CHAPTER I

GENERAL INTRODUCTION

Discovery of polyphenols

Since the 18th century, the Hungarian scientist Albert von Szent-Györgyi was the first who demonstrated that daily diets such as red pepper consists of phytochemicals including ascorbic acid and polyphenols (at that time called vitamin K) can cure from a serious haemorrhagic diathesis (increased permeability or fragility of the capillary wall) (1-4). Szent-Györgyi also showed that ascorbic acid and polyphenols can protect plant tissues from oxidative damage resulting a reversible interaction with a peroxidase-like enzyme probably involved in cellular respiration. He reported that the peroxidase oxidized the polyphenol to a quinone metabolite, which subsequently oxidized ascorbic acid by taking up both its hydrogen atoms (5). Szent-Györgyi showed that this polyphenol belonged to a large group of yellow phenol-benzol-γ-pyran plant dyes (including flavones, flavonols and flavanones). It became clear that the members of this group of plant dyes possessed great biological activity. In 1937, the importance of these findings was officially recognized when he was awarded Nobel Prize of Medicine for this pioneering work on vitamin C and vitamin P.

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"*I regret that I must conclude with many questions asked and none answered, but I hope to leave the reader with the impression that flavonoids represent one of the most exciting, broad, and hopeful fields of biological inquiry and I am glad to close on such an optimistic note*". These are the words the Nobel-Prize winning scientist closed a

lecture with in 1955 (6). And he was right, because his findings on the biological activity of polyphenols were the start of decades of research on polyphenols.

Health benefits of polyphenols particularly quercetin and its glycoside derivatives in cancer treatments

It was well documented that man has convinced and recognized the role of plant foods in maintenance of health since long time ago. Recently, huge data indicated that polyphenols derived from plants exhibit both *in vivo* and *in vitro* a wide variety of physiological and biological effects including antioxidants, anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic, anti-proliferative and anti-mutagens activities (7). Several reports have demonstrated that the polyphenolic compounds can be considered as therapeutic agents (8-13).

Among polyphenolic compounds, flavonoid is the most potential pharmaceutical molecules. The most studied flavonoid is quercetin (3,3',4',5,7-pentahydroxyflavone), which is a flavonoid that is commonly and abundantly found in plants, has been reported to have biological, pharmacological, and medical applications (14-16). Although the multiple activities of quercetin were believed to arise from its antioxidant properties, it was recently suggested that quercetin might behave as a cytotoxic and mutagenic agents (17,18,19). A recent study demonstrated that quercetin can act as both antioxidant and pro-oxidant, depending on the concentration and source of free radicals in the cell (20).

Quercetin is naturally found as bio-conjugation particularly with glycoside at different carbon atoms on ring A, B and C. For example quercetin (3,3',4',5,7-pentahydroxyflavone), quercitrin (Quercetin-3-L-rhamnoside, Quercetin 3-*O*-a-L-rhamnoside, 3-O-L-Rhamnopyranosyloxy-3', 4', 5,7-tetrahydroxyflavone) and rutin

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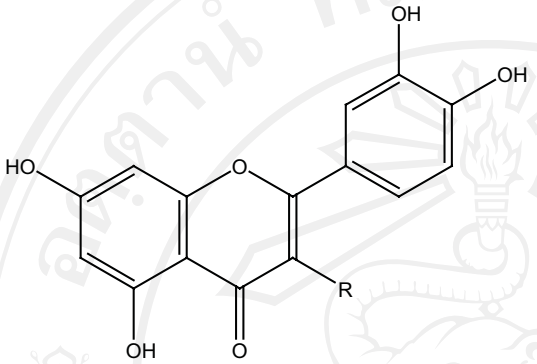
(quercetin-3-rutinoside, or quercetin 3-*O*-[β-L-rhamnosyl-(1→6)-β-D-glucoside]) **(Figure 1)** have been isolated from several natural sources in the flavor and color of many vegetables and fruits.



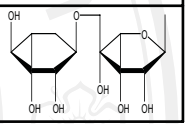
Flavonols	R
Quercetin	OH
Quercitrin	
Rutin	

Figure 1. Chemical structures of flavonols; quercetin, quercitrin and rutin.

Antioxidant might protect against carcinogenesis

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules.(21) Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (22). Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases (23). Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells (24). As oxidative stress might be

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an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. Antioxidants provide a source of protection against cancer-causing free radicals.

Huge data of *in vitro* studies demonstrate that flavonoids are good antioxidants (25-32). Structure-antioxidant activity relationships and the molecular parameters contributing to the antioxidant activity of the whole molecule was studied. These include a C_2=C_3-double bond, a C_4-keto group, a C_3-hydroxyl group and an *ortho*-diphenolic structure, also called catechol group, in the B-ring (25). Quercetin possesses all these structural elements (see Figure 1), and the potent ability of quercetin to scavenge reactive oxygen species (33-35), singlet oxygen (36), in addition to radicals of different origin (37-38) has been studied in various *in vitro* systems. However, it is still not clear whether the antioxidant activity seen *in vitro*, results in beneficial health effects *in vivo*.

The claims for health promotion particular protection against non-infectious chronic diseases was extrapolated based on *in vitro* studies of both chemistry and cancer cell systems, none was performed using normal cell system. This might be an origin of confusing hypothesis of health benefit of quercetin. On one hand, it was proposed that quercetin is a potent antioxidant (25), and might cause a decrease in oxidative stress levels, leading to abolish the development of certain cancers (39-40). Moreover, quercetin play role on cancer prevention via modulating the metabolism of carcinogens, through inhibition and/or induction of biotransformation enzymes of these carcinogens (41-42). On the other hand, the metabolism of quercetin yielded a

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catechol group which is thought to be involved in the mechanism underlying its carcinogenic effects.

Flavonoids, including quercetin, can induce both phase I and phase II biotransformation enzyme activities, the so-called “bi-functional inducers”, at the level of gene expression (43-44). Indeed, quercetin can stimulate the Electrophile Responsive Element (EpRE) (45), present as enhancer in the promoter region of certain genes encoding for phase II enzymes (46). Furthermore, quercetin directly modulates the activity of biotransformation enzymes. However in cancer cells with high activity of peroxidase and tyrosinase, 6-glutathionyl and 8-glutathionyl quercetin, the metabolites products of quercetin biotransformation were found only at outside of cells, signifying that a very low intracellular concentration of quercetin and/or the compartmentalization of these enzymes and/or quercetin do not submit to the biotransformation in cell system. In fact, it is the need to investigate the cellular distribution and biotransformation of quercetin in the living normal cells.

Quercetin is also known as a potent competitive inhibitor of certain cytochromes P450 (47-49) and sulfotransferases (50-51).

