CHAPTER I

GENERAL INTRODUCTION

Flavonoids have been considered to be potential pharmaceutical molecules due to their potent antioxidant and anticancer activities that have been extensively demonstrated *in vitro* and *in vivo*. Flavonoids efficiently scavenge and chelate the species which can undergo cellular oxidative stress or may serve as an intracellular electron donor for a transplasma membrane oxidoreductase, suggesting that the flavonoids exert beneficial effects under oxidative stress conditions (Miller and Paganga, 1997, Afanas'ev *et al.* 1989, Bors *et al.* 1990, Rifici *et al.* 1999). Recently, flavonoids have been demonstrated, particularly in cancer cells, to induce apoptotic cell death (Mouria *et al.* 2002, Kuntz *et al.* 1999, Wenzel *et al.* 2000). Mitochondria have been proposed to be the mechanisms by which this death occurs. The flavonoids could be promising compounds to overcome cancers and mediate their specific toxicity against cancer cells. This is due to the understanding that cancer cells are resistant to or have less control apoptosis pathway. Huge studies have been performed in this area in order to gain new generation anticancer molecules, but controversial results have been reported in international publications. According to most studies, research has always considered a given molecule as representative of all flavonoids and it is very difficult to compare results between one research group and another due to different experimental conditions. Indeed, flavonoids belong to a large family that

is different in chemical structure thus resulting in their differing efficiency and bioactivity.

1. Flavonoids

1.1 Description

Figure 1 Albert Szent-GyÖrgyi. The Nobel Prize in Physiology or Medicine 1937.

Flavonoids were discovered by Nobel Prize-winning biochemist Albert Szent-Gyorgi (Figure 1), who labeled them "vitamin P". Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Figure 2) and are classified according to substitutions (Figure 3). Flavonoids are C15 compounds composed of two phenolic rings connected by a three carbon units which consist of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled A, B, and C. The various classes of flavonoids differ in the level of oxidation and the pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Pietta 2000).

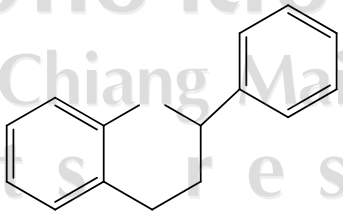


Figure 2 Nucleus structure of flavonoids

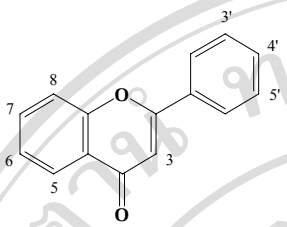
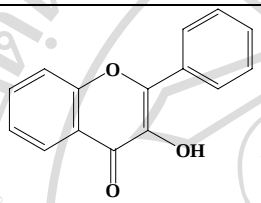
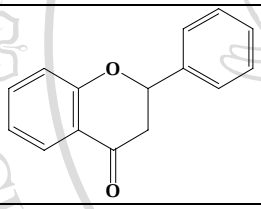
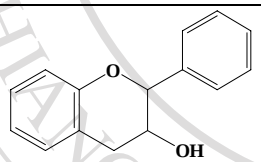
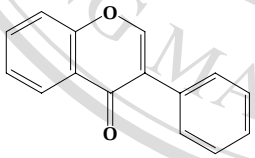
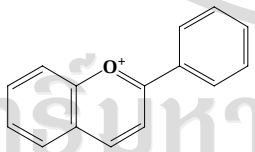
Class	General structure	Substitution Pattern								Name
		3	5	6	7	8	3′	4′	5′	
Flavone		H	OH	H	OH	H	H	OH	H	Apigenin
		OCH_3	OH	OCH_3	OCH_3	OCH_3	H	OH	H	WP279
		H	OH	OCH_3	OCH_3	H	OCH_3	OCH_3	OH	WP280
		OCH_3	OH	OCH_3	OCH_3	OCH_3	OH	OCH_3	H	WP283
Flavonol		OH	OH	H	OH	H	OH	OH	H	Quercetin
		OH	OH	H	OH	H	H	OH	H	Kaempferol
Flavanone		H	OH	H	OH	H	OH	OH	H	Eriodictyol
		H	OH	H	OH	H	H	OH	H	Naringrnin
Flavanol		βOH	OH	H	OH	H	OH	OH	H	(+)-Catechin
		αOH	OH	H	OH	H	OH	OH	H	(-)-Epicatechin
Isoflavone		H	OH	H	OH	H	H	OH	H	Genistein
		H	H	H	OH	H	H	OH	H	Daidzein
Anthocyanidin		OH	OH	H	OH	H	OH	OH	H	Cyanidin
		OH	OH	H	OH	H	H	OH	H	Pelargonidin

