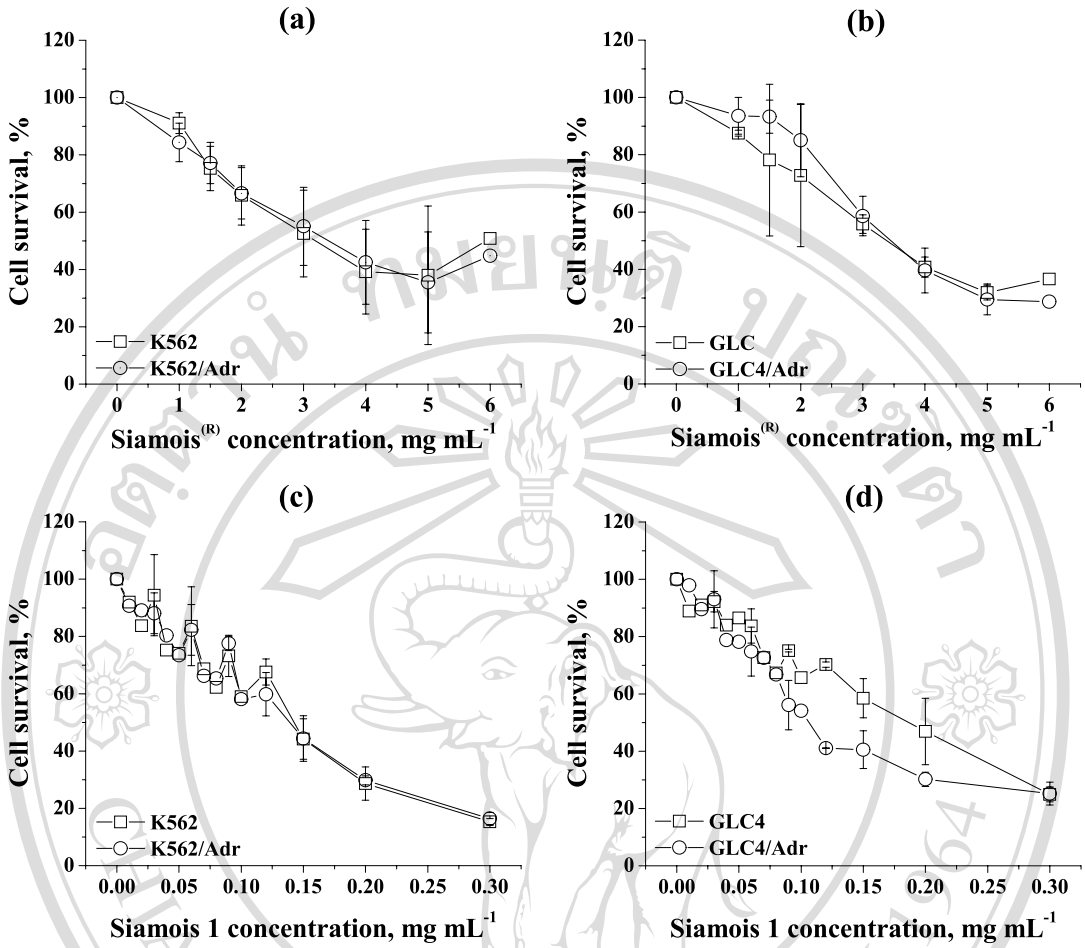

Figure 21. Effects of Siamois® on **(a)** K562 and K562/Adr, and **(b)** GLC4 and GLC4/Adr cell growth and Siamois 1 on **(c)** K562 and K562/Adr and **(d)** GLC4 and GLC4/Adr cell growth.

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Table 5. IC50 value of Siamois®, Siamois 1 and Siamois 2 crude extracts, quercetin, and doxorubicin in K562, K562/Adr, GLC4, and GLC4/Adr cells.

Cell lines	IC50*				
	Siamois® (mg mL^{-1})	Siamois 1 (mg mL^{-1})	Siamois 2 (mg mL^{-1})	Quercetin (μg mL^{-1})	Doxorubicin (μg mL^{-1})
K562	2.65 ± 0.21	0.06 ± 0.02	0.07 ± 0.02	7.8 ± 0.6	0.1 ± 0.02
K562/Adr	2.87 ± 0.11	0.03 ± 0.03	0.05 ± 0.01	9.7 ± 0.9	2.4 ± 0.6
GLC4	3.29 ± 0.18	0.06 ± 0.03	0.11 ± 0.01	6.6 ± 0.7	0.1± 0.01
GLC4/Adr	3.23 ± 0.90	0.02 ± 0.01	0.08 ± 0.02	3.3 ± 0.3	4.6± 0.07

* *IC_{50}, compound concentrations required to inhibit cell growth by 50%, at 72 h*

For overcoming the MDR phenomenon, the resistance factor (RF) which is ratio of IC_{50} of a compound in resistant cells and the IC_{50} in sensitive cells should be considered. When the RF is less than 1, it means that the compound exhibits more antiproliferative activity in resistant cells compared to sensitive cells. The RF values of Siamois®, Siamois 1, Siamois 2, quercetin and doxorubicin in both human erythromyelogenous leukemic and small cell-lung carcinoma cell lines are shown in Table 6. In this respect, Siamois®, Siamois 1, Siamois 2 and quercetin can overcome MDR compared to doxorubicin, a clinical used drug (Table 6).

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Table 6. Resistance factor (RF) of Siamois®, Siamois 1 and Siamois 2 crude extracts, quercetin, and doxorubicin in K562, K562/Adr, GLC4, and GLC4/Adr cells..

Cell lines	Resistance factor (RF)**				
	Siamois®	Siamois 1	Siamois 2	Quercetin	Doxorubicin
K562/Adr	1.1	0.5	0.7	1.2	24
GLC4/Adr	1	0.3	0.7	0.5	46

** *RF, ratio of the IC_{50} of a compound in resistant cells to that in the sensitive cells*

5. Effects of Siamois®, Siamois 1 and Siamois 2 crude extracts on cellular ATP, ADP and AMP levels in cancer cells

The Siamois® caused a decrease in rhodamine B accumulation in the cells. In order to identify the intracellular targets of the tested compounds, two series of experiments were performed in parallel. The cancer cells were incubated in the presence of the compounds for 24 h at 37 °C in a humidified incubator at 5% CO_2. In one series of experiments, the treated cells were examined under an inverted fluorescence microscope. Siamois 1 and Siamois 2 caused a dramatic decrease in bright yellow fluorescence intensity and the density of organelles, reflecting an alteration in mitochondrial function. In a second experimental series, the cells were collected for analysis of cellular ATP. Typical results of the variation in ATP, ADP and AMP levels in cancer cells treated with Siamois 2 are shown in Figure 22. These data indicated that Siamois 2 caused a dramatic decrease in the amount of ATP, ADP and AMP. Similar results were obtained for Siamois 1. Thus, both compounds affected energy levels within the cells.

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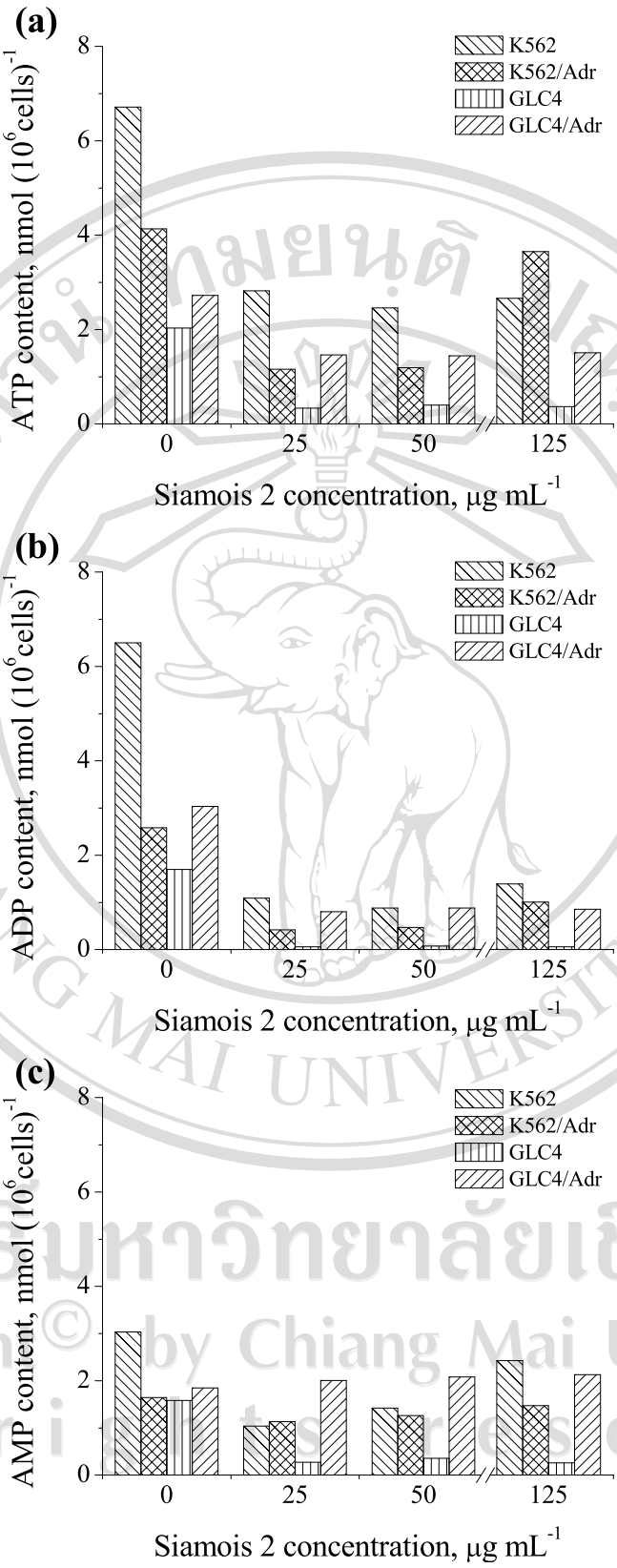

Figure 22. Effects of Siamois 2 on cellular **(a)** ATP, **(b)** ADP and **(c)** AMP levels in cancer cells.