Quercetin known as antioxidant and anticancer molecule

Many research groups including our group (PCMCB) have demonstrated that flavonoid particularly quercetin can be considered as therapeutic agents. An overview of such *in vitro* studies presently available is shown in Table 1. Several mechanisms for the anti-proliferative effect of quercetin have been proposed, including inhibition the activation of transcription factors such as NF-κB and AP-1 (11), induction of DNA strand breakage, cell cycle arrest, and/or apoptosis (52-54). The ability of quercetin to modulate the activity of enzymes involved in signal transduction, and cell growth and

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development, including phosphatidyl-inositol-3-kinase, protein kinase C and protein tyrosine kinase (55-56) might play a role in these effects. It should noted that mutations in the p53 gene in various cancer cell types that make these cancer cells resistant to apoptosis and, accordingly, to anticancer drugs (68). Recently, quercetin has been demonstrated, particularly in cancer cells, to induce apoptotic cell death (69, 70, 71). Indeed the mechanisms of the anticancer activity of quercetin are likely complicated, starting on metabolic changes or decreasing in IP3 concentration and down-regulation of oncogenes (c-myc and Ki-ras). The products of c-myc and Ki-ras oncogenes are required for induction of proliferation and of apoptosis (72,73). However, in our group we provided evidence that the intact molecule of quercetin also plays an direct action probably at the adenine nucleotide translocator (ANT) at mitochondria of cells. We thus proposed that mitochondria are the intracellular target by which this death occurs. The ability of quercetin to induce cellular apoptosis may depend on the extent and characteristics of the mitochondrial injury. Various forms of cellular stress result in mitochondrial alteration, mitochondrial membrane depolarization and release of cytochrome c from the intermembrane space to the cytosol (71, 74). In the presence of ATP, cytosolic cytochrome c interacts directly with apoptotic protease activating factor 1 and procaspase 9 to form an apoptosome, a macromolecular complex that cleaves procaspase 9 to active caspase 9, which, in turn, cleaves procaspase 3 to active caspase-3 (75, 76, 77) as indicated in Figure 2.

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Table 1. Summary of studies on the inhibiting effect of quercetin on cell proliferation

Cell line	Exposure time	Assay	IC_{50} (μM)[1)]	Reference
Breast				
MCF7	3 d	[^{3}H]-thymidine incorporation	15	(57)
MDA-MB-435	48 h	[^{3}H]-thymidine incorporation	32	(58)
		Cell counting		
	72h		10	(10)
MDA-MB-231	6 d	MTT	$2.4x10^{-6}$	(59)
MCF-7			$4.4x10^{-6}$	
T47D			$0.1x10^{-6}$	
Lung				
GLC4	72h	Cell counting	18	(9)
GLC4/adr				
Colon				
HT-29	48 h	Cell counting	13	(60)
Caco-2	48 h	Cell counting	45	(54)
HT29			53	
IEC-6			40	
HT-29	96 h	Cell counting	2.5	(61)
WiDr			0.06	
Colo-201			3.1	
LS-174T			0.7	
Leukemia				
TGBC11-TKB	72 h	Alamar blue reduction	5.9	(62)
K562	72h	Cell counting	23	(9)
K562/adr			23	
ALL	16 h	[^{3}H]-thymidine incorporation	2	(63)
AML			3	
Ovary				
OVCA-433	72 h	Cell counting	10	(64)
Prostate				
PC3	48 h	Cell counting	45	(65)
Skin				
OCM-1	48 h	Cell counting	18	(66)

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A549	72 h	Alamar blue reduction	1	(62)
B16-4A5			10	
CCRF-HSB-2			7.2	
TGBC11-TKB			5.9	
A431	72 h	Cell counting	21	(67)

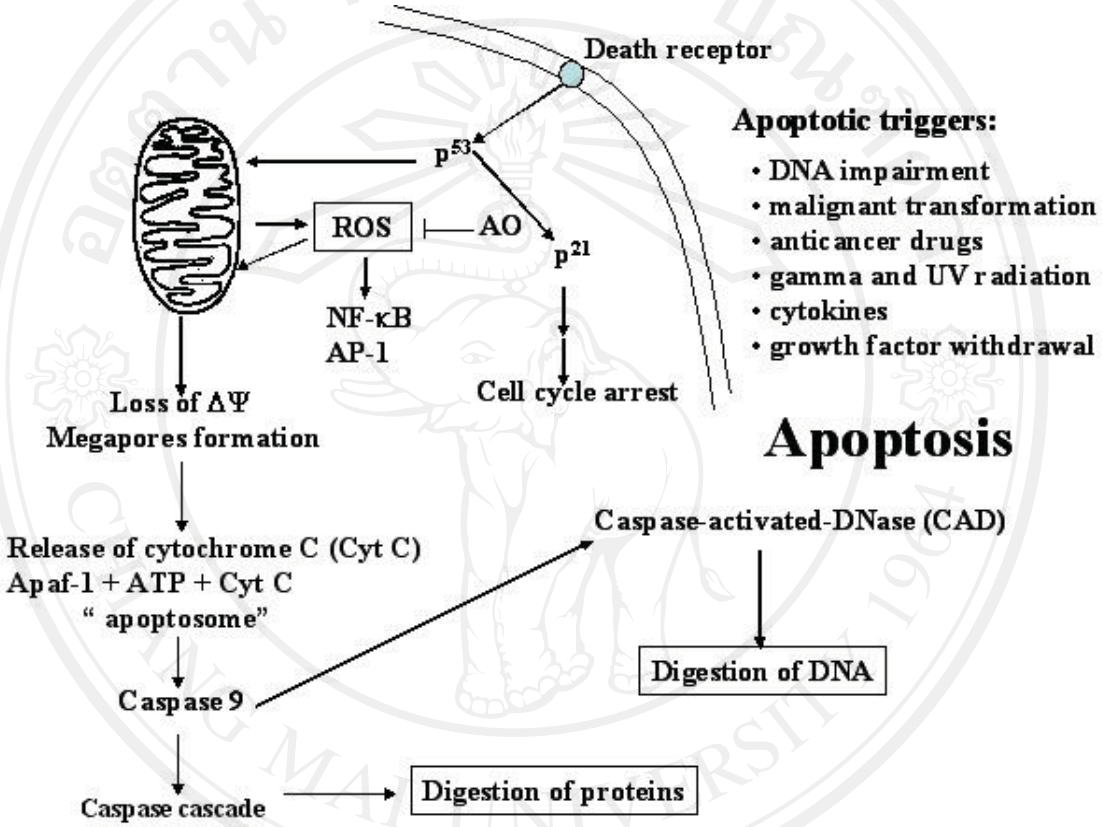

Figure 2. Intrinsic apoptosis pathway carried out by a multistage chain of reactions in which ROS act as triggers.

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Quercetin action in normal cells

By using a normal cell such as H9c2 cardiomyoblast cell, quercetin could protect hydrogen peroxide from inducing H9c2 cardiomyoblast cells to undergo apoptosis (78). It was also reported that quercetin showed higher value of antioxidant activity than of Vitamin C, Vitamin E and β-carotene on molar basis (79) and

probably due to the antioxidant action, it prevented the generation of reactive oxygen species by cyclosporine and thereby suppressed the cyclosporine-induced nephrotoxicity (80). These authors proposed that quercetin might play an expression modulator of Mn-SOD which is an enzyme located on the mitochondrial matrix. These results obtained from various normal cell types, reveal that quercetin is effective in protection of various cell types from oxidative injury and stimulate the damaged cells entering to apoptosis.