Figure 3 Classified of flavonoids

1.2 Physicochemical properties

The chemistry of flavonoids is complex. In plants, flavonoids frequently occur attached to sugars (glycosides) and polymers. Since these compounds are based on the flavan nucleus, the number, positions, and types of substitutions affect their biochemical and pharmaceutical properties.

The spatial arrangement of substituents usually enhances their antioxidant and anticancer activity compared with their flavan nucleus. Particularly both the configuration and total number of hydroxyl groups substantially influence the mechanisms of antioxidant action. For example, the hydroxyl substitution at the B-ring rendered their scavenging activity against both reactive oxygen species (Pannala *et al.* 2001, Burda and Oleszek, 2001) and reactive nitrogen species (Haenen *et al.* 1997, Kerry and Rice-Evans, 1999). It was reported that the hydroxyl groups on the B-ring donated a hydrogen atom and one electron to hydroxyl, peroxyl and peroxynitrite radicals thus stabilizing them. Li *et al* reported that luteolin, hydroxygenkwanin, quercetin and rutin possessed a 3′,4′-dihydroxyl group in the basic skeleton of the flavonoids. These results indicated that the 3′,4′-dihydroxyl on the B ring was a key functional group for scavenging DPPH. However, kaempferol that was without the 3′,4′ –dihydroxyl group, also showed a DPPH scavenging activity, suggesting that 3-OH in conjunction with the 4-carbonyl group on the C ring also could contribute to the anti-DPPH activity. The possible role of 3-OH in the anti-DPPH activity may be related to the transformation of the 3′, 4′-OH with 4-keto by keto-enolic tautomerism (Li *et al.* 2008). However, the antioxidant ability of the flavonoids containing hydroxyl substituents on A-ring is less clear and has been

claimed to be compared with the B-ring, as the A-ring correlates little with antioxidant activity.

It is well known that most flavones and flavonols exhibit two major absorption bands in the ultraviolet/ visible region, Band I in the 320-385 nm range representing the B ring absorption, and Band II in the 250- 285 nm range representing A ring absorption. An increase in the number of hydroxyl groups substituted on the flavan nucleus induces a red shift, for instance from 367 nm in kaempferol (with hydroxyl groups in positions 3,5,7,4') to 371 nm in quercetin (3,5,7,3',4') and to 374 nm in myricetin (3,5,7,3 ',4 ',5'). The absence of a 3-OH group in flavones (which distinguishes flavones from flavonols) means that Band I is always at a shorter wavelength by 20-30 nm than that in the equivalent flavonols, for example apigenin (5,7,4'): 337 nm, kaempferol (3,5,7,4'): 367 nm. The O-Methylation and glycosylation produce hypsochromic shifts (Rice-evans *et al* 1996).

Compared with polyhydroxylated flavonoids, the polymethoxylated flavonoids are more lipophilicity and their molecular planarity is changed. The steric change caused a dramatic decrease in antioxidant activity (Mora *et al.* 1990); i.e., the methoxyl substitution on the B-ring of quercetin yielded a suppression of antioxidant activity of the molecule. However, the methoxyl substitutions most likely enhanced anticancer activity. We have recently reported the advantage in their anticancer activity of the methoxyl compared with the hydroxyl substituted series, for example WP283 exhibited about 90 to 100-fold more efficacy than eriodictyol. This should be due to its higher solubility and the methoxyl group could protect an active site of molecules, particularly C4=O, C5-OH and C2=C3 (Tungjai *et al.* 2008).

