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Antimicrobial mechanism of flavonoids against *Escherichia coli* ATCC 25922 by model membrane study

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ABSTRACT

Antimicrobial mechanism of four flavonoids (kaempferol, hesperitin, (+)-catechin hydrate, biochanin A) against *Escherichia coli* ATCC 25922 was investigated through cell membranes and a liposome model. The release of bacterial protein and images from transmission electron microscopy demonstrated damage to the *E. coli* ATCC 25922 membrane. A liposome model with dipalmitoylphosphatidylethanolamine (DPPE) (0.6 molar ratio) and dipalmitoylphosphatidylglycerol (DPPG) (0.4 molar ratio), representative of the phospholipid membrane of *E. coli* ATCC 25922, was used to specify the mode of action of four selected flavonoids through Raman spectroscopy and differential scanning calorimetry. It is suggested that for flavonoids, to be effective antimicrobials, interaction with the polar head-group of the model membrane followed by penetration into the hydrophobic regions must occur. The antimicrobial efficacies of the flavonoids were consistent with liposome interaction activities, kaempferol > hesperitin > (+)-catechin hydrate > biochanin A. This study provides a liposome model capable of mimicking the cell membrane of *E. coli* ATCC 25922. The findings are important in understanding the antibacterial mechanism on cell membranes.

Introduction

Flavonoids are a group of plant-derived heterocyclic organic compounds, which are divided into 14 different subgroups [1] according to the chemical nature and position of substituents on the A, B and C rings. Many biological properties of flavonoids have been reported, including antimicrobial, antioxidant and vascular activities [2]. This family of compounds has undergone considerable antibacterial research and the common antimicrobial mechanism could be divided into two main aspects: (1) inhibition of the nucleic acid synthesis in bacteria: flavonoids with different substituent groups were reported to display different inhibition activities of DNA gyrase from *Escherichia coli* [3]; (2) cell membrane damage of bacteria: some flavonoids have been shown to inhibit bacteria by damaging the cell membrane which would cause change of membrane fluidity followed by outflow of some intracellular components. Sophoraflavanone G was reported to reduce the fluidity of bacterial cellular membranes [4]. The leakage of components such as intracellular enzyme, protein, ions and nucleotide was determined to illustrate damage to the cell membrane. Because of the complex composition of bacterial membranes, researchers have begun to study a model membrane as an alternative. A study using a liposomal model membrane concluded that epigallocatechin gallate damaged the membrane and induced the leakage of small molecules from cells [5]. Our research team reported a significant positive correlation between antibacterial activity and membrane rigidification effect of the flavonoids and demonstrated that the activity of the flavonoid compounds can be related to molecular hydrophobicity and charge on C atom at position C3 [6]. Other researches indicated specific groups on the ring of flavonoids also affected the membrane interactivity [7]. Although the relationship between flavonoids structure and membrane interactivity has been reported, there is little information on the mode of action of flavonoids on cell membrane.

On the basis of previous studies that flavonoids interact with phospholipids in bacterial cell membranes, the liposome model was selected to study how flavonoids alter the fluidity of membranes. Phosphatidylethanolamines (PEs) and phosphatidylglycerols (PGs) are main phospholipids in bacterial cell membranes. Dipalmitoylphosphatidylethanolamine (DPPE) is of a higher concentration in the inner membrane of Gram-negative bacteria,

Abbreviations: DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycerol-3-[phospho-*rac*-1-glycerol]; ATCC, American Type Culture Collection; PMB, polymyxin B; MIC, microbial inhibition concentration; TEM, transmission electron microscope; PBS, phosphate buffer solution; DSC, differential scanning calorimetry.

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for example *E. coli*, than dipalmitoylphosphatidylglycerol (DPPG). Mixtures of these phospholipids can approximate the natural membrane in some phase properties [8]. The DPPE–DPPG/water vesicles, with DPPE as the dominant constituent and DPPG in lower concentration, are reported to be reliable systems to mimic the cell membrane of Gram-negative bacteria [9].

In this work, four subgroups of flavonoids, biochanin A (isoflavone), hesperitin (flavanone), kaempferol (flavonol), (+)-catechin hydrate (flavanol), are represented. The four common flavonoids selected through testing show different antimicrobial activities which could reflect differences in cell membrane damage. Variation in antimicrobial activities had been reported [10–13]. The antibacterial property of the flavonoids and interaction between *E. coli* ATCC 25922 membrane and flavonoids were evaluated by determining the minimum inhibitory concentrations (MICs), leakage of bacterial proteins and observing the changes to the cell membranes through transmission electron microscopy (TEM). Then, fully hydrated liposomal vesicles were prepared to simulate the natural *E. coli* ATCC 25922 membrane. To determine the change of fluidity of the model liposome, effects of flavonoids as antimicrobials on model membrane structure were examined by Raman spectroscopy and differential scanning calorimetry (DSC). DSC was used to observe the changes in thermograms caused by chemicals and Raman method could determine the exact location of the chemicals on liposome. These two methods could jointly prove the interaction between chemicals and liposome as reported in previous studies [14–16]. This study constitutes the first contribution to the correlation between antimicrobial properties and liposome interaction activities of different flavonoids. An applicable liposome model to mimic *E. coli* ATCC 25922 cell membrane is provided.