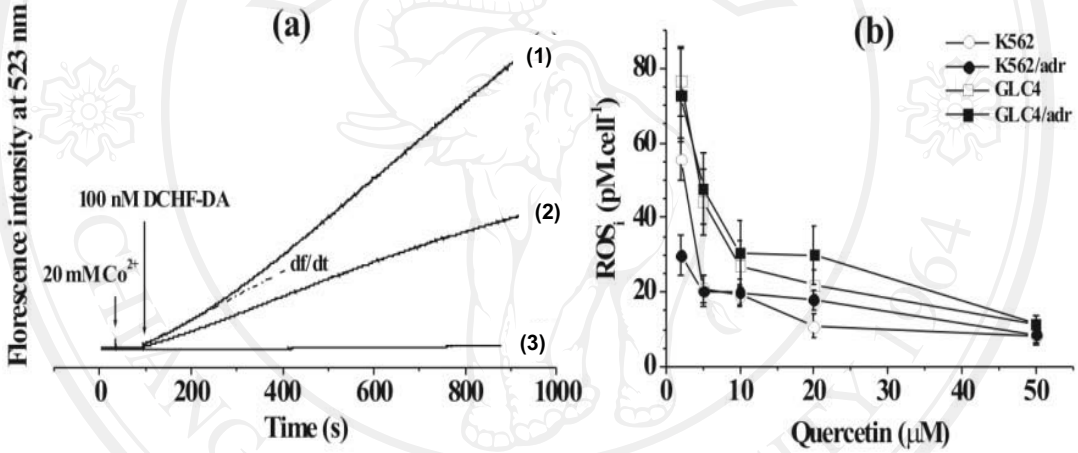

Figure 3. (a) Kinetics of DHCF-DA oxidation: cells ($2x10^5$) were suspended in 2 mL of HEPES-Na^+ buffer for 10 minutes before addition of (1) 20 mM $CoCl_2$ and 100 nM DHCF-DA, (2) of 20 mM $CoCl_2$, 100 nM DHCF-DA and 10 μM quercetin and (3) of 20 mM $CoCl_2$, 100 nM DHCF-DA and 10 μM quercetin without cell. The fluorescence intensity at 523 nm (excited 502 nm) were recorded as a function of time and the initial rate of an increase in DCF fluorescence intensity (Vi) was determined by the tangent to the curve of F = f(t) during a first 50s after addition of DHCF-DA. The determined ROSi is in $pM.cell^{-1}$ was calculated using the expression [ROSi] = a.Vi., where "a" is the ratio of ROSi/Vi.; (b) Variation of ROSi obtained from the

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series of experiments (a) as a function of flavonoid concentration used in (o) K562, (●) K562/adr, (□) GLC4 and (■) GLC4/adr cell. Data are the mean ± SD of three independent experiments(*).

Antioxidant ability of quercetin in cancer cell systems

Because flavonoids are very efficient antioxidants, their presence inside of cells could cause severe modification of the redox-status. We have established a spectrofluorometric method for quantitatively measuring the intracellular reactive oxygenspecies particularly for H_2O_2 in living drug-sensitive and its corresponding MDR cells. It should be noted that using our model of cell systems, there is existence of compartmentalization where the membrane acts as barrier to separate and preserve the differential physicochemical environments of each compartment. As can be seen in Figure 3a, immediately after addition of quercetin into the suspension of cells, the initial rate of DCF fluorescence intensity considerably decreased. This signified that the uptake of quercetin by cells was rapidly done since it is very potent antioxidant and has faster rate of reaction with ROS than DCHF. Quercetin diffused across the plasma membrane into cytosol of cells and immediately depleted the ROS_i content as time- and concentration-dependent manner. The antioxidant ability of quercetin in cell system was defined as the concentration of quercetin required for quenching the ROS_i by 50% assigned as $\rho_{0.5}$, which can easily graphical deterimine in Figure 1b. The $\rho_{0.5}$ values of quercetin determined in the four cell lines is very similar equal to 8 ± 2 μM.

The reaction of quercetin with ROS_i was proposed in Figure 4. Quercetin has extensively conjugated π-orbitals, it is theoretically expected that quercetin is able to donate electron or hydrogen from hydroxyl groups to free radicals. It is also well

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known that quercetin is able to chelate Fe^{2+}/Cu^{+}, inhibiting the formation of hydroxyl radical ($^{\bullet}OH$). Both $^{\bullet}OH$ and H_2O_2 were proposed to react with flavonoids at ring B. We thus imagined that the neutralization of both ROS_i species (reaction 1 and 2) yielded one and two water molecules and anion quercetin that can be protonated or prone to redox-cycling known as quinone specie.