The presence or absence of a double C2=C3 bond and C4=O has been found to distinguish the general structure of flavonoids. A double bond and carbonyl function in the heterocycle or polymerization of the nuclear structure increases activity by affording a more stable flavonoid radical through conjugation and electron delocalization.

Several studies have reported on the qualitative structure-activity relationships for flavonoid-mediated anticancer and antioxidant activities and have stressed the importance of (i) the catechol moiety in the B-ring, (ii) the 2, 3-double bond and a 4-keto function in the C ring, and (iii) the additional presence of 3- and 5-hydroxyl groups in the flavonoid molecular structure to achieve an efficient antioxidant action.

Flavonoids are hydrophobic and weak acid molecules. Generally, the ionizing rate constant (pKa) of the hydroxyl moieties is always lower than physiological pH except for C3-OH and C5-OH. This indicated that hydroxyl moieties at all other positions may be sensitive to deprotonation at physiological pH. The deprotonation of hydroxyl moieties at C4′ or C7 is facilitated to the possibility for charge delocalization in the anion to the C4-keto moiety. It has been demonstrated that hydrogen bonding of the C3-OH or C5-OH with the C4=O carbonyl group hampers deprotonation (Lemańska *et al.* 2001, Teixeira *et al.* 2005, Castro *et al.* 2005). From the studies of the structure-activity relationship (SAR) of flavonoids, it has been shown that the dissociation of the hydroxyl function occurs in the following sequence: 7-OH > 4′-OH > 5-OH (Lien *et al.* 1999).

1.3 Bioactivity

1.3.1 Antioxidant activity

Flavonoids clearly showed antioxidant properties *in vitro* as chain breakers or radical scavengers depending on their chemical structures, which also affected their antioxidant power. A hierarchy has been established for the different flavonoids within each class on the basis of their capability to protect lipids, proteins or DNA against oxidative injury. Consequently, many of their biological actions have been attributed to those antioxidant properties (Foti *et al.*, 2005; Jeyabal *et al.*, 2005). This concept however, appears now to be a simplistic way to conceive their activity. First of all, pro-oxidant effects of flavonoids have also been described (Labieniec *et al.*, 2003) to have opposite effects on basic cell physiological processes: for example, if as antioxidants they improve cell survival, as prooxidants they may indeed induce apoptosis, cell death and block cell proliferation (Galati and O'brien, 2004). It should be noted that the intracellular redox status, which is influenced by antioxidants, can regulate different transcription factors, particularly NFκB, which in turn regulates various cell activities (Kabe *et al* , 2005; Crack and Taylor , 2005).

1.3.2 Anticancer activity

Many researchers have conducted *in vitro* studies on the potential anticancer activity of flavonoids in diverse cell systems. The inhibitory properties of flavonoids against carcinogenesis have also been reported. Hirano *et al.* examined anticancer efficacy of 28 flavonoids on the human acute myeloid leukemia cell line HL-60, and compared the differences between antiproliferative activity and cytotoxicity of these compounds with those of four clinical anticancer agents. Eight of the 28 flavonoids showed considerable suppressive effects on HL-60 cell growth with IC_{50}s ranging

from 10-940 ng/ml. The flavonoid genistein had the strongest effects which were almost equivalent to the effects of current anticancer agents with little cytotoxicity against HL-60 cells, whereas the regular anticancer agents had potent cytotoxicity (Hirano *et al.* 1994). Kuntz *et al.* screened more than 30 flavonoids for their effects on cell proliferation and potential cytotoxicity in human colon cancer cell lines Caco-2 and HT-29. Almost all compounds displayed antiproliferative activity without cytotoxicity. There was no obvious structure-activity relationship in the antiproliferative effects either on basis of the subclasses (i.e.,isoflavones, flavones, flavonols, and flavonones) or with respect to kind or position of substituents within a class (Kuntz *et al.*1999). An array of 55 flavones having a variety of substituents was evaluated by Cushman and Nagarathnam for cytotoxicity in five cancer cell cultures, A-549 lung carcinoma, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, SKMEL-5melanoma, and MLM melanoma. Fifteen of the 55 favone derivatives were significantly active against at least one of these cell cultures (Cushman and Nagarathnam 1991).