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6. Apoptosis-inducing activity of Siamois® crude extract

Because the chemical composition of Siamois® crude extract can change with time, the apoptosis-inducing activity of Siamois® crude extract was evaluated as a function of wine age (0, 8, 12 months). The series of experiments were performed using annexin V-FITC and PI staining in K562, K562/Adr, GLC4 and GLC4/Adr cell. The result showed that Siamois® exhibited similar apoptosis-inducing activities in the four cell lines, as indicated in Figure 23 and apoptosis levels appeared to be independent of the age of the wine (50% total apoptosis at 15 mg mL^{-1} for 24 h).

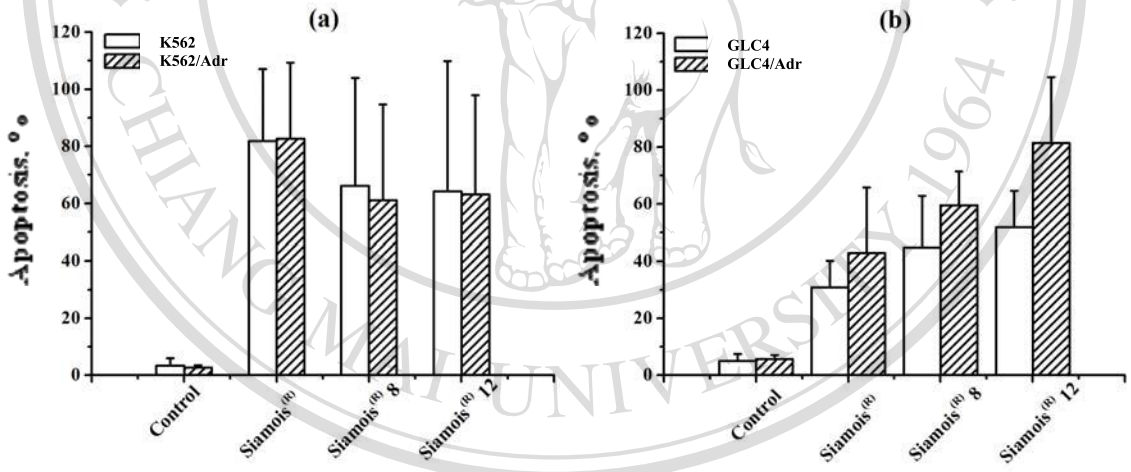

Figure 23. 24 h apoptosis-induced activities of Siamois® crude extract at 15 mg mL^{-1} against **(a)** K562 and K562/Adr and **(b)** GLC4 and GLC4/Adr cells.

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7. Putative active polyphenols in Siamois®, Siamois 1 and Siamois 2 crude extracts

According to the analytic chromatographic profiles (Figure 14), we believed that the most potent active molecules of these crude extracts should be flavonoids particularly the most abundant such as quercetin, kaempferol, apigenin and eriodyctiol.

8. Modulation of mitochondrial membrane potential ($\Delta\Psi_m$) and apoptotic induction by quercetin

Modulation of absolute value of $\Delta\Psi_m$ ($|\Delta\Psi_m|$) in K562, K562/Adr, GLC4, and GLC4/Adr cells in the presence of quercetin was investigated as a function of time. At basal level (without quercetin), $|\Delta\Psi_m|$ was 160 ± 1.0, 145 ± 1.2, 154 ± 2.3, and 160 ± 2.1 mV in K562, K562/Adr, GLC4, and GLC4/Adr cells, respectively. Typical results of quercetin- induced $\Delta\Psi_m$ changes as a function of time are shown in Figure 24 a and b. After adding quercetin (10 μM), $|\Delta\Psi_m|$ increased slightly to reach a maximal value within 1.0 and 0.5 h, then progressively decreased by 5.5 and 3.8% of the initial value at 3 h for K562 and K562/Adr cells, respectively. For GLC4 and GLC4/Adr cells, $|\Delta\Psi_m|$ elevated to reach a maximal value within 2 h, then progressively declined and revert to be almost the same level as the initial value at 6 h. An increase or a decrease in $|\Delta\Psi_m|$ after addition of quercetin, due to an increase or a decrease in mitochondrial matrix rhodamine B concentration, which depends on the mitochondrial energetic state, is not a result of direct inhibition of P-glycoprotein function by quercetin. This suggests that quercetin mediates action at mitochondrial level in K562 and K562/Adr cells but not in GLC4 and GLC4/Adr cells. The decrease in $|\Delta\Psi_m|$ correlated with an increase in percentage of early apoptotic cells that can be detected at 1 h in quercetin treated K562 and K562/Adr cells (Figure 24 a and c). In case of GLC4 and GLC4/Adr cells, the maximal percentage of early apoptotic cells was reached at 24 and 72 h, respectively associated with a delayed reduction of the $|\Delta\Psi_m|$ (more than 6 h) as shown in Figure 24 b and d.

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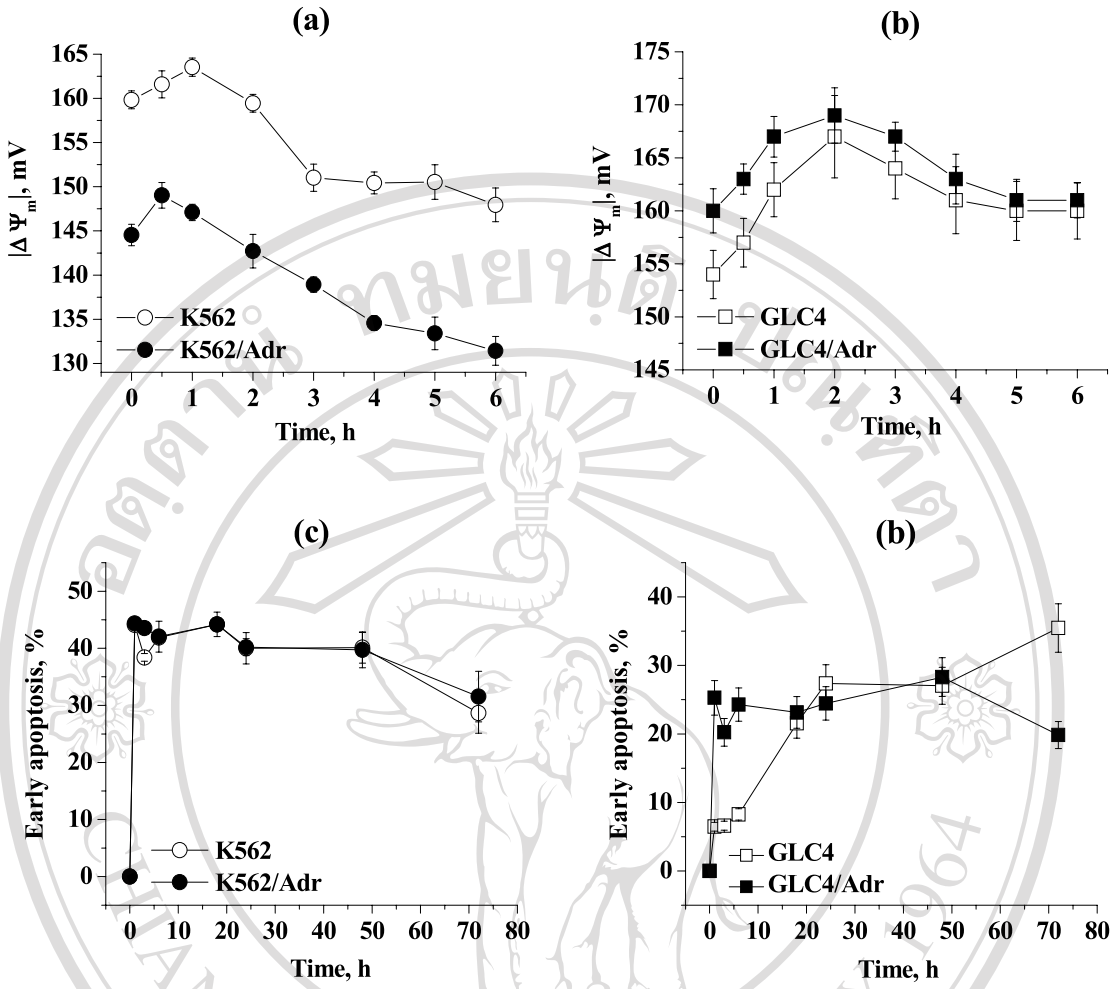

Figure 24. Spontaneous change of absolute value of $\Delta\Psi_m$ and apoptosis-induced activity in the presence of 10 μM quercetin. **(a-b),** $|\Delta\Psi_m|$ values; **(c-d),** percentages of early apoptotic cells were determined as a function of time in K562, K562/Adr, GLC4, and GLC4/Adr cells in the presence of 10 μM quercetin.

