CHAPTER III

INTERACTION OF FLAVONOIDS WITH MYRISTYL MYRISTATE AND TWEEN20 BILAYER MEMBRANE: RELATION BETWEEN THE CHEMICAL STRUCTURES AND MEAN RATE INFLUX COEFFICIENT

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Interaction of flavonoids with myristyl myristate and Tween20 bilayer membrane : relation between the chemical structures and mean rate influx coefficient

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Abstract

This study aims to investigate their interaction and to determine the mean rate influx coefficient of two series of flavonoids including the hydroxyl and methoxyl group substitution with artificial bilayer membrane of multilamella vesicles. All molecules studied underwent a reversible ketalisation with keto group of the head of myristyl myristate yielded hemiketal and ketal molecules that affected the head volume of lipid as consequence dramatically decrease in gel-fluid phase transition temperature. A molar fraction of flavonoids passively diffused through out then resided at hydrophobic zone of the membrane. Among the flavonoids studied, the k_+ values varied from the slowest for eriodictyol (0.5×10^{-5} s^{-1}) to the fastest for quercetin was by factor 8. The results also clearly shown that both C2=C3 and C3-OH are essential functional groups that facilitate the flavonoids to diffuse across the lipid bilayer membrane. Compared with apigenin, their polymethoxylated derivatives passively diffused through out the membrane faster become similarly to that of quercetin. In addition, these methoxyl substitutions could protect the flavonoids to undergo ketalisation with the ketal group of the membrane to preserve the molecule in intact form.

Keywords: Flavonoids, micromultilamellar vesicles, mean rate influx coefficient (k_+), 1,6-diphenyl-1,3,5-hexatriene (DPH), gel-fluid phase transition temperature (T_t)

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Introduction

Flavonoids 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* [Tungjai *et al.* 2008, Kothan *et al.* 2004, Ng *et al.* 2005] and *in vivo* [Dechsupa *et al.* 2007, Yuan et al. 2006, Benkovic *et al.* 2007] conditions to triggering apoptotic program cell death via mitochondrial pathway [Kothan *et al.* 2004, Lee *et al.* 2005, Yeh *et al.* 2007, Lee *et al.* 2008, Zhang *et al.* 2008]. Kothan reported that quercetin, apigenin, kaempferol and eriodictyol exhibited advantage cytotoxicity against multidrug resistant K562/adr with overexpression P-gp and GLC4/adr with overexpression MRP1 compared with their corresponding parental cell lines [Kothan *et al.* 2004]. 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 previously characterized the speciation of catechin, eriodyctyol, apigenin, kaempferol and quercetin compared with those molecules substituted at various carbon atom positions such as WP279, WP280 and WP283 in a physiological solution. The hydroxyl or 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 [Tungjai *et al.* 2008].

Huge studies reported the interaction of flavonoids with artificial membrane systems [Ollila *et al.* 2002, van Dijk *et al.* 2000, Azize *et al.* 1992, Yuan *et al.* 2006]. 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.* [Saija *et al.* 1995] reported that flavonoid incurporation into liposomal membrane caused stress in the 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.

This study rigorously investtigated 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 Tween20 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 [Andrich and Vanderkooi, 1976, Lakowicz, 1999]. 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.

This study clearly shown that the lipid bilayer of myristal myristate-Tween20 membrane system was suitable as model for determining the mean rate influx of non-fluorescence molecules such as flavonoids. This membrane system also provided the data of flavonoid interacted and resided on the head and hydrophobic zone, respectively. This is very important parameters for developing the molecules as anticancer drugs.

Materials and Methods

Construction of the multilamellar mysistyl-myristate-Tween20® vesicles:

Myristyl myristate and poly-oxyethyene (20) sorbitan monolaurate (Tween20) was purchased from Fluka Chemie (Fluka Chemie GmbH, Switzerland). Myristyl myristate was used as the lipophilic phase and Tween20 was used as surfactant. Twice distilled water was used as hydrophilic phase.

Myristyl myristate (0.073 g, wax form) was dissolved in 1.5 mL chloroform in a 500 mL round flask then added 400 μL Tween20 and, after brief and vigorous mixing (mm:tween 20=1:4.22).The solvents were removed under a nitrogen flow in a rotary evaporator yielding thin film. Then thin film was freeze-dried under vacuum to remove the residual solvents. Liposomes were obtained by adding to the film 20 mM HEPES-Na^+ buffer (pH 4), then heated at a temperature above that of the gel-liquid crystalline phase transition (60°C) and mixed three times for 1 minute using vortex. The resulting vesicles were filtered using 5 and 1 μm pore size of cellulose nitrate membrane filters and the exterior pH of vesicle was adjusted to 3, 7.3 and 8.2 using 0.1 N HCl or 0.1 N NaOH before use.

In order to labeling the hydrophobic zone of myristyl myristate-Tween20 vesicle, 1 μM 1,6-diphenyl-1,3,5-hexatrine (DPH) was added to the mixture solution of myristyl myristate and Tween20 prior to constructing the thin film.