Quercetin induces an increase in apoptosis followed by a decrease in mitochondrial membrane potential value depending on its concentration. A decrease in the mitochondrial membrane potential value is associated with an increase in the percentage of early apoptotic cells (Kothan *et al.* 2004). The tea polyphenol, (-),-epigallocatechin gallate effected growth, induced apoptosis, and inhibited telomerase activity in cervical cell lines (Yokoyama *et al.* 2004). Dechsupa *et al.* showed that Quercetin, Siamois 1 and Siamois 2 induce apoptosis in human breast cancer MDA-mB-435 cells xenograft *in vivo*. As such, the first report on the apoptosis-inducing

effects of quercetin, Siamois 1 and Siamois 2 on the MDA-MB 435 cell *in vitro* were effectively extrapolated to the *in vivo* situation (Dechsupa *et al.* 2007).

1.3.3 Intracellular targets

Because flavonoids are very efficient antioxidants, their presence inside cells could cause severe modification of the redox-status. Recent evidence has added a newer dimension to the effects of elevated intracellular ROS by demonstrating that a pro-oxidant state amplifies cell proliferation either via direct stimulation of cell division and activation of transcription or indirectly by inhibiting the execution of the cell-death signal (Shi *et al.* 2003, Tatla *et al.*1999, Martin *et al.* 2002). These findings are consistent with the observations that certain cell types, in particular cancer cells, generate ROS which functions as an autocrine growth stimulator. As well as the pharmaceutical or endogenous inhibition of intracellular supperoxide anion radical production which enhances cancer sensitivity to drug-induced apoptosis (Martin *et al.*2002, Lehert *et al.* 2002, Miura *et al.* 2003). We have previously reported that there is a correlation between the mitochondrial membrane potential ($\Delta\Psi m$) as incubation time changed during quercetin induced cellular apoptosis in K562 and K562/adr cell. The ability of flavonoids 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 (Schimizu *et al.* 1999, Hengartner *et al.* 2000). 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 (Kroemer *et al.* 2000, Salvesen *et al.*1997, Raff M. 1998, Green DR. 1995).

1.3.4 Interactions of flavonoids with membranes

Depending on their chemical structures, flavonoids can partition in the hydrophobic core of membranes. Among different subclasses of flavonoids, their relative hydrophobicity measured as the partition coefficient between water and olive oil, is higher for the flavones and flavanones than for the flavanols. At the same hydroxylation degree, flavones are more hydrophobic than flavanones. The capacity of these compounds to affect transmembrane potential and pH differences is restricted to the more hydrophobic flavones and flavanones, while the flavonols, quercetin and morin, have no effect on these parameters (van Dijk *et al.* 2000). Ollila et *al.* showed an inverse correlation between the capacity of acacetin, apigenin, n-propylgallate, luteolin, quercetin and myricetin to induce membrane permeabilization (the release of calcein entrapped in liposomes) and their retention in a phosphatidylcholine coated column. The authors reported that this correlation was related to the relative hydrophobicity of the compounds (presence of the methoxy group and distribution of hydroxyl groups on the B-ring) (Ollila *et al.* 2002). The capacity of cinnamic acid and p-coumaric acid (4-hydroxy-cinnamic acid) to promote liposome permeability was studied by differential scanning calorimetry. The more hydrophobic compound: cinnamic acid which affected membrane permeability while its hydroxylated derivative, with a higher polar character, had no effect (Castelli *et al.*1999). Enhancing the hydrophobicity of cinnamic acid by decreasing the pH of the media (which causes the protonation of the carboxyl group) enhancing its permeabilizating effect. Similarly, the embedding of quercetin in bilayers depends on the pH of the