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9. Pure polyphenols present in Siamois® crude extractdose dependently inhibited NFκB-driven reporter gene expression in murine L929sA cells

As anti-cancer properties of various polyphenols have been linked to inhibition of the inflammatory transcription factor NFκB, we first compared potential anti-inflammatory properties of the Siamois® polyphenols quercetin, kaempferol, eriodictyol,

and WP283 in NFκB-driven reporter gene assays. We performed a dose-response experiment in L929sA cells, stably transfected with a TNF-inducible NFκB-driven reporter gene construct with a minimal IL6 promoter ($p(IL6\kappa B)_3$-50hu.IL6P-luc+) and a constitutively expressed reporter gene construct (pPGκBGeobpA) controlled by the phosphoglycerokinase promoter (187) for normalization of reporter gene expression. Upon TNF treatment (2000 IU mL^{-1} for 4 h), significant promoter induction can be observed with the NFκB-driven reporter gene construct, which can be reversed with quercetin, kaempferol, eriodictyol, or WP283 in a dose-dependent manner. The inhibitory concentration for NFκB-driven reporter gene expression by 50% of the different polyphenols was in the range of 30 to 50 μM (Figure 25).

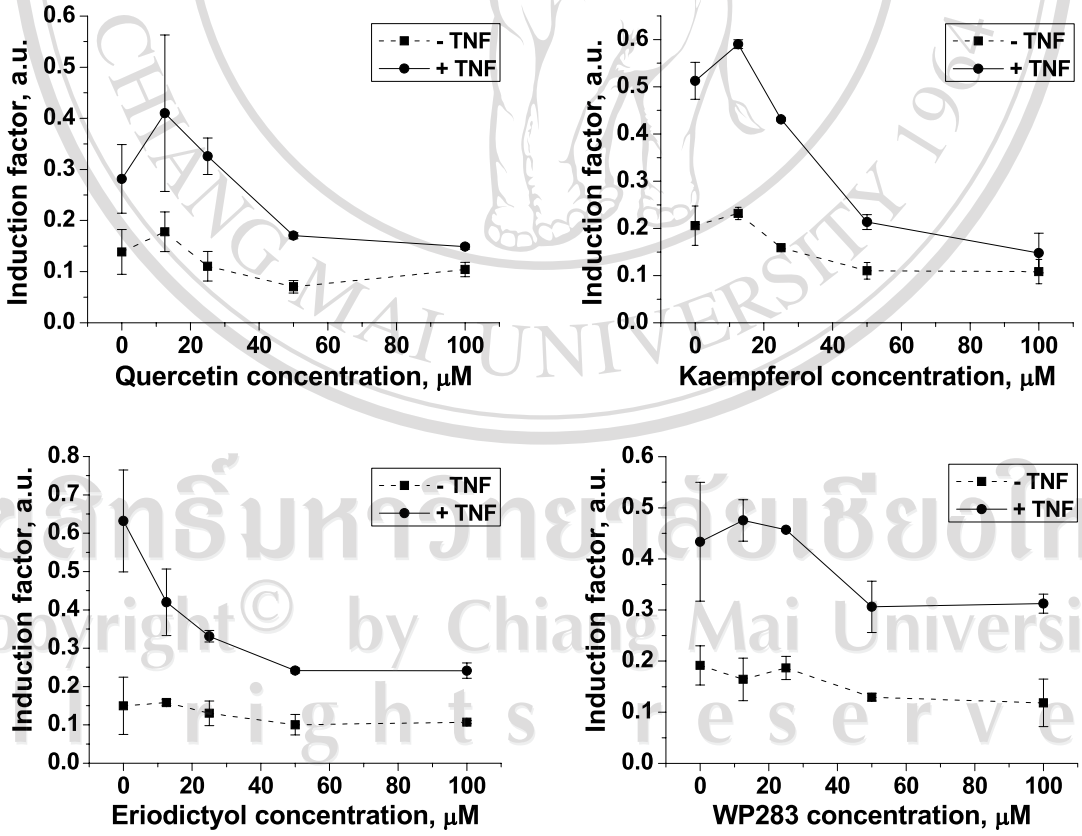

Figure 25. Pure polyphenols present in Siamois® crude extract dose-dependently inhibited NFκB-dependent reporter gene expression in murine L929sA cells.

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10. Pure polyphenols present in Siamois® crude extract inhibited similarly endogenous NFκB target gene expression in K562 and K562/Adr cell lines

To extend our reporter gene expression results in a more specific cancer settings, we further studied pure polyphenols present in Siamois® crude extract effects in K562 and K562/Adr cells, which may demonstrate different NFκB activation status related to doxorubicin sensitivity (194). Since NFκB hyperactivation is involved in chemoresistance, we next evaluated whether different types of NFκB inhibitors may have different effects on endogenous NFκB target genes in K562 and K562/Adr cells, involved in inflammation and metastasis (IL6, IL8, MCP1, A20), cell cycle (cyclin D1), angiogenesis (VEGF), multidrug resistance (mdr1/P-gp) and apoptosis (A1/Bfl1). Cells were pretreated with pure polyphenols present in Siamois® crude extract for 2 h, either or not following 3 h treatment of PMA, after which RNA was isolated and mRNA levels of interest were quantified by Q-PCR with specific primers. As illustrated in Figure 26, NFκB target genes are potently induced by PMA in both cell types. NFκB target genes are differentially expressed in K562 as compared to K562/Adr cells. In contrast to IL6, IL8, MCP1, and A1/Bfl1 which revealed stronger transcription in K562 cells, the A20, cyclin D1, VEGF, and mdr1/P-gp genes are preferentially expressed in K562/Adr cells. Furthermore, repression of PMA inducible NFκB target genes can be observed in K562 and K562/Adr cells, irrespective of mdr1/P-gp expression. Interestingly, although NFκB inhibitors can completely reverse PMA-inducible effects of P-gp in K562/Adr cells, basal transcription levels cannot be reversed to the background levels, as observed in K562 cells. Finally, efficacy of target gene repression seems also to be compound- and target gene-specific. Altogether, these results demonstrate selective inhibitory effects of pure

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polyphenols present in Siamois® crude extract on target genes involved in inflammation, metastasis, cell cycle, angiogenesis, multidrug resistance, and anti-apoptosis in doxorubicin-sensitive and -resistant K562 cells.

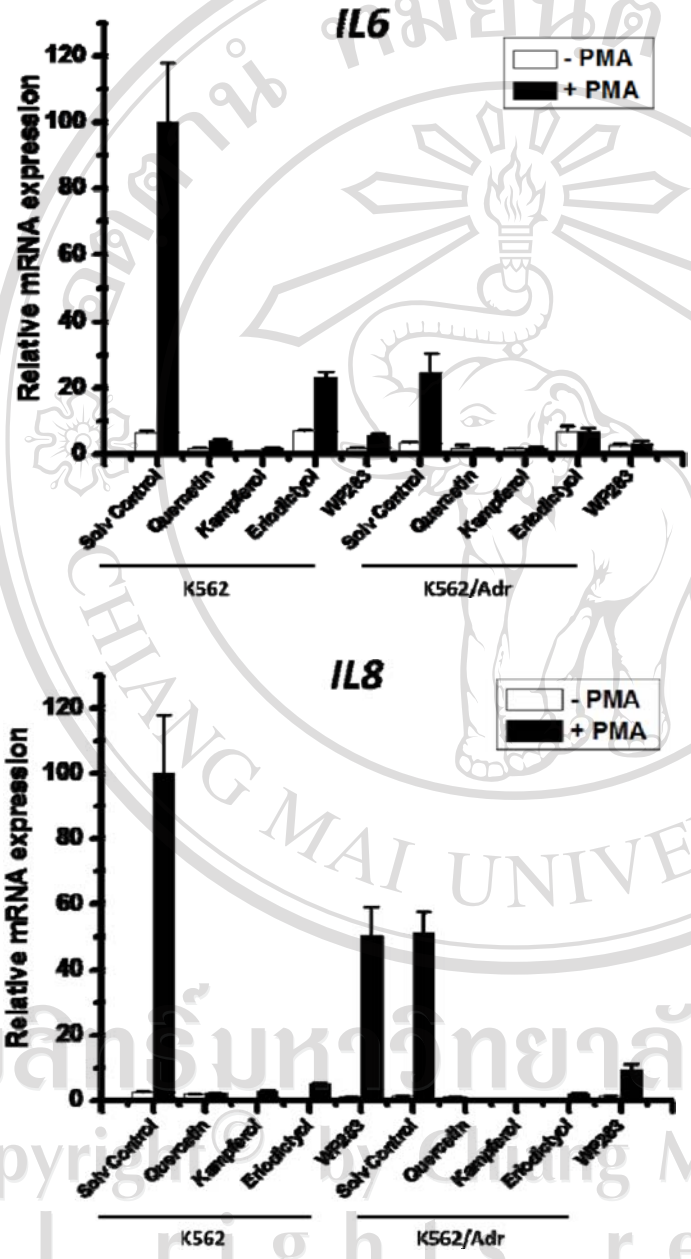

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Figure 26 Pure polyphenols present in Siamois® crude extract inhibited endogenous NFκB-dependent transcription in K562 and K562/Adr cells in the absence or presence of 100 μM of each pure polyphenols for 5 h.