Micrograph of liposomes

The micrograph of liposomes was recorded using an inverted microscope (CETI, Belgium) combine with an Avervision 300P (Aver media, Taiwan). The liposomes (10 μL) were injected into the hemocytometer. The diameter of the liposomes was obtained using the photoshop Microsoft, by counting the number of liposomes in the area of 1 mm^2. The diameter of liposomes in μm unit was plotted as a function of the number of liposomes and the data was computed using Gaussian equation (data analysis software Origin 6 (OriginLab Corp., Nortampton, Mass.).

Measurements of doxorubicin entrapped in liposomes

Doxorubicin, an intrinsic fluo-rescence molecule can be used to assess the existence of lipid bilayer that can separate the outside from the inside of vesicle. Because doxorubicin is a weak base (pKa =8.4 at 37 °C)

[Frezard and Garnier-Suillerot, 1990], thus in an aqueous solution at a given pH, it is found in positive charge (-NH_3^+ the will call DH^+) form equilibrate to neutral (-NH_2 the will call D°) form. It is generally accept that only D° can passively diffuse from outside (alkalinize pH) through membrane into inside (acidic pH) and, the weak base doxorubicin readily protonates to DH^+; at pH 4, more than 99% doxorubicin was found in positive charge form. The so-called auto-quenching effect which corresponds to a significantly decrease in doxorubicin fluorescence intensity was observed at high concentration (millimolar range). The so-called "auto quenching" can be used to assess the efficiency of the entrapping and accumulation of doxorubicin inside liposome.

An experiment was conducted in 1 cm quartz cuvettes containing 1 μM Doxorubicin and 2 ml of HEPES-Na^+ buffer, vigorously stirred at 37 °C in a spectrofluorometer (Perkin Elmer spectrofluorometer, model LS55). The emission spectrum of doxorubicin when excited at 480 nm was recorded. Then an incremental concentration of liposome was added into the solution prior to recording their emission spectra.

Measurements of the mean rate influx coefficient (k_+) of flavonoids:

Fluorescence quenching was recorded in a Perkin Elmer spectrofluorometer, model LS55 equipped with stirrer system and thermoregulated cell holders. The emission spectrum of liposome-DPH when excited at 356 nm was appeared between 380 nm to 550 nm with the maximum fluorescence intensity at 429 nm. The maximum fluorescence intensity decreased without any change in spectral shape in the presence of flavonoids. For measuring the influx of flavonoid through out the bilayer membrane, an 1-cm quartz cuvette containing 2 mL HEPES-Na^+ pH 7.3 at 37°C and the fluorescence intensity at 429 nm when excited at 356 nm was recorded as a function of time. Vesicles (1×10^7) were added into the cuvette, the fluorescence intensity immediately increased then stable and assigned as F_0. After addition of the precisely flavonoid concentration the fluorescence intensity at 429 nm progressively decreased as a function of time.

Measurement the critical temperature of lipid bilayer:

The heating and cooling system consists of a Peltier element located directly below the sample. The liquid cell is placed on top of the sample and reference. The temperature is monitored by a IQ3000 combines handheld computer power with 2-channel thermocouple thermometer (IQ Scientific Instruments, Inc., USA) of type K maintained directly inside the cell of sample and reference. The measured temperature is a relative temperature with respect to the temperature of reference, which is monitored separately with a thermometer. This experimental setup allows us to follow the thermotropic behavior of lipid bilayer of liposome aqueous suspension in a real-time continuous acquisition.

Theoretical approach

The interaction of flavonoids with keto group, acyl chain of saturated lipid and their distribution in myristyl myristate-Tween20 bilayer affected the gel-fluid phase transition temperature of the lipid bilayer. Temperature has been used to trigger lipid phase transitions. With increasing temperature the following

conformational changes of lipid at the transition temperature, T_t are: (a) formation of rotational isomers within the lipid hydrocarbon chain, (b) the onset of lipid lateral diffusion of the lipids molecules (ordered to fluid transition), and (c) a considerable expansion of the bilayer area. 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 $\Delta H = T_t \Delta S$, where ΔS denotes the entropy change and ΔH the enthalpy change, or the heat absorbed at the ordered $\rightarrow$ fluid transition. ΔH may be written as a sum of a pure lipid term, ΔH°, and a mixture of flavonoid-lipid term $\Delta T = T_{(fluid)} - T_{(ordered).}$. The change in T_t caused by flavonoid effects is given by $\Delta T_t = T_t - T_t^0 = \frac{\Delta T}{\Delta S}$, where T_t is the observed transition temperature and $T_t^\circ = \frac{\Delta H^\circ}{\Delta S}$ is the value of T_t in the absent of flavonoid interactions.

The temperature difference between the sample and the reference cell as a function of temperature used for trigger lipid phase transitions was recorded and the transition temperature can be determined as the peak of the first derivative of the curve. Enthalpies were evaluated from the peak areas using data analysis software Origin 6 (OriginLab Corp., Nortampton, Mass.), permitting the choice of different baselines and ranges of integration.