media. At acidic pH, quercetin is deeply embedded in planar lipid bilayers, while at physiological pH it interacts with the polar head groups at the water–lipid interface (Terao *et al.* 1994, Movileanu *et al.* 2000, Pawlikowska-Pawlega *et al.* 2003). The flavanones, naringenin and rutin, and a series of isoflavones partition in the hydrophobic portion of liposome bilayers, inducing the loss of membrane fluidity. The use of fluorescent probes that test lipid packing at different depths in the hydrophobic core showed the highest ordering effect of the flavonoids in the deepest region of the membrane (Arora *et al.*2000). At pH 7.4, quercetin, hesperetin and naringenin decreased the transition temperature of dipalmitoylphosphatidylcholine liposomes. This result indicates that at neutral pH, both flavonoids (hesperetin and naringenin) that are distributed in the hydrophobic portion of the membrane, and quercetin, which interacts with the surface of the bilayer, can affect the transition temperature of a membrane (Saija *et al.*1995).

2. Distribution and localization

2.1 Anatomy and physico-chemical properties of lipids

Lipids are amphiphilic molecules, contain both a polar hydrophilic, (water-liking) and a non-polar hydrophobic, (water-disliking) part (Figure 4). The former is soluble in water and the latter in organic solvents. Therefore, amphiphilic molecules are often present at interfaces (2-dimensional self assembly) where they lower the surface energy. For reasons that will be explained, amphiphilic molecules also self-assemble when they are completely surrounded by water, (3-dimensional self assembly,). For amphiphilic molecules to self-assemble, a hydrocarbon chain length of at least 10-12 carbon atoms is normally required (Bergethon 1998).

Figure 4 An amphiphilic lipid

Self-association in aqueous surfactant and lipid systems leads to the formation of a wide range of crystalline, liquid-crystalline, gel, and micellar phases. The formation of a particular phase depends on the geometry of the surfactant or lipid molecule, water content, pressure and the temperature of the system.

Let us consider, adding a drop of lipid to a container of water. The organic solvent, chloroform, will evaporate and lipids will form a monomolecular layer on the surface area in contact with water when water surface is sufficient. Due to the amphipphilic property of lipid molecules of which the head part (water-soluble) contacts to water and the tail part (oil-like) is exposed to air (see fig 5). Due to the fact that van der Waals envelope of hydrocarbon chain R1 and R2 in amphiphilic is cylinder shaped, the lipids in solution will form a lipid vesicle by bimolecular layer (Bergethon 1998, Nelson and Cox 2000).

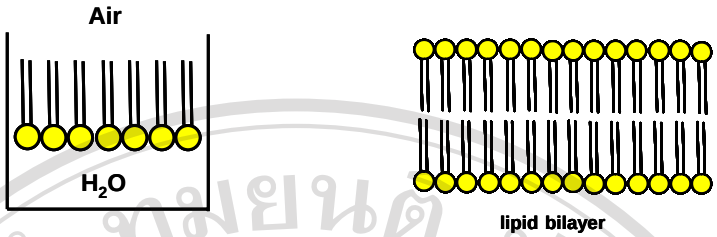


Figure 5 The monomolecular layer of lipid on surface area in contact with water

When lipids are dissolved in a polar solvent, there will be less contact between the lipid and the polar solvent. The head part of the lipid will be in contact with the solvent continuously. When water is added to the lipid solution, the lipid will continuously form a bilayer in which the polar head of the lipid contacts to the water and the tail of the lipid contact to the tail of another lipid. This lipid extends itself in water to form a sheet which then curls into a vesicle (see fig 6) (Bergethon 1998, Nelson and Cox 2000).