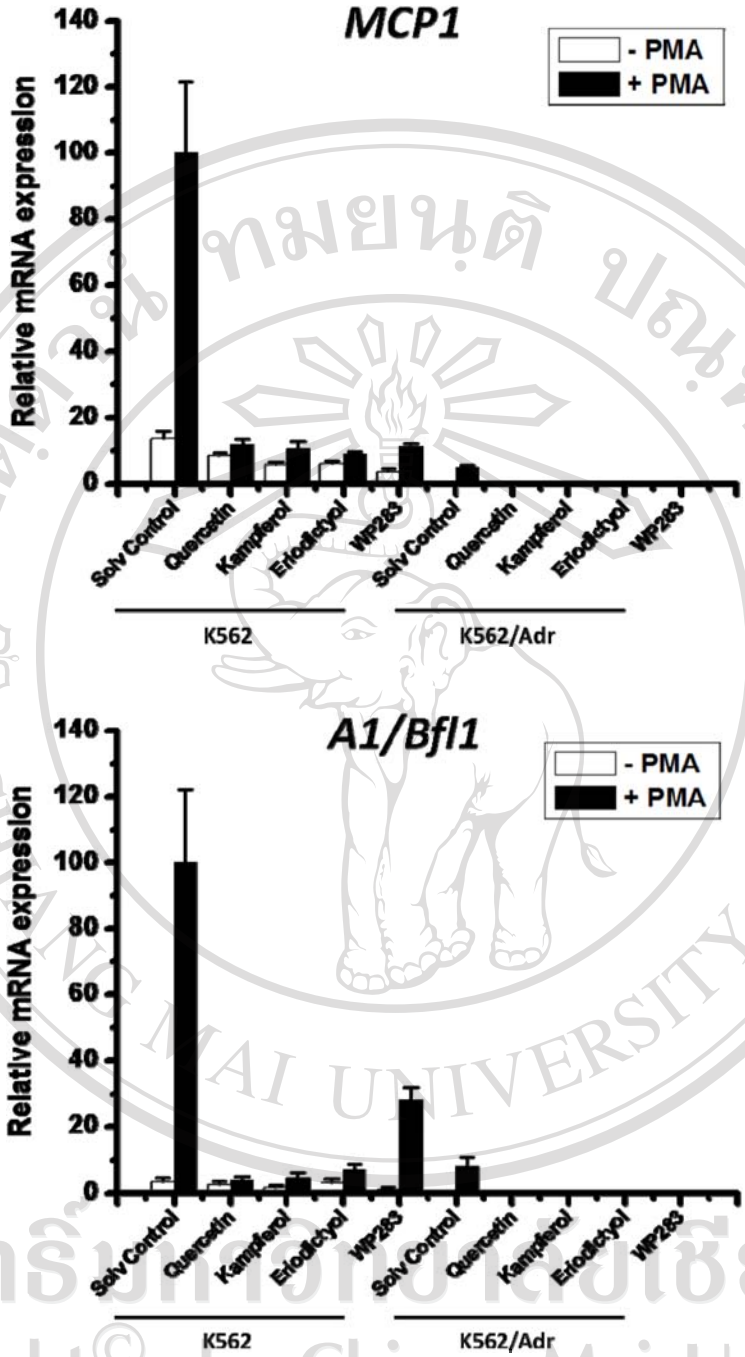

Figure 26 Pure polyphenols present in Siamois® crude extract inhibited endogenous NFκB-dependent transcription in K562 and K562/Adr cells in the absence or presence of 100 μM of each pure polyphenol for 5 h (continued).

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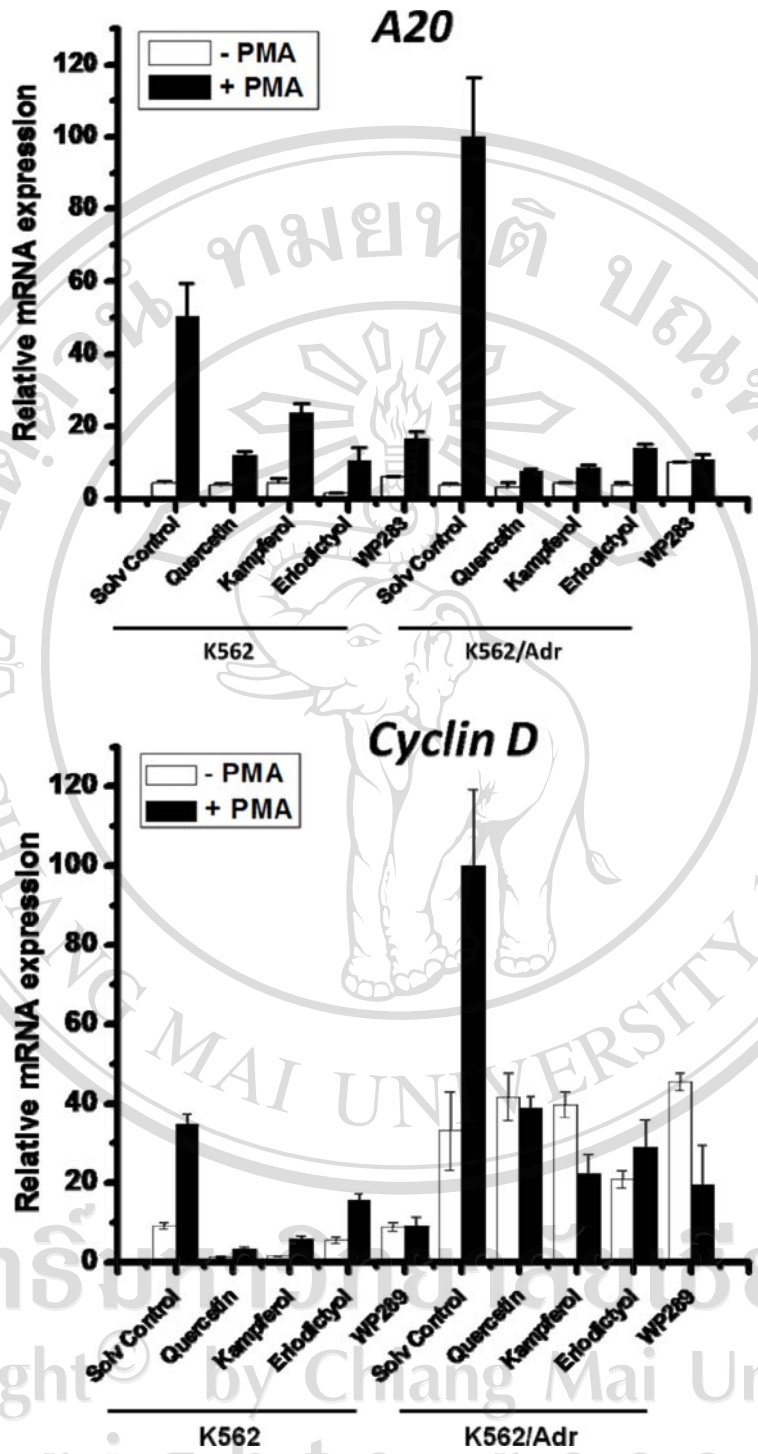

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Figure 26 Pure polyphenols present in Siamois® crude extract inhibited endogenous NFκB-dependent transcription in K562 and K562/Adr cells in the absence or presence of 100 μM of each pure polyphenol for 5 h (continued).