Results

Measurements of doxorubicin accumulation and size of liposome:

The micrograph demonstrates that the vesicles obtained using freezed/thaw technique and filtrated using syringe filter was regularly in size and form. The normal distribution pattern of vesicles was computed using Gaussian equation and have the center of peak at 8.006 μm and the full width at half of maximum (FWHM) was equal to 2.1157 μm (Figure 1a and 1b). The multilamellar myristyl myristate vesicles can be classed as micro-vesicle and were stable when suspended in an aqueous solution during experiments under vigorously stirring at least 48 h at room temperature.

The permeability of the bilayer membrane and its ability to separate the environment of the outside from the inside of the vesicles was evaluated. The liposomes were firstly created by adjusting the inside and outside pH to 4 , then the pH of outside of liposome was adjusted to 7.3 or 8.2 and the stability of vesicles in these news conditions was rechecked again by using microscope.

Before evaluating the doxorubicin entrapped inside the liposomes, it was verified that an addition of incremental concentration of myristyl myristate lipid solution or the solutions containing liposomes having the equal outside and inside pH into the solution containing 1 μM doxorubicin yielded slightly decreased in doxorubicin fluorescence intensity without any changes in emission spectral shape. While an addition of incremental content of liposomes containing the pH inside equal to 4 against the pH outside 7.3 or 8.2 resulting a significant extinction of doxorubicin fluorescence intensity the so-called autoquenching that signifies doxorubicin entrapping inside liposome (Figure 2a and 2b). The overall doxorubicin accumulation was 0.027 μM and 0.040 μM per 10^6

vesicles/μL at pH 7.3 and 8.2, respectively. These signified ability of the lipid membrane to separate the different environment, outside against inside of vesicles.

Measurement of the mean rate influx coefficient (k_+) of flavonoids:

The rate of flavonoid diffused through out the myristyl myristate-Tween20 bilayer was measured by recording the rate of DPH fluorescence quenching. The hydrophobic zone of the myristyl myristate and Tween20 bilayer was successfully labeled using DPH. The acyl chain-DPH fluorescence quenching after addition of flavonoids could be interpreted as followed: (i) flavonoid from out side of liposomes passively diffused through the polar zone of lipid bilayer to reach its hydrophobic zone where DPH resides and (ii) flavonoid mediates a collision with DPH that can facilitate non-radiative transitions to the ground state, resulting an immediately DPH fluorescence extinction. It was verified that using the absolute ethanol as solvent, the rate of DPH fluorescence quenching in the presence of flavonoid is very fast compared with those in the presence of liposome. The progressive quenching of acyl chain-DPH fluorescence quenching after addition of flavonoids was due to passive diffusion of the molecule through out the membrane is the rate limiting step of the entire process.

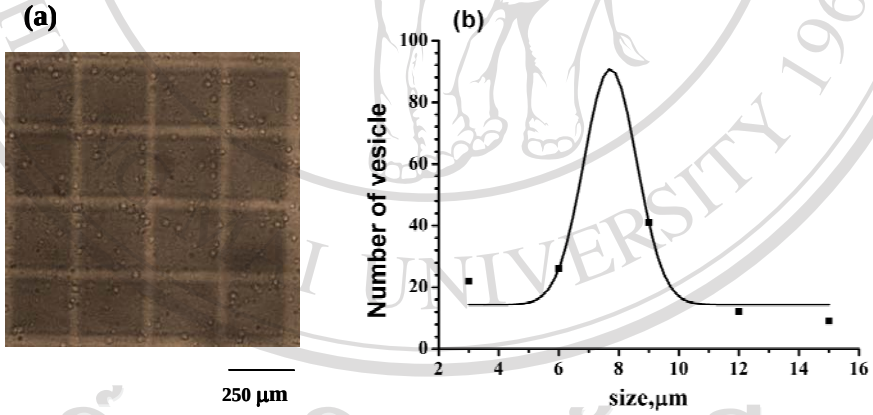


Figure 1. Micrograph of multilamellar vesicles (a) and histogram of vesicular size distribution: 10 μL vesicles suspension was injected into the chamber of the hemocytometer then placed on the sample holder and took the micrograph. The diameter of vesicles was measured using the photoshop Microsoft and the numerized for 1 mm^2. The data was computed using the Gaussian equation; the center of peak found at 8.006 μm and full width at half of maximum equal to 2.1157 μm.