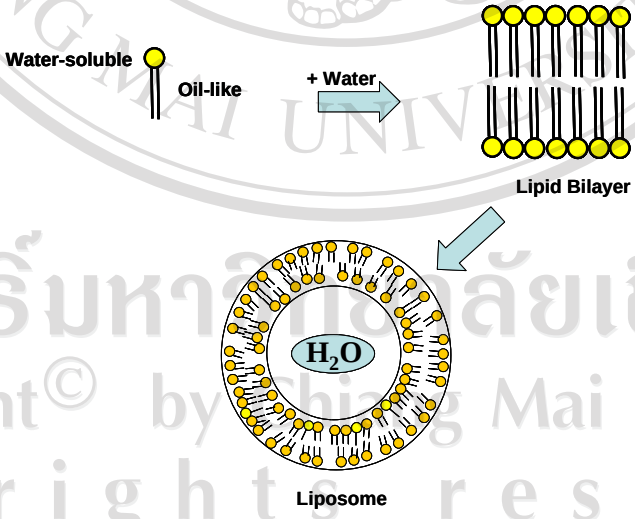


Figure 6 Scheme of vesicle formation

2.2 Enthalpy/Entropy

2.2.1 Phases of lipid

Amphiphilic molecules can form a variety of aggregate structures in the presence of water. Generally the geometrical shape of the lipid is cylindrical, and hence aggregation into bilayers is preferred. Bilayer forming tends to have a low solubility owing to their comparably long hydrocarbon chains. Lamellar phases exhibit several thermotropic phase transitions. The phase transition of the lipid depended on the enthalpy (ΔH), entropy (ΔS) and temperature. For a reversible phase transition at constant pressure, the molar free energies of the two states are equal at the critical temperature T_t. Therefore $\Delta H = T_t \Delta S$. Fig. 7 demonstrated that at low temperature, the bilayers are in a lamellar with a dense gel-crystalline structure. The symbol gel-crystalline phase β indicates a phase with tilted lipids. As the temperature is increased, conformational chain excitations increase and at a certain temperature, the sub-transition temperature, the bilayer is transformed into a lamellar gel phase. In this phase the lipids are less tightly packed than in the gel-crystalline phase, but there is still considerable chain order, with most of the chains in the all-trans configuration. Continuing to increase the temperature further increases the motion of the aliphatic chains until the order is lost. The lipids are said to melt and the bilayer transforms into the liquid-crystalline phase α , in a cooperative manner at the so-called gel to liquid crystalline phase transition temperature T_t. The α phase is characterized by trans-cis isomerizations or all-cis configuration (Bergethon 1998, Nelson and Cox 2000, Huang and Li 1999).

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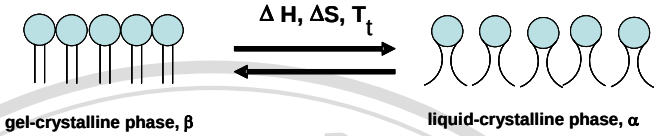

Figure 7 Phase transition

The diagram which shows the enthalpy in the system related to temperature is shown in figure 8. This diagram demonstrates that at initial temperature (T_i.), lipids were ordered conformation. When temperature is increased until reaching transition temperature (T_t), lipids achieved equilibrium between the gel-crystalline phase and the liquid-crystalline phase. At the final temperature (T_f), the lipids were disordered conformation. Therefore, when T_t is decreased, this represents that the lipid is in gel-crystalline phase and if T_t is increased that the represent lipid is in liquid-crystalline phase.

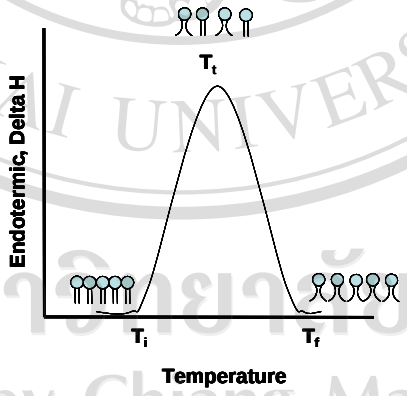

Figure 8 This diagram represents the energy transfer in the system related to temperature. T_i, T_t, and T_f represent initial, transition and final temperature for phase transition. Gel-crystalline phase β is shown at T_i and liquid-crystalline phase α is shown at T_f.