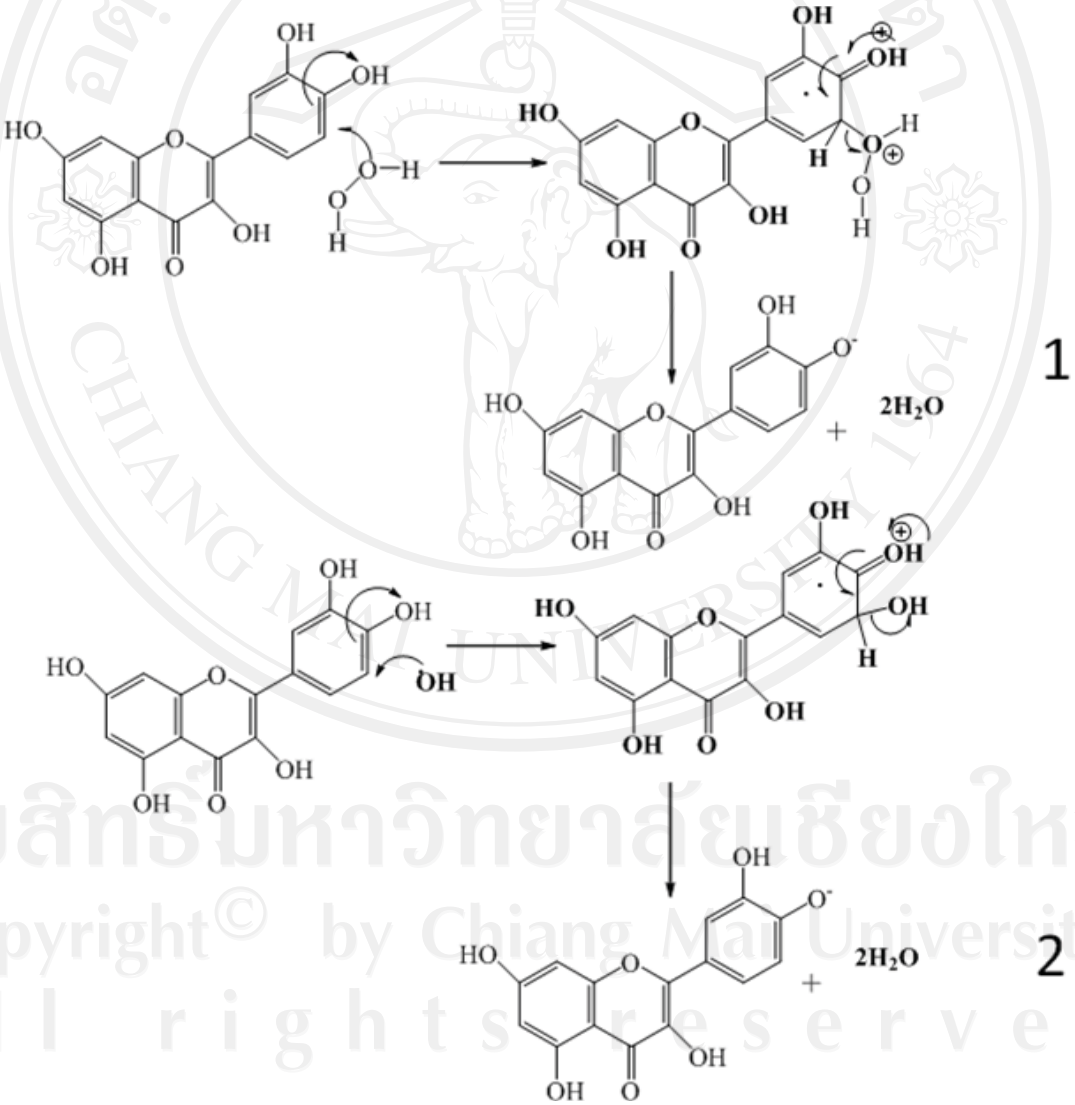

Figure 4. Reaction of quercetin with hydrogenperoxide (1) and with hydroxyl radical (2)**.**

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Inhibition of NF-κB and AP-1 oxidation

We have recently reported that quercetin and other flavonoids can selectively block nuclear NF-κB transactivation specific NF-κB target genes independently their estrogenic activity (11). As previously mentioned quercetin is very potent antioxidant react with free radical with very fast, we firstly asked a question that what the chronological consequence of depletion of ROS_i in the presence of quercetin in these cancer cells. Since it was well documented that ROS_i is predominant factor controls the activation of transcription factors such NF-κB and AP-1. A depletion of ROS_i should be yielded a down regulation of both NF-κB and AP-1 activation.

In order to get further information the chronology of events, the activation of NF-κB and AP-1 was measured in the presence of varied concentration of quercetin as a function of time. The activation process of the activation of NF-κB was summarized in Figure 5. The activation of this transcription factor can be achieved by using immune-fluorescence staining using antibody of with NF- p65 antibody. To help understand better the molecular mechanisms associated with NF-κB activation in response to quercetin, we also examined the expression pattern of various genes known to be regulated by NF-κB activation as a function of dose and incubation time.

The fluorescence micrographs of cells stained using NF-p65 antibody demonstrated the activated and inactivated NF-κB cells after exposed to 200 μM H_2O_2 for 1 hour for K562 cells (Figure 3a). The activated NF-κB was enumerated as

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a function of incubation time. The percentage of activated NF-κB cells was slowly increased and reached to the maximal values 30 ± 3% at 1 hour then progressively decreased to reach the basal level at 4 hours. Contrary the same concentration of H_2O_2 caused slightly NF-κB activation in K562/adr cells. Similar results were obtained with GLC4 and GLC4/adr cells.

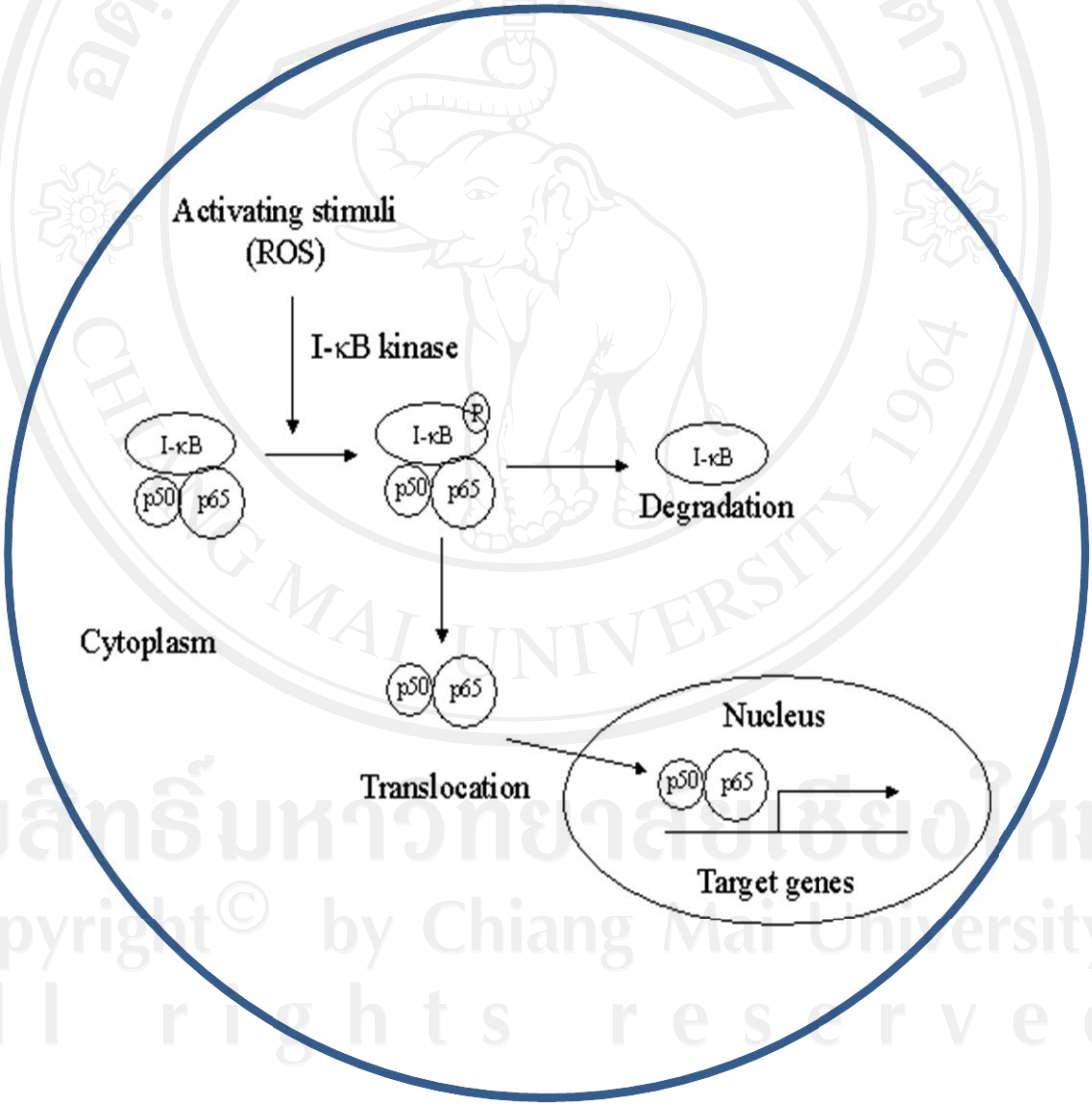

Figure 5. A scheme indicting reactive oxygen species induced activation and translocation of NF-κB into nucleus.