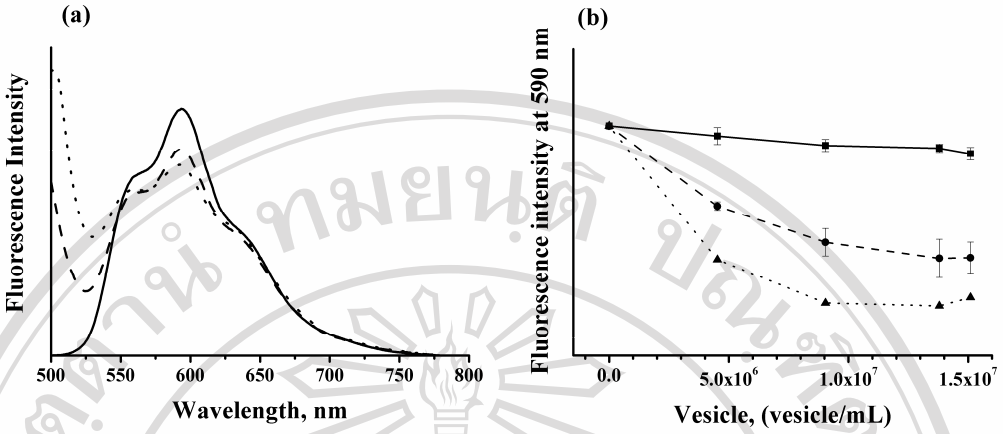


Figure 2. Emission spectra of doxorubicin (1 μm) (a) and variation of fluorescence intensity at 590 nm as a function of liposome contents (b). Experiments were conducted in 1-cm quartz cuvette containg 2 mL HEPES-Na^+ buffer and 1 μM doxorubicin, vigorously stirred at 37 °C in a spectrofluorometer. The emission spectrum when excited at 480 nm of doxorubicin was recorded. Then a successive addition of a small volume yielding the indicated vesicle concentration was added into the system, then let further incubation for 30 minutes prior to record the emission spectra.

Under these conditions, the unidirectional influx of flavonoid into liposome can be written as:

$$\vec{J} = PC_T^{\circ}$$

where P is the permeability coefficient that depends on flavonoid and the myristyl myristate-Tween20 bilayer. C_T° is a final flavonid concentration. When 1 mole of flavonoid moves from outside the liposome to its hydrophobic zone where DPH resides, and the variation of the fluorescence per mole is:

$$\delta F = F_0 - F_{flavonoid}$$

where the F_0 and $F_{flavonoid}$ are the DPH fluorescence intensity before and after addition of flavonoid to the system, and during Δt, n moles of flavonoid move from the outside of liposome through the bilayer, resulting in a modification of the fluorescence intensity, ΔF :

$$n = V_+ \Delta t \text{ or } n = PC_T^{\circ}.\Delta t$$

$$\Delta F = n\delta F$$

Thus,

$$\frac{\Delta F}{\Delta t} = PC_T^{\circ}(F_0 - F_{flavonoid})$$

During the first 30 seconds after the addition of flavonoids, $\frac{\Delta F}{\Delta t}$ was determined by $\frac{\partial (F)}{\partial t}$, the slope of the tangent of $F = f(t)$, corresponding to V_i, the initial rate of the DPH fluorescence quenching.

$$V_i = PC_T^{\circ}(F_0 - F_{flavonoid}) = k_+ \Delta F$$

The k_+ value determined for all flavonoids studied was indicated in Table 1.

Thermal properties of the myristyl myristate and Tween20 bialyer:

In order to get further more insight the interaction between flavonoid and lipid, the temperature was applied to trigger lipid phase transitions. The reference and sample 1-cm quartz cuvette were filled with 2 mL HEPES-Na^+ buffer pH 7.3 at 37°C and let to reach an equilibrium state for 30 minutes before measuring. In order to test the effect of pH an liposome formation, the HEPES-Na^+ buffer and the outside pH of liposomes was initially adjusted to pH 3.0, 7.3 or 8.0 at 37°C. The ΔT ($\Delta T = T_S - T_R$) was recorded as a function of time or temperature. A small volume of liposomes to yield a final concentration 3.0×10^6 /mL was injected into the sample cuvette, resulting a progressive increase in ΔT. It was check that such a small volume of buffer solutions used did not cause any increase in ΔT. Figure 3a demonstrates that the change of ΔT after injection of liposomes by varying the out side pH of 3.0, 7.3 or 8.0 as a function of temperature. The pattern of ΔT change of the three series of experiments is similar; slightly decreased at lower temperature following sharply decreased then reached a pseudo-plateau. The particular observation was that for the series of experiments using the pH at outside 3.0 and 8.0 results perfectly shown the same pattern of ΔT change while those using the pH at outside 7.3 shown the extend of reflect curve. The first derivative of the curve was calculated using the data analysis software Origin 6 as indicated in Figure 3b. Figure 3b clearly demonstrated the temperature that induces the initial transition T_i, the temperature transition T_t and the temperature at end point T_f of acyl chain of the lipids. The T_t value of the acyl chain of the bilayer of lipid membrane of the liposome with the pH at outside 3.0, 7.3 and 8.0 was 30°C, 63°C and 30°C, respectively.

Flavonoids affected the thermal properties of the myristyl myristate-Tween20 bialyer:

In order to assess the effects of molecules studied on the thermal properties of lipid bilayer, the reference and sample of 1-cm quartz cuvette were filled with liposomes (final concentration = 1.0×10^7) suspended in 2 mL HEPES-Na^+ buffer pH 7.3 at 37°C and let to reach an equilibrium state for 30 minutes before measuring. It was check that neither such a small volume nor solvent used caused an increase in temperature difference. The typical results of the flavonoids affected the thermal properties of the membrane were indicated in Figure 4 and 5.