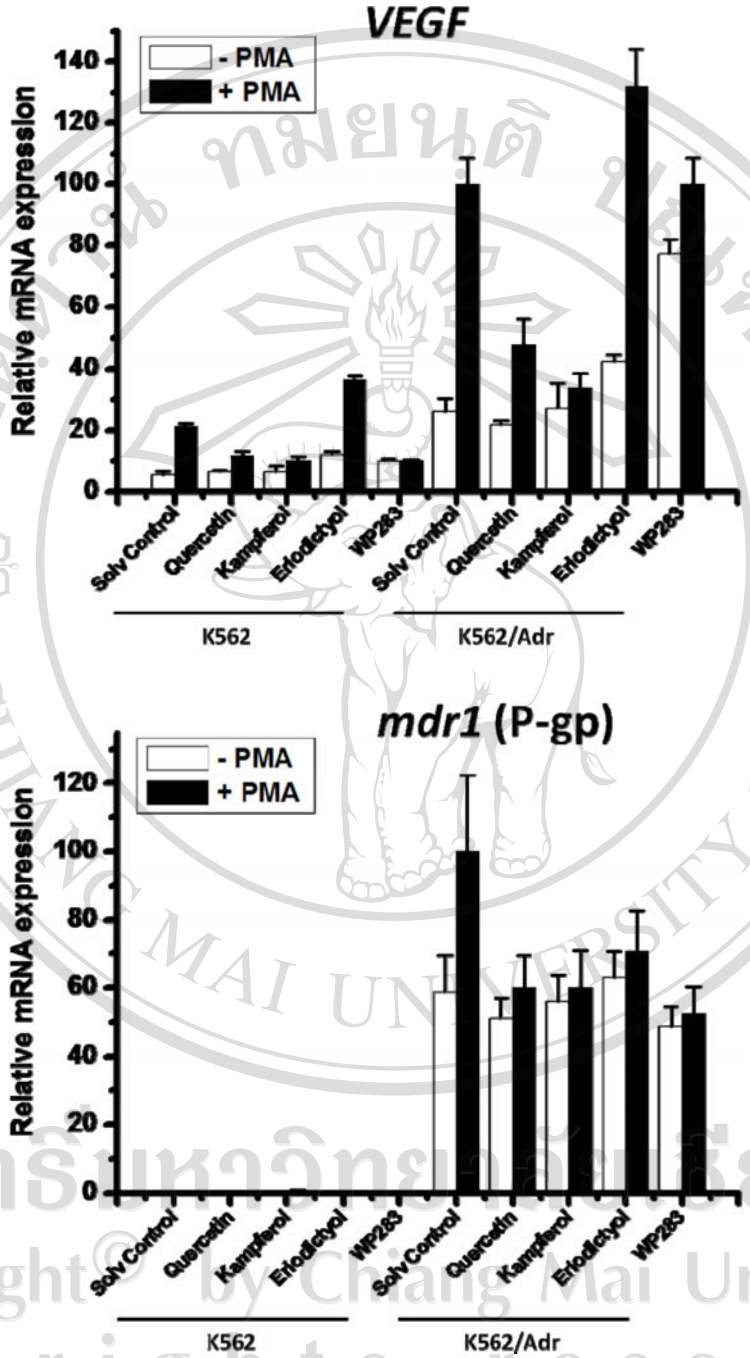

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Figure 26. Pure polyphenols present in Siamois® crude extract inhibited endogenous NFκB-dependent transcription in K562 and K562/Adr cells in the absence or presence of 100 μM of each pure polyphenol for 5 h (continued).

11. Pure polyphenols present in Siamois® crude extract all prevent IκBα degradation but selectively interfere with p38, ERK MAPK, MEK1 and Akt kinase activation

As NFκB target gene expression encompasses multiple regulatory steps, including IκBα degradation, NFκB translocation, NFκB/DNA binding and NFκB transactivation we next examined which regulatory steps are affected by pure polyphenols present in Siamois® crude extract in K562 and K562/Adr cells. Since IκBα degradation is required for liberation and subsequent translocation of NFκB to the nucleus, we determined Pure polyphenols present in Siamois® crude extract effects on PMA-induced IκBα protein degradation in K562 and K562/Adr cells. As maximal degradation of IκBα is observed between 15-30 minutes after PMA treatment, we measure effects of Pure polyphenols present in Siamois® crude extract on IκBα degradation following 2 h pretreatment and 30 minutes cotreatment with PMA. From Figure 27 a, it can be observed that in both cell types 30 minutes of PMA treatment is able to reduce IκBα levels. Furthermore, all tested compounds reduce IκBα degradation in both cell types (Figure 27 b). Along the same line, all tested compounds significantly reduce basal and/or PMA inducible p65 Ser 536 phosphorylation in both cell types. Altogether, these results suggest that activation of NFκB and subsequent translocation of NFκB for gene induction is significantly reduced in presence of pure polyphenols present in Siamois® crude extract.

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As target gene-specific effects are also depending on p65 phosphorylation status and epigenetic settings, dynamically controlled by multiple kinase pathways, i.e. Akt, MAPK, MSK, PKA, we next measured the phosphorylated levels of the following species: P-Akt, P-p38, P-ERK, P-MEK1 in the various experimental conditions in both

cell types. A significant reduction of basal and PMA-induced P-Akt and P-p38 levels can be observed upon treatment with quercetin and kaempferol in both K562 cell types (Figure 27 b), whereas P-ERK levels do not reveal significant inhibition (Figure 27 c). Western analysis against p38 and ERK protein levels confirms equal protein loading in the various experimental setups (Figure 27 b and c). Interestingly, pure polyphenols present in Siamois® crude extract demonstrate increased MEK1 phosphorylation in K562/Adr cells, suggesting that uptake of compounds is not impaired in P-gp overexpressing K562/Adr cells. Altogether, besides significant inhibition of IκBα degradation and NFκB p65 Ser 536 activation by pure polyphenols present in Siamois® crude extract, compound-specific regulation of p38, ERK, Akt, and MEK1 kinases could be observed, which may further interfere with nuclear transcriptional regulation of NFκB target genes (195-197).

(a)

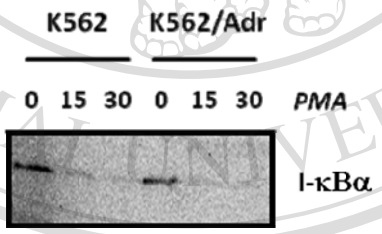

Figure 27. Selective effects of pure polyphenols present in Siamois® crude extract on the NFκB signaling pathway in the absence or presence of 100 μM of each pure polyphenol for 2.5 h. Quer, quercetin; Kaem, kaempferol; Erio, eriodictyol; WP, WP283.

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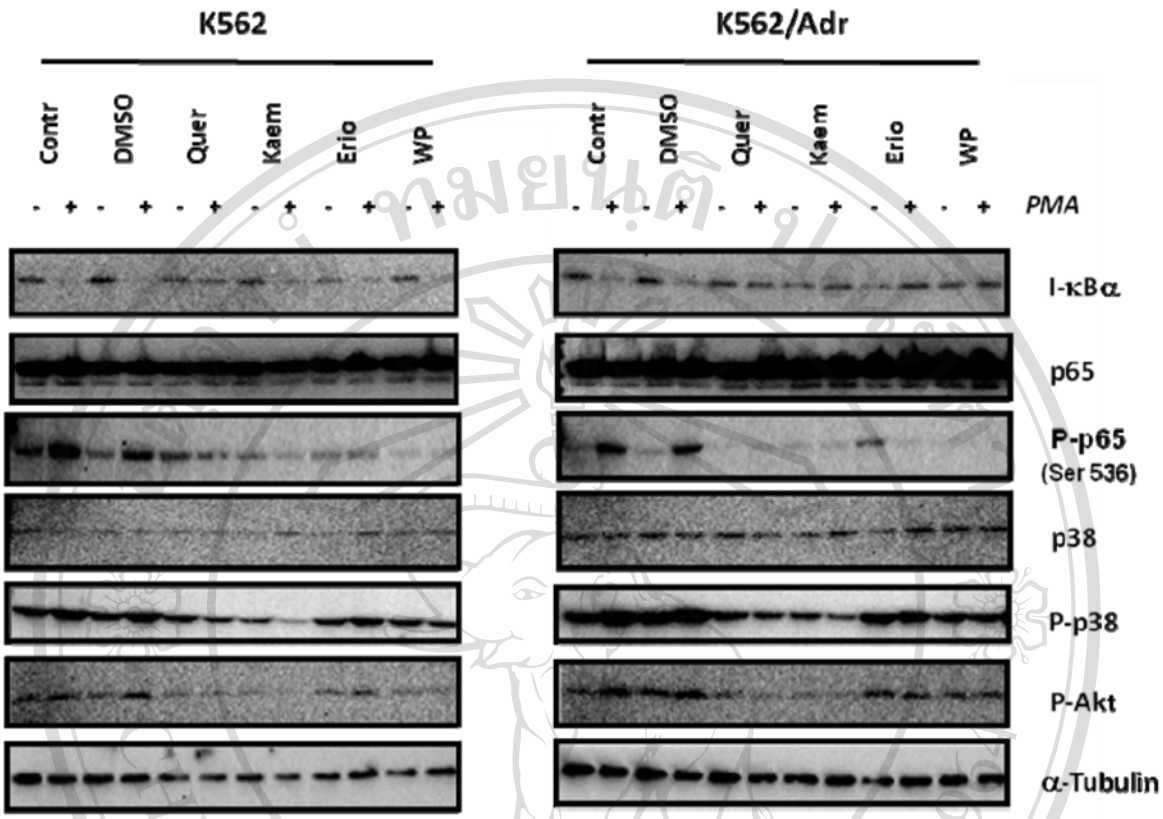