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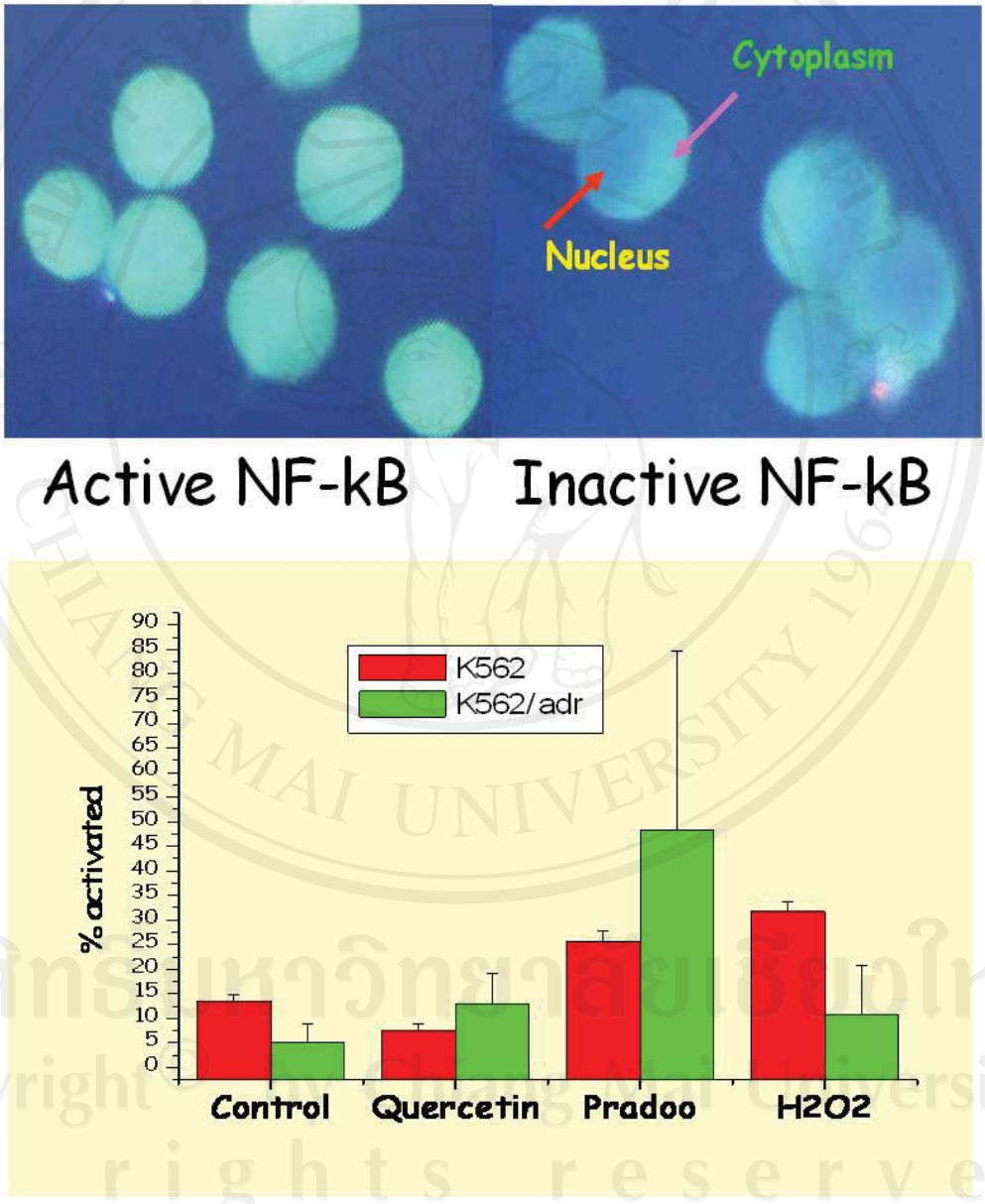

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Figure 6. Fluorescence micrograph of cells (upper panel) and enumeration of NF-κB activated cells induced by various stimuli (lower pannel). Cells (10^6/mL) were incubated in RPMI–1640 medium supplemented with 10 % fetal calf serum, in the

presence of 60 μM quercetin or 200 μg/mL Pradoo-wood's crud extraction or with 200 μM H_2O_2 at 37 ^{o}C in a humidified incubator of 95% air, 5% CO_2 for 1 hour. The cells were collected and immunofluorescence staining with NF- p65 antibody.