Figure 4a demonstrated that the first derivative of the temperature difference after addition of 50 μM, 100 μM or 200 μM apigenin (final concentration) into the sample cuvette of the series of experiments performed using the pH equal to 7.3 as a function of temperature. The addition of 50 μM apigenin caused a broad single transition at 39 °C and this peak became sharper and increase in intensity when 100 μM and 200 μM was added signifying an increase in apigenin concentration bound to lipid bilayer. The results clearly shown that apigenin efficiently decreased in T_t of the lipid from 63 °C to be 37 °C. Two series of experiments with similar conditions were performed using HEPES-Na^+ buffer pH 3 and 8 at 37°C, respectively.

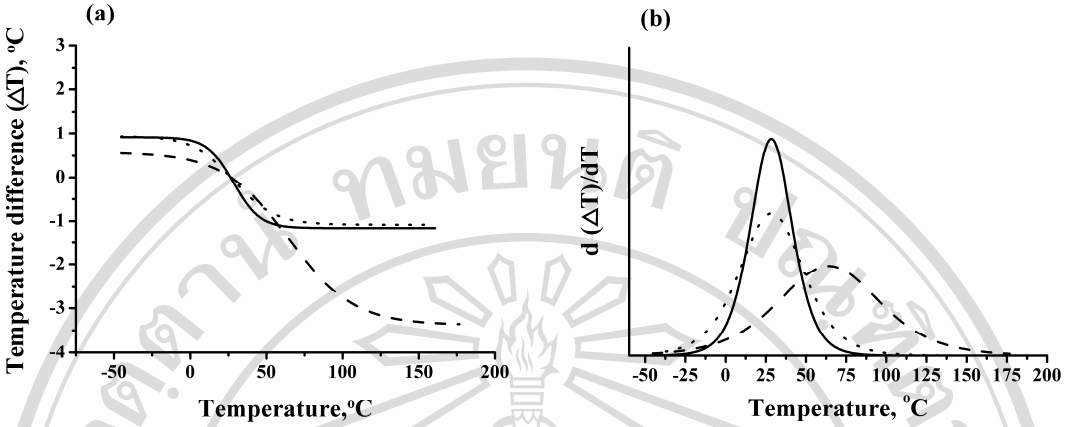


Figure 3. Variation of temperature difference of myristyl myristate-Tween20 bilayer membrane (a) and first derivative spectra of the data (b). One-cm quartz cuvettes were placed on the heating and cooling system. The temperature is monitored by a IQ3000 combines handheld computer power with 2-channel thermocouple thermometer of type K maintained directly inside the cell of sample and reference. The measured temperature is a relative temperature with respect to the temperature of reference, which is monitored separately with a thermometer. One-cm quartz cuvette of reference and sample was filled with 2 mL HEPES-Na+ buffered initially adjusted to pH 3 (point line), 7.3 (dashed line) and 8 (bold line) at 37 °C and let to reach equilibrium state for 30 minutes before starting a record. A small volume of liposomes to yield a final concentration 3.0×10^6 /mL was injected into the sample cuvette, and the temperature difference was recorded as a function of temperature and the transition temperature can be determined as the peak of the first derivative of the curve.

Figure 4b demonstrated the partition of the neutral form of apigenin (pH 3) caused a broad single transition at 33 °C and at higher concentration such as 200 μM, apigenin trends to caused a decreased in T_t from 30°C to 26 °C. Figure 4c demonstrated the partition of charge form of apigenin (pH 8) caused a broad single transition compared with those of pH 7.3 and, apigenin caused an increase in T_t from 30°C to 41 °C, 43 °C and 36 °C for 50 μM, 100 μM and 200 μM, respectively.

Figure 5 demonstrated that the first derivative of the temperature difference after addition of 50 μM, 100 μM or 200 μM WP 283 (final concentration) into the sample cuvette of the series of experiments performed using the pH equal to 7.3, 3 and 8 as a function of temperature. The addition of 50 μM WP 283 caused a broad single transition at 24 °C and the center of the peak was shifted to 19 °C and dramatically decreased in ampitude when 100 μM and 200 μM, respectively, was added (Figure 5a). The results clearly shown that WP 283

efficiently decreased in T_t of the lipid in the HEPES-Na^+ buffered pH 7.3 dependent upon the concentration added. It should be noted that such the effects did not obviously registered with the series of experiments performed in acidic and alkalinized conditions (Figure 5b and 5c).

The gel-to-fluid phase transition temperature difference ($\Delta T_t = T_t - T_t^0$) before and after addition of flavonoids performed using HEPES-Na^+ buffer the initial pH of 3, 7.3 and 8 were indicated in Table 1.