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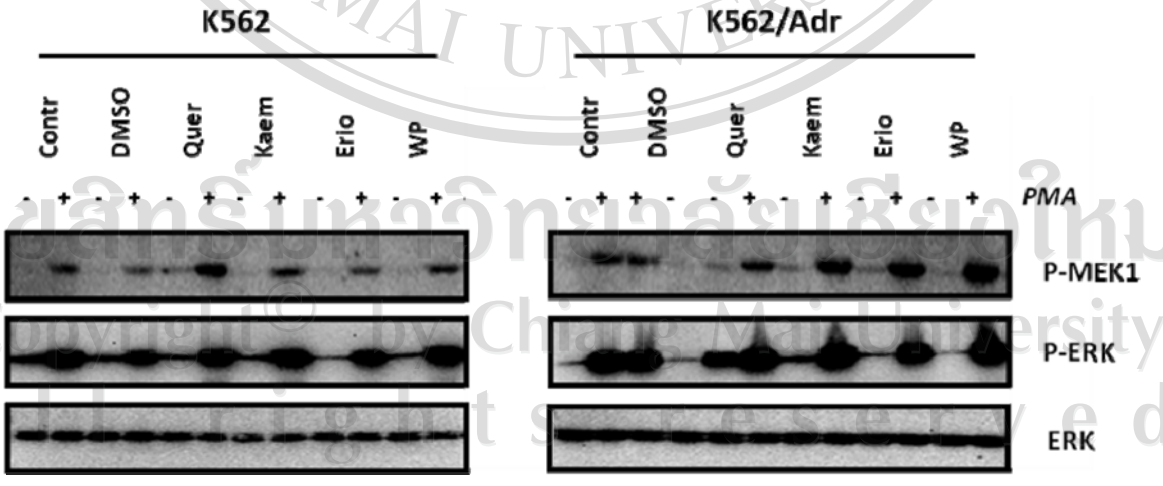

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Figure 27. Selective effects of pure polyphenols present in Siamois® crude extract on the NFκB signaling pathway in the absence or presence of 100 μM of each pure polyphenol for 2.5 h. Quer, quercetin; Kaem, kaempferol; Erio, eriodictyol; WP, WP283 (continued).

12. K562 and K562/Adr cells reveal distinct nuclear regulation of NFκB, AP1, Nrf2 and Sirt1 proteins

As K562 and K562/Adr cells demonstrate differential regulation of NFκB target genes, we next explored whether both cell types may show different nuclear regulation of potential cooperative transcription factors (i.e. AP1, Nrf2) or cofactors (Sirt1) which might coregulate NFκB target genes. As can be observed from Figure 28, basal levels of nuclear NFκB p65, AP1 c-Jun, Jun D and Fra1 are significantly increased in K562/Adr as compared to K562 cells, in contrast to c-Rel and Rel B. This agrees with previous observations on doxorubicin-resistant MCF7 cells, in which AP1 transcription factors were demonstrated to be responsible for upregulation of P-gp/mdr1 (198). Furthermore, PMA treatment significantly increases nuclear levels of NFκB p65, Rel B, c-Rel. Of special note, increased nuclear levels of Nrf2 upon PMA treatment are more pronounced in K562/Adr than in K562 cells. Only recently, the involvement of Nrf2 has been demonstrated in chemoresistance (199). Also in line with previous studies on the role of Sirt1 in chemoresistance, basal Sirt1 levels are slightly increased in doxorubicin-resistant K562/Adr cells. Particularly, Sirt1 was found to positively contribute in P-gp/mdr1 expression (200). Altogether, our results demonstrate that activities of NFκB p65, AP1 c-jun, jun D, Fra1, Nrf2 transcription factors and Sirt1 cofactors are increased in doxorubicin resistant K562/Adr cells.

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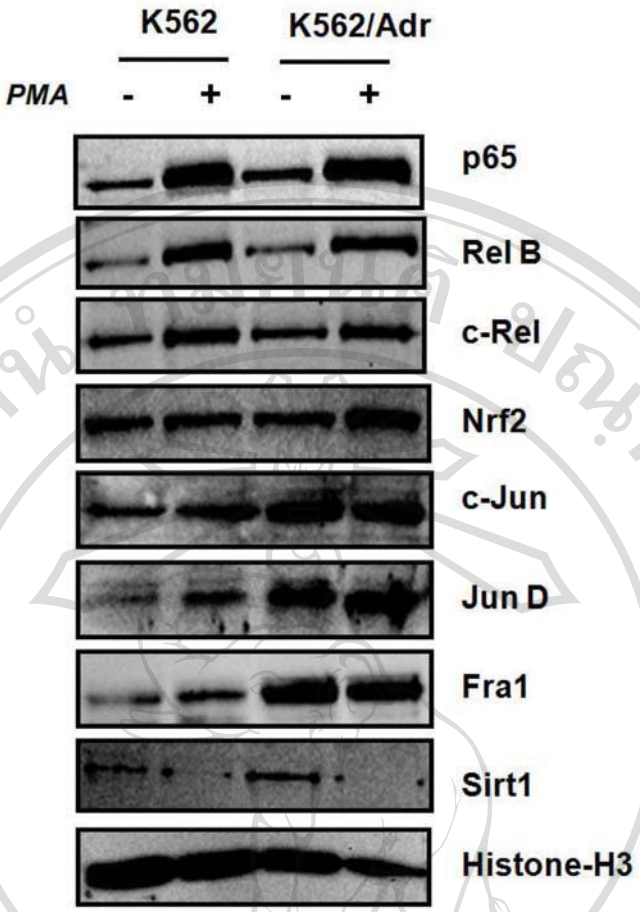

Figure 28. K562 and K562/Adr cells reveal different nuclear regulation of NFκB, AP1, Nrf2 transcription factors and Sirt1 cofactors.

13. NFκB, AP1/DNA-binding profiles in K562 and K562/Adr cells show qualitative and quantitative differences

To compare DNA-binding properties of NFκB, AP1 in K562 and K562/Adr cells, we performed competitive and and supershift analysis of electrophoretic gel shift mobility assays (EMSA) in response to PMA stimulation. Figure 29 a reveals that both cell types showed inducible NFκB/DNA-binding, whereas basal NFκB/DNA-binding is slightly elevated in doxorubicin-resistant K562/Adr cells, in line with observations that doxorubicin can elevate basal NFκB activation via DNA damage pathways (201). Interestingly, supershift analysis reveals subtle differences in the heterodimer/homodimer

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composition of DNA-bound NFκB- and AP1-binding complexes in both cell types. Supershift analysis reveals at least three different NFκB/DNA-binding complexes including p65-p65, p50-p65, and p50-p50. Basal constitutive p50-p50 and p50-p65 NFkB/DNA-binding activity in K562/Adr is increased as compared to K562 cells. Similarly, increased basal and inducible AP1-binding is detected in K562/Adr cells in comparison with K562 cells, in line with increased levels of nuclear AP1 members. Furthermore, although both cell types demonstrate PMA-inducible NFκB/DNA-binding, K562 cells show higher intensity of p65-p65 homodimers but comparable amounts of p50-p65 and p50-p50 DNA-binding complexes in comparison to K562/Adr cells (Figure 29 a). Concerning AP1-binding complexes, increased Fra1 levels can be detected in K562/Adr cells as compared to K562 cells. EMSA competition with excess unlabelled NFκB or AP1/DNA-binding motifs further demonstrates specificity of the DNA-bound NFκB, RBP-Jκ and AP1-binding complexes.

14. Quercetin and eriodictyol both present in Siamois® crude extract strongly inhibit DNA binding of NFκB, AP1 and Nrf2

To verify whether transcriptional repression of target genes involved in inflammation, anti-apoptosis, angiogenesis, metastasis, drug resistance by pure polyphenols present in Siamois® crude extract may be the consequence of inhibition of NFκB, AP1 or Nrf2 TF/DNA binding in K562 and K562/Adr cells, we performed EMSA experiments with nuclear extracts from cells treated with PMA alone, or following pretreatment with Siamois® polyphenols. As shown in Figure 29 b, basal constitutive p50-p50 and p50-p65 NFκB/DNA-binding activity in K562/Adr is increased as compared to K562 cells. PMA stimulation again increases p50-p50 and p50-p65 NFκB/DNA

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binding in both cell types whereas p65-p65 homodimers demonstrate stronger DNA binding in K562 only. Furthermore, treatment with different pure polyphenols present in Siamois® crude extract causes strong to moderate inhibition of the basal and inducible NFκB-and AP1-DNA binding complexes, as shown in Figure 29 b (188). Along the same line, Nrf2/DNA binding is increased in K562/Adr cells as compared to K562 cells, whereas Siamois® polyphenols are able to reduce basal and PMA inducible Nrf2 binding in both cell types (199, 202). Of the different pure polyphenols present in Siamois® crude extract tested, quercetin and eriodictyol show the strongest inhibition of TF/DNA binding, whereas Kaempferol and WP283 are less effective. Nevertheless, transcriptional inhibition of the various target genes by pure polyphenols present in Siamois® crude extract is regulated at multiple levels and depends on DNA-binding properties of NFκB, AP1, Nrf2 transcription factors, nuclear cofactor dynamics, as well as epigenetic settings (196, 203-205). Of special note, although pure polyphenols present in Siamois® crude extract are able to reverse inducible NFκB/DNA-binding in K562/Adr cells, basal NFκB/DNA-binding levels cannot be further decreased to levels, as observed in K562 cells.