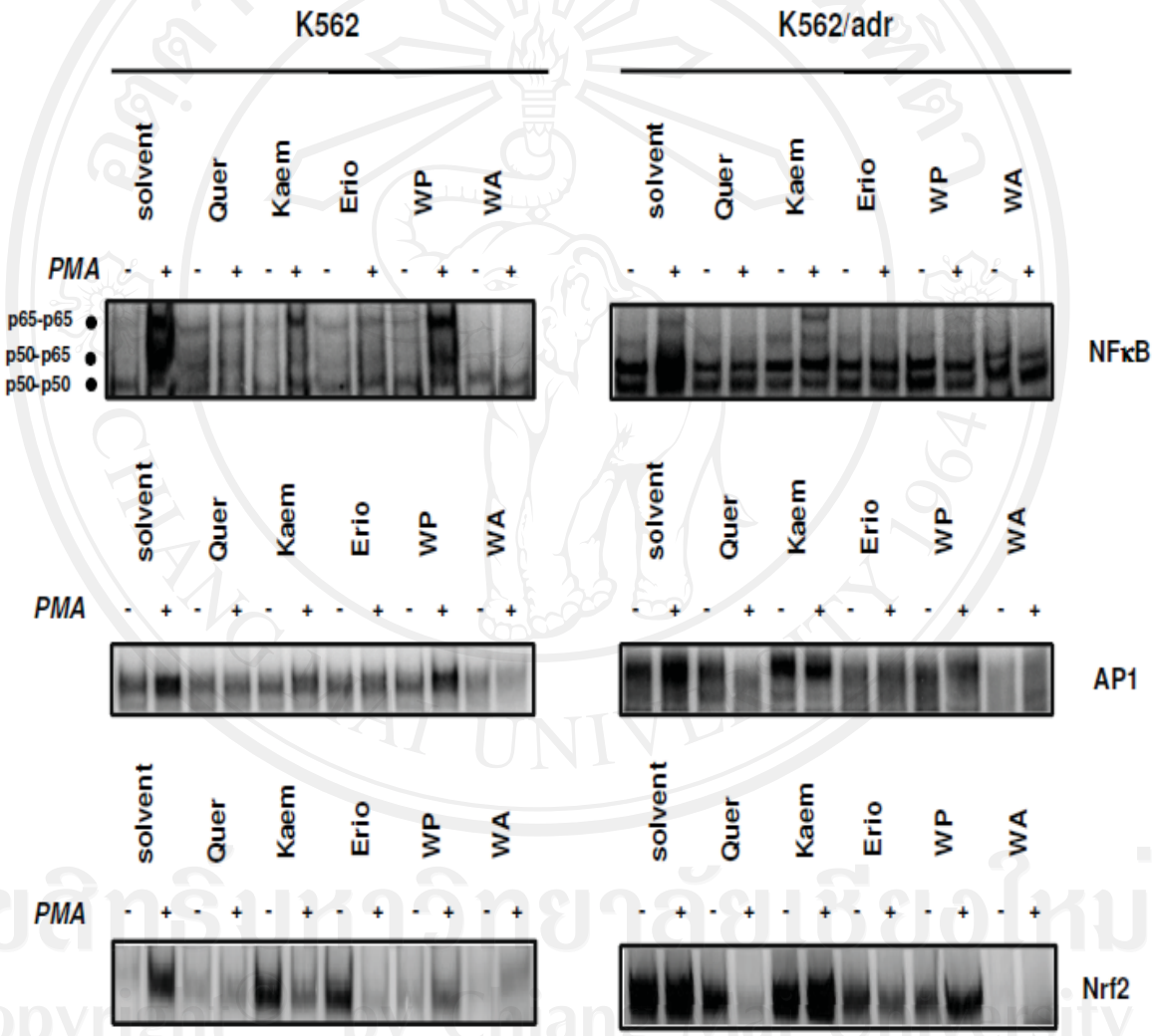

Figure 7. K562 and K562/Adr cells show qualitative and quantitative differences in NF-**κ**B and AP1 DNA binding profiles. K562 and K562/Adr cells were pretreated with 100 μM of quercetin, kaempferol, eriodictyol, WP283, or 6 μM of withaferin A for 2 hours followed by incubation with PMA (0.1 μg/ml) for 30 minutes. Cell lysates

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were fractionated for cytoplasmic and nuclear extracts which were analyzed for NFκB, AP1, or Nrf2-dependent DNA binding with specific radiolabeled probes. Binding complexes formed were analyzed by EMSA (11).

The fluorescence micrographs of cells stained using NF-p65 antibody demonstrated the activated and inactivated NF-κB cells after exposed to 200 μM H_2O_2 for 1 hour for K562 cells (Figure 3a). The activated NF-κB was enumerated as a function of incubation time. The percentage of activated NF-κB cells was slowly increased and reached to the maximal values 30 ± 3% at 1 hour then progressively decreased to reach the basal level at 4 hours. Contrary the same concentration of H_2O_2 caused slightly NF-κB activation in K562/adr cells. Similar results were obtained with GLC4 and GLC4/adr cells.

These results were consistency to those reported for bone marrow cells isolated from irradiated mice (81). In fact we know that irradiation using an ionizing radiation is the best way to select free radicals which now known as ROS_i in living organisms. It was clearly shown that there was a significant increase in NF-κB activation in bone marrow cells isolated 1 hour post-exposure of mice to 10 and 100 cGy**.** In samples isolated 4 hours post-irradiation, however, no significant activated NF-kappa B signal was detected. A significant level of expression of several genes was detected as early as 1 hour post-irradiation in samples exposed to a high dose level (100 cGy). In contrast, a high level of gene expression in samples collected from mice exposed to 5 or 10 cGy was observed at 4 hours post-irradiation. The results indicate a delay in expression of genes in samples from mice exposed to low doses of ^{137}Cs gamma rays.

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The action of quercetin on NF-κB activation is dose- and time dependent manner. It should be noted in the presence of 60 μM quercetin with certitude of experimental error did not affect the NF-κB activation in K562, K562/adr, GLC4 and GLC4/adr cells. While a significantly inhibition of NF-κB activation was observed when the higher concentration (≥ 100 μM) of quercetin was applied.

As indicated in Figure 3a, only 8±3% not all treated cells were identified as activated NF-κB, it is of prime important to investigate the effects of the transcription factor activation on endogenous NF-κB targeted gene, involved in inflammation, metastasis (IL6, IL8, MCP1, A20), cell cycle (cyclinD1), angiogenesis (VEGF), multidrug resistance (mdr1/P-gp), and apoptosis (A1/Bfl1) as indicated in Figure 5.

The results clearly showed that for cancer cells, factor-kappa B (NF-κB) is a key transcription factor that regulates expression of numerous genes (coding for cytokines, chemokines and adhesion molecules) involving in cellular response to DNA damaging agents.