Discussion

This study vigorously determined the interaction of flavonoids including apigenin, eriodictyol, kaempferol, quercetin, WP 279, WP 280 and WP 283 with artificial lipid bilayer membrane. The myristyl myristate and Tween20 were chosen for constructing the micro-multilamellar vesicle because of their simple chemical structure particularly its saturated acyl chains. Indeed these micro-mutilamellar vesicles are very useful model for studying the reaction between flavonoid and keto head of myristyl myristate and, its passive diffusion ability through out the lipid bilayer and its distribution on the membrane. In order to use lipid bilayer models for measuring the permeability of the bilayer membrane against flavonoid molecules, the micro-multilamellar vesicles were constructed with incorporating DPH into the hydrophobic zone of the membrane. In fact the distribution of DPH in lipid bilayer was well characterized by neutron diffraction (Pebay-Peroula *et al.*, 1994*).* The same authors showed that there were two DPH populations found in the hydrophobic zone. The first group of DPH was oriented parallel to the acyl chains, at a 30° angle to the bilayer normal, buried deep within the hydrophobic region. And the second group of DPH was oriented parallel to the membrane surface resided between the two leaflets and was parallel to the surface, with a wider distribution. We had verified that DPH was almost nonfluorescent in water and most of the DPH emission originated from membrane bound DPH. This was consistency to those reported by Lakowicz (Lakowicz, 1999). The acyl chain-DPH fluorescence quenching after addition of flavonoids predominantly caused by the molecule at the hydrophobic zone underwent a collision with DPH that can facilitate non-radiative transitions to the ground state, resulting in an immediately DPH fluorescence extinction. It was verified that the rate of DPH fluorescence quenching performed in ethanol without micro-multilamellar vesicles was very fast compared with the rate of lipid-DPH fluorescence quenching (data not shown). Indeed, the membrane permeability is the rate limiting step of the entire process that lowering the flavonoids to reach at the hydrophobic zone of membrane. Thus, the rate of DPH fluorescence extinction reflects the diffusion rate of flavonoid across the polar head into the hydrophobic zone. The results indicated that the lipid bilayer labeled with DPH is a suitable model for measuring the transport parameters of non-fluorescence molecules, particularly flavonoids.

The flavonoids used in this study was divided into two groups regarding to their k_+ values through out the myristyl myristate-Tween20 bilayer; the first group consisted of apigenin, eriodictyol, kaempferol and quercetin

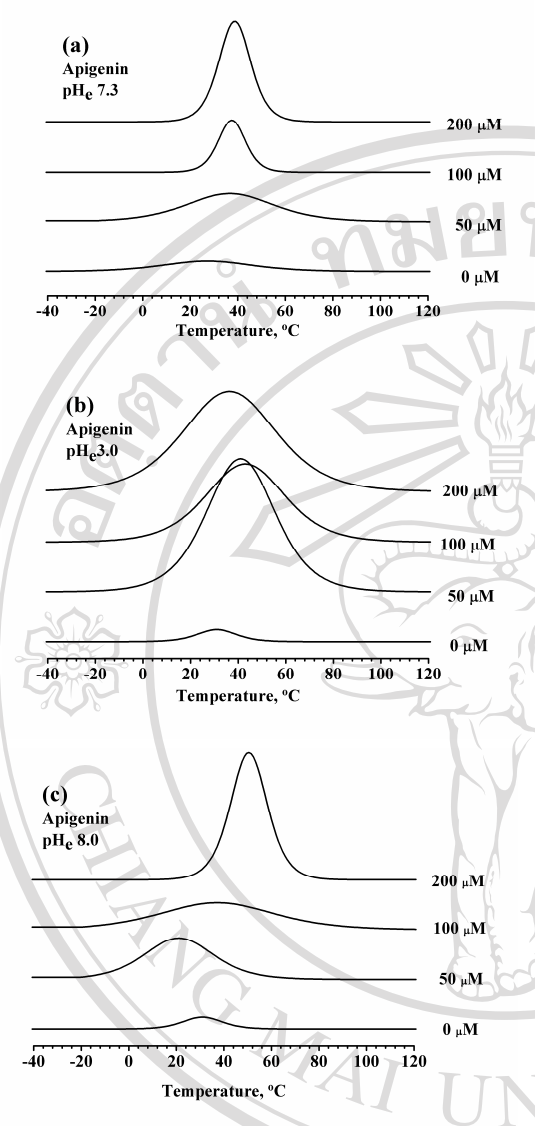


Figure 4. A typical results of apigenin caused the thermal pattern of myristyl myristate-Tween20 bilayer changes. The series of experiments conditions and procedures were the same as cited in Figure 3, the outside pH of vesicles equal to 7.3 (a), 3.0 (b) and 8.0 (c).