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(a)

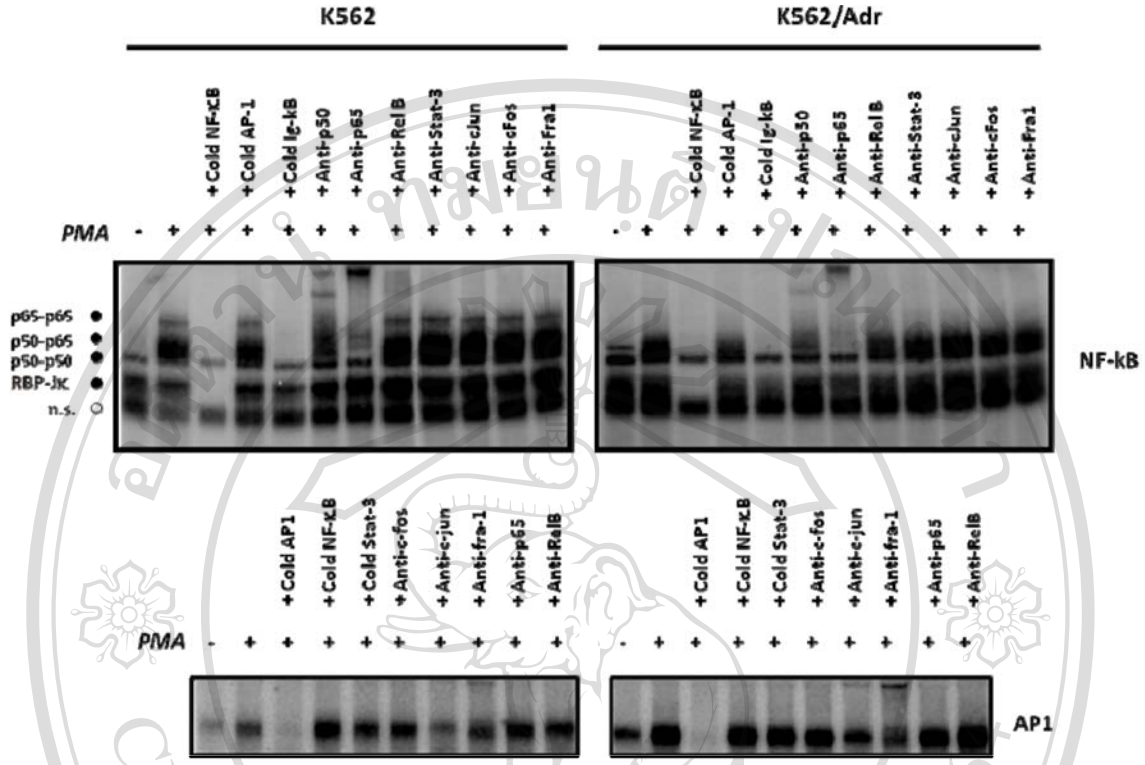

Figure 29. K562 and K562/Adr cells show qualitative and quantitative differences in NFκB and AP1/DNA-binding profiles including **(a)** compettetive and supershift EMSA and **(b)** EMSA for pure polyphenols present in Siamois® crude extract treatment (100 μM for 2.5 h). Quer, quercetin; Kaem, kaempferol; Erio, eriodictyol; WP, WP283.

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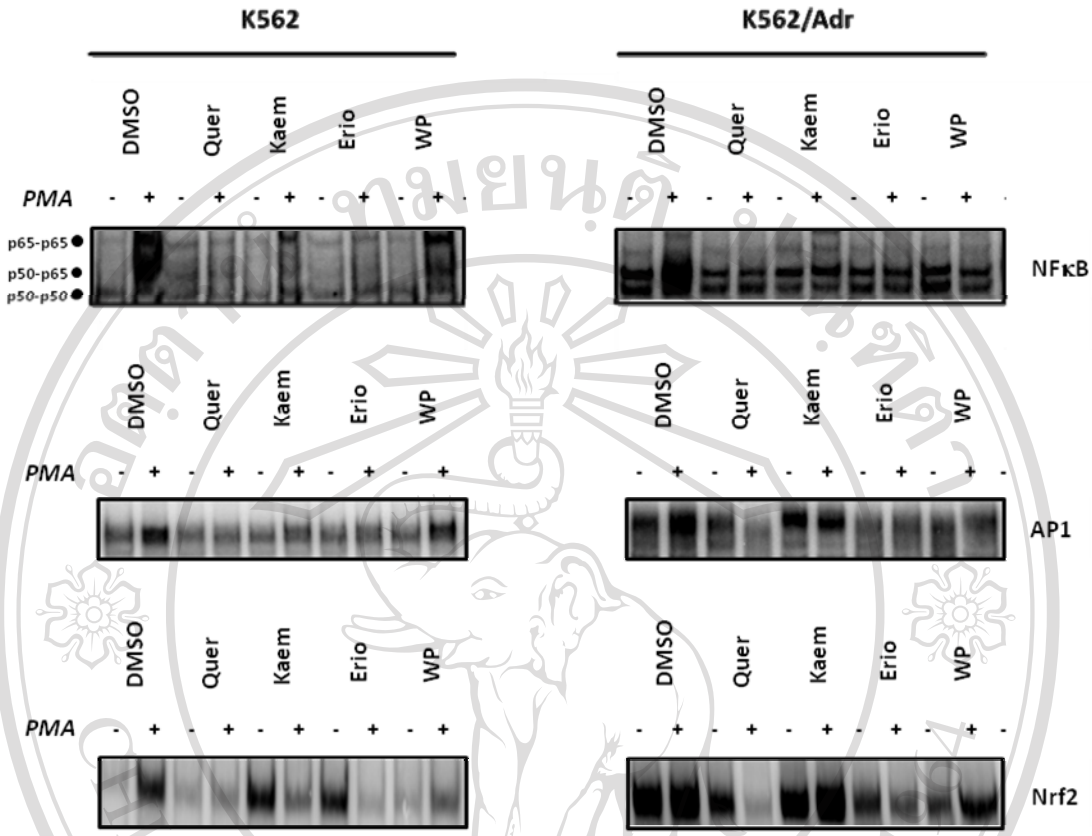

Figure 29. K562 and K562/Adr cells show qualitative and quantitative differences in NFκB and AP1/DNA-binding profiles including **(a)** competetive and supershift EMSA and **(b)** EMSA for pure polyphenols present in Siamois® crude extract treatment (100 μM for 2.5 h). Quer, quercetin; Kaem, kaempferol; Erio, eriodictyol; WP, WP283 (continued).

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15. Pure polyphenols present in Siamois® crude extract reduce cell viability in both K562 and K562/Adr cells

K562 and K562/Adr cells which are sensitive are resistant to doxorubicin respectively, were incubated with doxorubicin, or pure polyphenols present in Siamois®

crude extract, including quercetin, kaempferol, eriodictyol, WP283 to evaluate cytostatic and/or cytotoxic activity of the various compounds. After 72 h, cell survival was determined by the MTT cell viability assay and the IC50 values are summarized in Table 7. Among pure polyphenols present in Siamois® crude extract, WP283 and eriodictyol exhibit the strongest and weakest effects in mitochondrial reduction of tetrazolium salts to formazan. Interestingly, K562 and K562/Adr cells reveal comparable sensitivity to pure polyphenols present in Siamois® crude extract, whereas IC50 values for doxorubicin show a 20-fold higher sensitivity for K562 cells, compared to K562/Adr cells. These results indicate a pronounced cellular resistance for doxorubicin as compared to pure polyphenols present in Siamois® crude extract.

Table 7. IC50 values of pure polyphenols present in Siamois® crude extract in K562 and K562/Adr cells after 72 h incubation.

Compounds	**IC50 (μM)**	
	K562	**K562/Adr**
Quercetin	26 ± 2	32 ± 3
Kaempferol	39 ± 3	60 ± 4
Eriodictyol	> 100	> 100
WP283	0.03 ± 0.02	0.03 ± 0.02
Doxorubicin	0.2 ± 0.04	4.1 ± 1.0

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16. Pure polyphenols present in Siamois® crude extract induce early apoptosis in both K562 and K562/Adr cells but only late apoptosis in the former

K562 and K562/Adr cells were incubated for 48 h with pure polyphenols present in Siamois® crude extract, followed by annexin V-FITC in the presence of the nuclear stain propidium iodide (PI) and FACS analysis to quantify early (annexin V-FITC positive) and late (annexin V-FITC/PI double positive) apoptotic cells. The relative percentage of apoptotic versus living cells in the different experimental setups in K562 and K562/Adr cells, following 48h treatment are represented as a bar graph in Figure 30. Interestingly, although both cell types show comparable early apoptotic cell populations in presence of the different pure polyphenols present in Siamois® crude extract, late apoptotic cells only accumulate in K562 cells. Furthermore, although the concentrations applied of the different pure polyphenols present in Siamois® crude extract closely relate to the IC50 values determined in MTT assay, FACS analysis reveals significant variation in apoptosis efficacy between the different polyphenolic compounds. The latter suggests significant discrepancies between MTT cell viability assays revealed by mitochondrial reduction of tetrazolium salts and cell survival score measured by annexinV-FITC/PI apoptosis FACS assay. Indeed, it is of utmost importance to perform multiple, methodologically unrelated assays to quantify dying and dead cells (206).