2.2.2 Mono lipids system

The main phase transition behavior of one-component lipid bilayers is well known to be influenced by many internal and external factors. Internal factors refer to the changes in the lengths of the two acyl chains, the chain length difference between the sn-1 and sn-2 acyl chains, chain unsaturation, back bone modification, and head group structures. External factors include pressure, pH, chemical composition and the temperature of the aqueous medium. Of the two thermodynamic parameters (T_t and pH) associated with the gel-to-liquid-crystalline phase transition, which can be determined directly from the thermal analysis experiment, the value of T_t can be determined with considerably greater accuracy. (Huang and Li 1999)

2.2.3 Lipid mixture system

Lipid bilayer membranes are composed of two types. The ones with low transition temperatures have acyl chains that are short or that cannot pack closely due to double bonds or side chains and Lipids with high transition temperatures have long, saturated acyl chains. Once these undergo phase separation they will be in both gel and liquid-crystalline phases between the chain transition temperatures (T_t) of the two components. Similar phase behavior is observed in mixtures of lipids with different headgroups, in mixtures of saturated and unsaturated lipids (Veatch and Keller 2005) and in mixtures of saturated and saturated lipids (Abes *et al.* 2007)

2.3 Transition phase

The effects of three flavonoids (quercetin, hesperetin and naringenin) upon the gel-to-liquid crystalline phase transition of model membranes is constituted by L-a-dipalmitoylphosphati-dylcholine (DPPC) vesicles. All tested flavonoids interacted with DPPC liposomes causing different shifts, toward lower values, of the

transition temperature typical for DPPC multilayers; the enthalpy changes (ΔH), related to the calorimetric peak area, remained nearly constant. However, no change in Tm of DPPC dispersion was observed in the presence of rutin. (Saija 1995, 1995) Hendrichl *et al* studied isoflavones (mononetin, irisolidone,licoisoflavone A and 6,8-diprenylgenistein) and noted that they alter the thermotropic phase behavior of DPPC in lipid–isoflavone mixture system. All four isoflavones caused the temperature of phase transition and their enthalpies to decrease, while the transition peaks were broadened in the presence of all isoflavones in a concentration-dependent manner. Transition peaks was apparently biphasic: for the isoflavone: DPPC, molar ratios up to 0.1 a concentration-dependent peak broadening was observed, but for the highest molar ratio (0.2) a resharpening of transition was recorded. The enthalpy of the DPPC phase transition was also altered in the presence of all studied isoflavones. (Hendrich *et al.* 2002)

OBJECTIVES

The aims of the study are:

1. To investigate the behavior and speciation of flavonoids in an aqueous physiological buffer.
2. To determine the interaction of flavonoids with myristyl myristate and Tween20 bilayers membrane, in terms of permeability and distribution on those bilayers.
3. To determine the relationship among chemical structure, anticancer activity and mean rate influx coefficient.

THE ORGANIZATION OF THE THESIS DISSERTATION

In this study, 7 flavonoids including catechin, eriodictyol, apigenin, kaempferol, quercetin, 5,4′-dihydroxy-3,6,7,8-tetramethoxyflavone (WP279), 5,5′-dihydroxy-6,7,3′,4′-tetramethoxyflavone (WP280) and 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxy- flavone (WP283) (for chemical structure see Figure 2) were selected and their molecular physical properties, such as aggregation, deprotonation rates and lipophilicity, were vigorously studied. This study also investigated the interaction of these flavonoids with micro-multilamellar vesicles made from myristyl myristate and Tween20. The distribution and localization of flavonoids onto the lipid membrane can be studied by using the thermotropic behavior of the lipid bilayer. The hydrophobic zone of the bilayer was successfully labeled using 1,6-diphenyl-1,3,5-hexatrine (DPH). Using the micro-multilamellar vesicle labeled DPH allowed the determination of the mean rate influx coefficient (k_+) of flavonoids.

The thesis has been edited and presented in article research formats, chronologically demonstrated from determination of solubility, lipophilicity of flavonoids and their anticancer properties in drug-sensitive and -resistant cancer cells. Thus, the establishment of thermal analysis method for the determination of the transition temperature of lipids in the membrane and in the molecular interaction of flavonoids with membrane has been achieved.

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