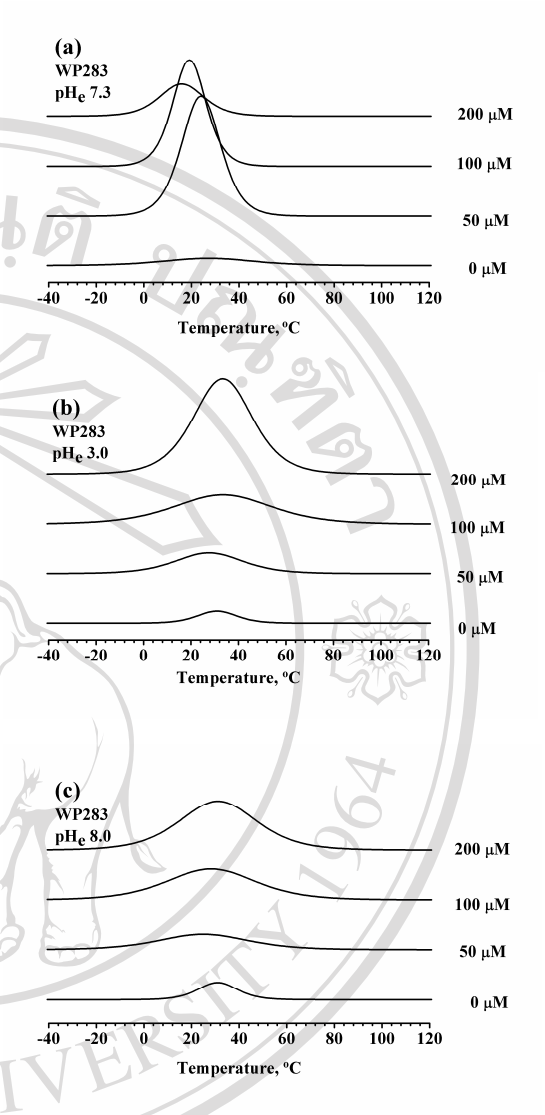


Figure 5. A typical results of WP 283 caused the thermal pattern of myristyl myristate-Tween20 bilayer changes. The series of experiments conditions and procedures were the same as cited in Figure 3, the outside pH of vesicles equal to 7.3 (a), 3.0 (b) and 8.0 (c).

and the other group is the flavonoids substituted with methoxyl groups at various carbon atoms of the molecules included WP 279, WP 280 and WP 283. Among the flavonoids studied, the k_+ values varied from the slowest for eriodictyol to the fastest for quercetin is by factor 8 (Table1). The results also clearly show the relationship between chemical struc- ture and the k_+ values. Eriodictyol, apigenin, kaempferol and quercetin is very similar in their chemical structures. As previously mentioned, eriodictyol that lacking in double bond at C2=C3 and hydroxyl group at C3 has k_+ value slower than apigenin that contains double bond at C2=C3 but lacking in hydroxyl at C3 by factor 4. The particular observation was eriodictyol has k_+ value slower than quercetin that contains double bond at C2=C3 and hydroxyl at C3 by factor 8. The results clearly show that both C2=C3 and C3-OH are essential facilitate the flavonoids to translocation across the lipid bilayer membrane.

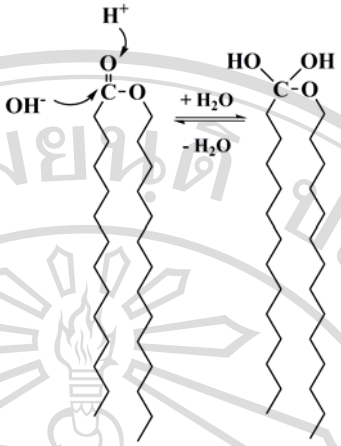
Comparison with eriodictyol, WP 280, the methoxyl groups substitution at C6, C7 , $C3^{/}$, $C4^{/}$ and hydroxyl group at $C5^{/}$ but lacking in C3-methoxyl group caused an increased in k_+ value by factor 6. The substitution of methoxyl groups at various positions of carbon atoms on ring A, B and C (WP 279 and WP283) caused an increase in k_+ value by factor 8. As indicated in Table 1 showed that the methoxyl group substitutions in place of hydrogen atoms and/or hydroxyl groups at various positions of carbon atoms in ring A, B and C resulted in an increase in the neutral form, solubility and lipophilicity of molecules [Tungjai *et al.* 2008] but these substitution did not cause an increase, contrary slightly decrease in k_+ value when compared with quercetin.

In this work we demonstrated that the micro-multilamellar vesicles were stable in HEPES-Na^+ buffered in a wide range of pH comprised from 3 to 8. However, the thermal properties of the bilayer in solution pH 3 and 8, particular the gel-fluid phase transition temperature was the same and, 33°C decreased compared with those performed in pH 7.3. Because the myristyl myristate contains keto group that serves as polar or head of the lipid. In an aqueous solution, the keto group easily undergoes hydration by H^+ or OH^- catalysis and this is reversible process (Scheme 1). Both the electrophilic addition to oxygen atom and the nucleophilic addition to carbon atom following an intramolecular rearrangement of the lipid yields the hydrate molecules. The hydration of keto group resulted in an increase in polar head volume thus the distance between the lipid-lipid molecules as the consequence of lowering hydrophobic forces. These should be the predominant phenolme- non responsible to a considerable decrease in the transition temperature of the lipid bilayer.