Next, as apoptotic threshold in compound-treated K562/Adr cells may be higher due to elevated basal anti-apoptotic activity of NFκB, AP1 and Nrf2, we wanted to further evaluate whether increasing activity of NFκB, AP1 and Nrf2 by PMA treatment in K562 cells could similarly protect compound-treated K562 cells from late apoptosis in analogy to K562/Adr cells. However, although the relative number of late apoptotic cells decreases upon cotreatment of K562 cells with PMA and Siamois® inhibitors, execution of apoptosis is not completely blocked because pure polyphenols present in Siamois® crude extract are able to partially counteract PMA effects on NFκB, AP1 and Nrf2. Along

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the same line, pure polyphenols present in Siamois® crude extract cannot overcome late apoptosis block in K562/Adr cells, despite efficient inhibition of NFκB, AP1 and Nrf2. This suggests that late apoptosis in K562/Adr cells is only in part determined by transcriptional activity of NFκB, AP1 and Nrf2.

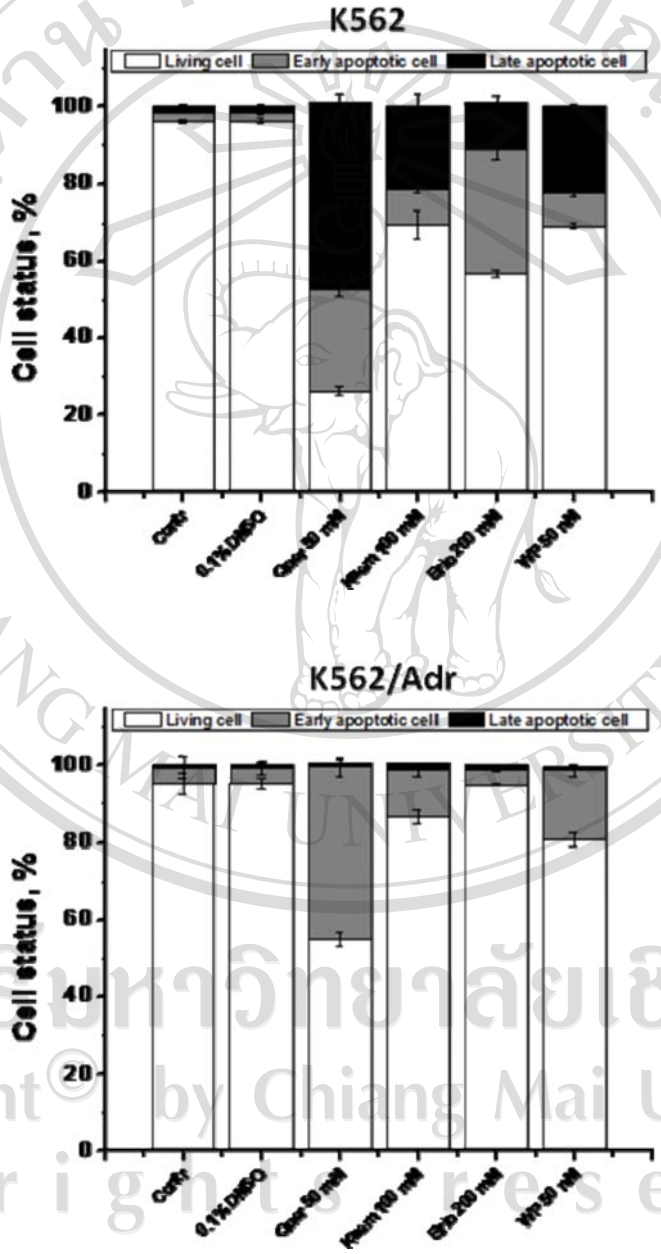

Figure 30. Apoptosis induced-activities of pure polyphenols present in Siamois® crude extract in K562 and K562/Adr cells after 48 h treatment and in the presence of 10 ng mL^{-1} PMA.

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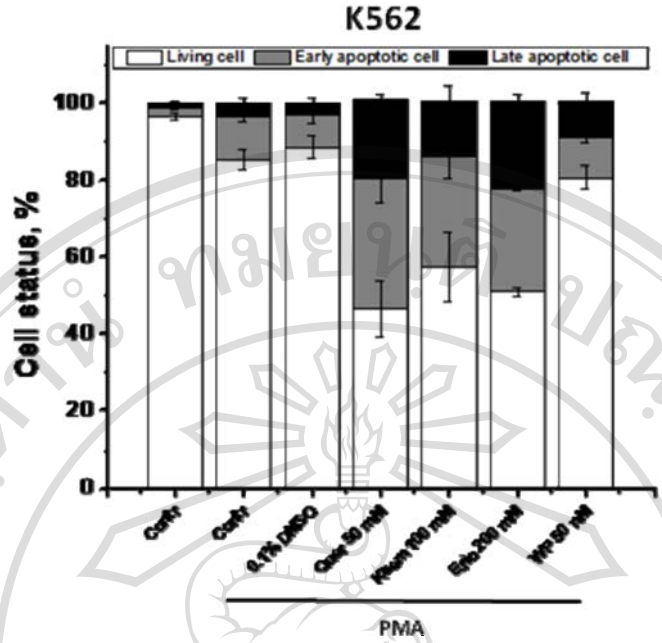

Figure 30. Apoptosis induced-activities of pure polyphenols present in Siamois® crude extract in K562 and K562/Adr cells after 48 h treatment and in the presence of 10 ng mL^{-1} PMA (continued).

17. Pure polyphenols present in Siamois® crude extract induce caspase-3 activation in K562 but not in K562/Adr cells

In addition to propridium iodide as a late apoptotic FACS marker, we measured biochemical activation of the executioner caspase-3 in K562 and K562/Adr cells exposed to PMA, pure polyphenols present in Siamois® crude extract. Analysis of caspase-3 activation in K562 cells exposed to quercetin as a function of incubation (2-72 h treatment) time revealed a maximal caspase-3 activity after 12 h (data not shown) in a fluorescent caspase substrate assay. In addition, K562 and K562/Adr cells were treated for 12 h with PMA and pure polyphenols present in Siamois® crude extract, after which caspase-3 activity present in the cell lysates was determined. Figure 31 shows that pure polyphenols present in Siamois® crude extract only increased caspase-3 activation in

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K562 cells but not in K562/Adr cells, which agrees with the lack of late apoptosis observed in K562/Adr.

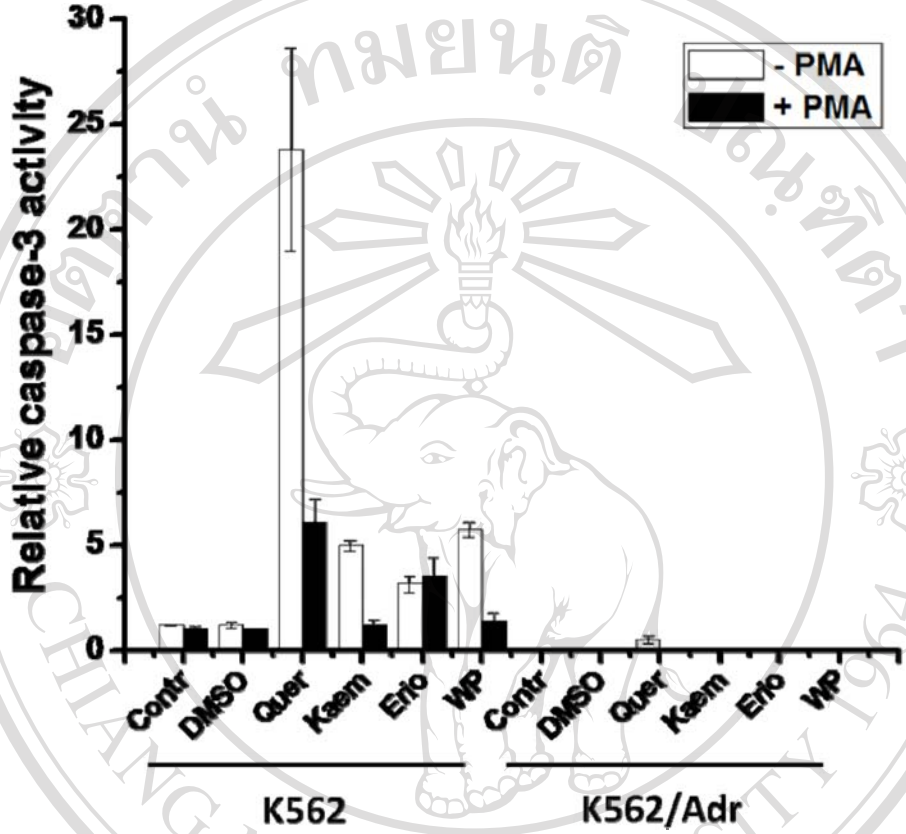

Figure 31. Caspase3 activation by pure polyphenols present in Siamois® crude extract in K562 and K562/Adr cells in the presence of 100 μM pure polyphenols and 10 ng mL^{-1} PMA for 12 h. Quer, quercetin; Kaem, kaempferol; Erio, eriodictyol; WP, WP283.

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