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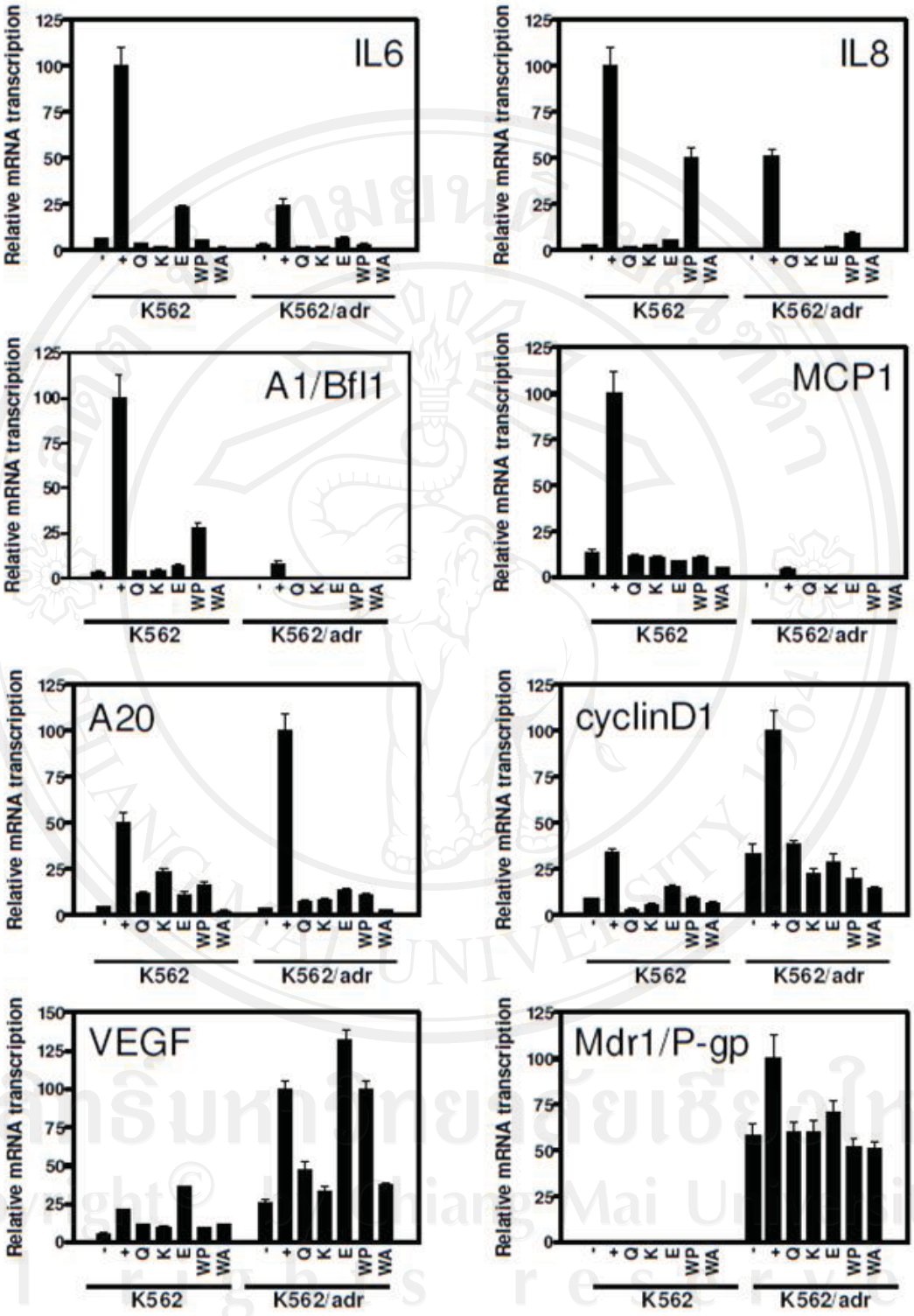

Figure 8. Siamois polyphenols and withasteroids inhibit endogenous NF-**κ**B dependent transcription in K562 and K562/Adr cells.

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Figure 8. (continued) K562 and K562/Adr cells were pretreated with 100 μM of quercetin, kaempferol, eriodictyol, WP283, or 6 μM of withaferin A for 2 hours followed by incubation with PMA (0.1 μg/ml) for 3 hours. Total RNA was isolated and mRNA was converted into cDNA. Relative mRNA levels were quantified by QPCR by specific primer sets for IL6, IL8, A1/Bfl1, MCP1, A20, cyclin D1, VEGF and mdr1/P-gp. Specific mRNA transcription levels were normalized by transcription levels of cells incubated in control medium with 0.1% DMSO. Data of two independent experiments, each done in triplicate, are presented as mean ± S.E.M

Anticancer action of intact molecule of quercetin

Indeed an immediately depletion of ROS_i known as a major regulator of NF-κB activation, there was only small fraction of cancer cells were responded even the concentration of quercetin used is higher than the IC_{50} values. These results suggested that it is possible that other mechanisms should be intervened the cytotoxicity of quercetin. Since we found that the reaction product of quercetin with hydroxyl radical and H_2O_2 is quercetin anion that can be protonated in protonic environments. We thus proposed that beside antioxidant action, the intact molecule should predominately play role in antiproferation via direct interaction at the mitochondria of cancer cells. Flavonoids particularly are now reconsidered as a promise new generation of anticancer molecules because various molecules belong to the flavonoids family such as quercetin, apigenin, kaempferol, etc. efficiently induced various cancer cell types both *in vitro* and *in vivo* (82-89) conditions to triggering apoptotic program cell death via mitochondrial pathway (90-92). However, the efficacy of these flavonoids was limited by their physicochemical properties, such as solubility, stability and susceptive to undergo reaction with oxidant species found in their environments. We have

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previously characterized the speciation of catechin, eriodyctyol, apigenin, kaempferol and quercetin compared with those molecules substituted with methoxyl groups in place of hydroxyl groups at various carbon atom positions such as WP279, WP280 and WP283 in a physiological solution (for chemical structure see Figure 6). The methoxyl group substitutions at various positions of carbon atoms in ring A, B and C, particularly for WP 283, resulted in a protection of a neutral form to undergo an aggregation or increase in its solubility and its Log P value, thus increasing its anticancer efficacy. The predominant active molecules should be the neutral form and the active site for anticancer activity of molecules are found in ring A and C, specifically C4=O, C5-OH and C2=C3.

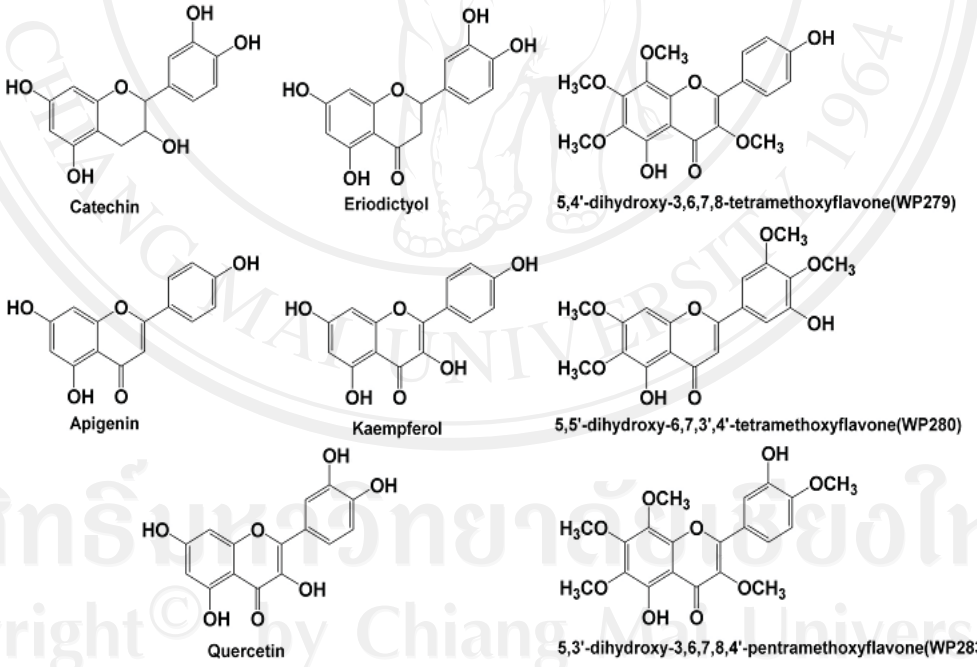

Figure 9. Chemical structure of catechin, eriodictyol, apigenin, kaempferol, quercetin, 5,4′-dihydroxy-3,6,7,8-tetramethoxyflavone (WP 279), 5,5′-dihydroxy-6,7,3′,4′-tetramethoxyflavone (WP 280) and 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone (WP 283).