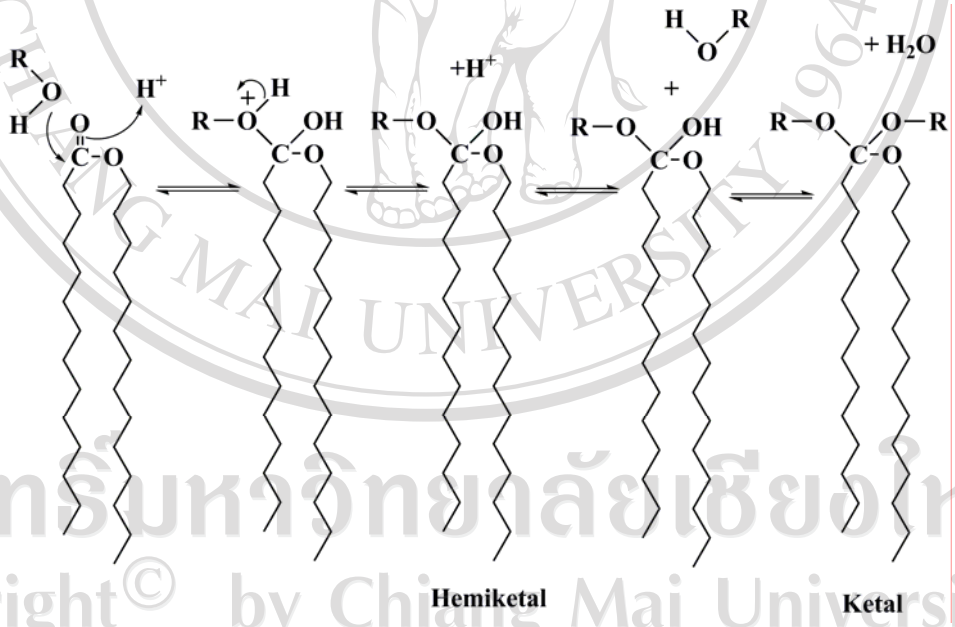
Flavonoids probably play two distinct actions on the lipid bilayer membrane, first, they undergo reversible ketalisation with the keto head of lipid and its neutral form passively diffused into the hydrophobic zone of the membrane that caused a strengthen in its hydrophobic force. Indeed the chemical structure of given flavonoid is similar to cholesterol that is well known as cement of the natural membrane. The ketalisation yielded

Table 1 The effects of flavonoids studied on the gel-to-fluid phase transition of the lipids the initial pH of 3, 7.3 and 8

Molecules	k_+ , s^{-1}	$\Delta T_t = T_{to}-T_{tf}$, °C			*IC_{50}, μM* [Tangjai *et al.* 2008]			
		pH = 7.3	Neutral form	Charge form	K562	K562/ *adr*	GLC4	GLC4/ *adr*
Apigenin	2×10^{-5}	+ 24	- 6	- 21	9.0 ± 5	9.0 ± 5	4.0 ± 2	6.0 ± 0.4
Eriodictyol	0.5×10^{-5}	+ 25	-10	- 5	9.0 ± 2.5	> 100	10 ± 3	10 ± 2.5
Kaempferol	4×10^{-5}	+ 23.5	+ 12	+ 1	8.5	7.0	6.5 ± 0.7	16.8 ± 9.6
Quercetin	4×10^{-5}	+ 26	+ 7	+ 18	6.0 ± 1.4	7.8 ± 0.4	6.0	11.5
WP 279	3×10^{-5}	+ 29.5	0	0	0.8	0.8	0.5	0.7
WP 280	3×10^{-5}	+ 37.5	+ 5	0	4.0 ± 3.0	1.0 ± 0.1	1.1 ± 0.6	1.0 ± 0.7
WP 283	4×10^{-5}	+ 47.6	+ 3	+ 1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1



Scheme 1. Hydration of keto group of myristyl myristate-Tween20 bilayer membrane catalysis by acid or base.



Scheme 2. Ketalisation of flavonoids with keto group of myristyl myristate-Tween20 bilayer membrane

hemiketal and ketal molecules that caused considerable an increase in polar head volume (Scheme 2). The results clearly demonstrated that flavonoids significantly caused a decrease in T_t of the lipid bilayer (Table 1 for pH 7.3), signifying the predominant reaction was the ketalisation of flavonoids with keto group and these were reversible process. A molar fraction of flavonoids passively diffused through out the membrane and at steady state a molar fraction of molecules resided in the hydrophobic zone of the membrane. The study also shown that the neutral and charge form of flavonoids interacted with the ketal group of lipid except for the series of molecules with methoxyl substitution, particularly for WP279.

This study clearly shown 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 through out the myristyl myristate-Tween bilayer. The k_+ values of the planar flavonoids with hydroxyl substitution depends upon the C2=C3 and C3-OH. The substitution of methoxyl groups caused slightly a decrease in the k_+ value of molecules. However, all compounds studied affected he thermal properties of the membrane via ketalisation as the predominant reaction and this reaction is reversible process. We have recently preported that the advantage in their anticancer activity of the methoxyl compared with the hydroxyl substituted series, for example WP 283 exhibited about 90 to 100-fold more efficacy than eriodictyol. This should be due to its higher solubility [Tungjai *et al.* 2008] and the methoxyl group could protect the active site of molecules particularly C4=O, C5=OH and C2=C3.

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