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Huge studies reported the interaction of flavonoids with artificial membrane systems. It was reported that the glycosylated flavonols and their aglycons incorporated in the artificial planar membranes caused an increase in the specific membrane conductance linearly with their concentration. These authors porposed that flavonoids are protonophores. Saija et al. (93,94,95) stress in the reported that flavonoid incorporation into liposomal membrane caused packaging of the bilayer, thereby altering its barrier functions. It should be noted that these authors performed the series of experiments using very high concentration of flavonoids. However, the interaction of these flavonoids with lipid bilayer membrane particularly aiming to determine the mean influx coefficient (k+), their distribution and localization on the lipid bilayer membrane have been under-exposed. In fact, the transport parameters of molecules across the membrane are very important, especially the mode of transport. We thus rigorously investigated the interaction of eriodyctyol, apigenin, kaempferol and quercetin, WP279, WP280 and WP283 and particularly focus on their behavior to translocation through the vesicle membrane. For these purposes, the myristyl myristate and Tween® were used for constructing the multilamella liposomes. The hydrophobic zone of the bilayer was successfully labeled using 1,6-diphenyl-1,3,5-hexatrine (DPH). DPH is widely used as a probe for the hydrophobic regions of the phospholipids bilayers because of its favorable spectral properties (96). The quenching of DPH fluorescence intensity can be quantitatively determined and calculated to flavonoid concentration in molar unit. The distribution and localization of flavonoids onto the lipid membrane can be studied by using thermotropic behavior of lipid bilayer of liposomes suspension in a real-time continuous acquisition.

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Our results clearly show for the first time that flavonoids at least the two series of hydroxyl and methoxyl substitution at various carbon atoms on nuclear ring A, B and C passively diffused throughout the myristyl myristate-Tween® bilayer. The k+ values of the planar hydroxyl substitution flavonoids depends upon the C2=C3 and C3-OH. The substitution of methoxyl groups caused a decrease in the k+ value of molecules. However, these methoxyl substitutions protect the flavonoids to interact with the acetal group of the membrane to preserve the molecule in intact form. The advantage in their anticancer efficacy of the methoxyl compared with the hyoxyl substituted series, for example WP 283 exhibited about 90 to 100-fold more efficacy than eriodictyol. This should be due to its higher solubility (97) and the methoxyl group can protect the active site of molecules particularly C4=O, C5=OH and C2=C3.

Determination of apoptosis against cancer cells

It is clear that all compounds used induced apoptotic cell death even after a very short exposure time (30 min). The early apoptotic cells (%) increased with the time to reach a pseudo plateau at about 24 h after incubation when 10 μM of each flavonoid was used. We observed that quercetin for example; an increase in the early apoptotic cells (%) occurred at the first hour (42 ± 2 % in K562 and K562/adr cells) and then no further change until 24h. For longer times, the late apoptotic cells (%) and necrotic cells (%) slightly increased consistency with a slowly decreasing in early apoptotic cells (%) to 32 ± 2 % at 72h. However, the early apoptotic cells (%) increased with flavonoid concentration. The efficacies of apoptotic cell induction by these flavonoids from high to low activity is quercetin > kaempferol > apigenin > eriodictyol.

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Decrease in the ROS_i and alteration of $\Delta\Psi_m$ of cancer cells

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

State of the art of for new anticancer drug development

Quercetin exhibits an anti-proliferation of a broad range of cancer cell lines, with IC_{50} values ranging from 0.1 pM to 45 μM, depending on the cell type and experimental conditions used (Table 2). The mechanism by which quercetin mediated its specific anticancer action is debating. Of course quercetin encounters with a variety of biomolecules once found in cytosolic of cells, various biochemical reactions should be disturbed. However, these biochemical reactions are dose and time dependent-manner. As we have known that the pharmacological effects of molecule depends upon its intracellular target concentration. Although, the mode quercetin transport through the lipid bilayer vesicles were studied in our group. However, the partition of quercetin into drug-sensitive and drug-resistant cancer cells especially in the conditions of cytotoxicity assay did not perform yet. We hypothesized that serum albumin contained in the culture medium could play role as binder and spontaneously release of quercetin. This should be limiting step of a low intracellular concentration of quercetin, contrary maintain the retention time and constant dose. Indeed, an explanation and find the limiting step of *in vitro* cytotoxicity and *in vivo* testing is the need in order to break through the obstacle of preclinical study.

Quercetin is known as apoptosis inducer and MDR modulator. Experimental approaches aiming to determine the direct interaction between flavonoids and P-

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glycoprotein were studied by many groups. On one hand, studied the fixation of flavonoids at vicinal ATP-binding site. They measured the resonance-energy transfer of the tryptophan intrinsic fluorescence of H6-NBD2, a highly soluble recombinant protein, from mouse P-gp and flavonoids [98]. Similar results were obtained from the series of experiments dealing with the inhibition of photolabeling of ATP analogues on the ATP-binding site within the C-terminal nucleotide-binding domain of mouse P-gp. The results of using 30 flavonoids (99) De Wet *et al.* showed the structure-activity relationships of 30 flavonoids on their ability to bind the vicinal ATP- and steroid-binding site. On the other hand, Phang *et al*. reported that flavonols (quercetin, kaempferol, and galangin) were potent stimulators of the P-gp-mediated efflux of 7,12-dimethylbenz (a)-anthracence in multidrug-resistant breast cancer cells (100). Consistently with previously cited data, Critchfield *et al*. found that galangin, kaempferol, and quercetin reduced [^{14}C] ADR accumulation and this phenomenon was blocked by verapamil, vinblastine, and quinidine in HCT-15 colon cells (101). In fact, the efficiency of inbitory of drug efflux by quercetin might be dependant on the nature of both the drug and inhibitor molecules. It should also be noted that rare studies reported on the inhibition of Pglycoprotein or MRP1 function related to the chemical structures of flavonoids in living MDR cells.

Objectives and outline of this thesis

The objectives of the thesis were (1) to determine the role of serum albumin as limiting step and subcellular distribution of flavonoids particularly for the cytotoxicity assay conditions and (2) to elucidate the direct interaction of flaonoids with multidrug resistant protein such as P-glycoprotein and MRP1 protein in living cells. To achieve the objective, quercetin and its glycoside derivatives (Figure 1) which possess all the

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structural elements of the active site of molecules particularly C4=O, C5=OH and C2=C3 were selected in the thesis.

The thesis edited and presented in article research formats, chronologically demonstrated from Chapter I general introduction, chapter II materials and methods and chapter III BSA found in the culture medium can serve as a carrier and spontaneously and constantly release source of flavonoids *in vitro* cytotoxicity assay. Chapter IV quercetin, quercetrin except rutin potentially increased pirarubicin cytotoxicity by non-competitively inhibiting the P-glycoprotein and MRP1 function in living K562/adr and GLC4/adr cells. Finally, Chapter V summarizes the results of this thesis and presents a potential anticancer drug development, based on the findings described in this work.

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