Materials and methods

Chemicals

Flavonoids (biochanin A, hesperitin, kaempferol, (+)-catechin hydrate) and the selected antibiotic polymyxin B sulfate (PMB) were purchased from Aladdin Industrial Co. (Shanghai, China). Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dipalmitoyl-*sn*-glycerol-3-[phospho-*rac*-1-glycerol] (DPPG) of high purity (>99%) were purchased from Xi'an Ruixi Biological Technology Co. Ltd. (Xi'an, China). Coomassie brilliant blue kit was purchased from NanJing JianCheng Bioengineering Institute (Nanjing, China). Other chemicals of analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Antibacterial activity and mechanism

Bacterial strain and cultures

E. coli ATCC 25922 used to evaluate antibacterial properties was purchased from China Center for Type Culture Collection and cultured on nutrient broth (NB, microbial reagent, Hangzhou, China) at 37 °C for 12 h. Then the bacterial strain was maintained in tubes with 50% (v/v) glycerol at −80 °C according to the stock method described by a previous study [17].

Active cultures were achieved by dispensing a tube of cells into 100 mL of nutrient broth and incubating at 37 °C for 10 h. The turbidity of the cell suspension was adjusted to the initial concentration of 10^8 cfu/mL according to the McFarland standard [18].

Microbial inhibition concentration

The minimum inhibitory concentrations (MICs) were determined according to the micro-dilution in broth method described by previous study [19]. First the antimicrobial agents were diluted in duplicate using nutrient broth by serial twofold dilution method to obtain the range of concentrations and added to each well of 96-well plates. Next, the cell suspensions of *E. coli* ATCC 25922 containing 10^8 cfu/mL in nutrient broth were added, so that each well contained 100 μL antimicrobial agent and 100 μL cell suspension. Background tubes containing broth and chemical agent, negative control tubes containing broth and bacterial suspension and the positive control (PMB and cell suspensions) were set up. The plates were incubated in a shaker incubator at 37 °C for 16 h, then the absorbance was measured at 600 nm by Microplate Reader (Multiskan Spectrum, Thermo Co., Waltham, Massachusetts, USA). The MIC_{90} was defined as the lowest concentration of antimicrobial agent which could inhibit the growth of the bacteria by 90% [20]. The inhibition ratio (%) was calculated as follows: percent inhibition = 100% − [(absorbance of the test sample − absorbance of control)/absorbance of control] × 100.

Bacterial proteins release

In order to determine loss of membrane integrity, amount of protein released from the cells was determined in supernatants. 0.5 mL cell suspensions (10^8 cfu/mL) in 4 mL broth were shaken at 37 °C in the presence of the antimicrobial agents at the concentration of MIC_{90} values (the concentration of biochanin A was the same as (+)-catechin hydrate). The suspensions with PMB and flavonoids were set as positive control and treated samples, respectively. The bacterial suspension in the absence of the antimicrobial agents was the negative control. The samples were collected at 0, 1, 2, 3, 4 h and centrifuged at 2000 × *g* (4 °C, 20 min). The supernatants were stored at 4 °C until analysis according to a modified method of Coomassie brilliant blue protein assay [21]. Samples containing 0.1 mL supernatant with 6 mL Coomassie brilliant blue reagent were prepared to determine the UV absorption at 595 nm after 10 min.

Transmission electron microscope (TEM)

TEM method was used for observing cell membrane damage. Suspensions of the logarithmic growth phase cells of *E. coli* ATCC 25922 were cultured for 3 h with antibacterial agents at a concentration of more than 10-fold MIC values according to the method described by previous study [22]. The concentration of biochanin A was the same as (+)-catechin hydrate. The negative control, without antibacterial agents, was prepared in a similar manner. The samples were harvested by centrifuging at 8000 rpm for 10 min at 4 °C and washed three times with 0.01 M phosphate buffer solution (PBS, pH 7.4). The pellet was resuspended in 2.5% glutaraldehyde at 4 °C for 12 h and fixed in 1% osmium tetroxide for 2 h, then dehydrated by graded ethanol and acetone, embedded with SPI-812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under Tecnai G^2 20 TWIN TEM of USA FEI Co. (Hillsboro, Oregon).

Model lipid membranes

Preparation of liposome

The liposomal vesicles were prepared by the method of thin-film hydration ultrasound method reported by previous study [23] with some modifications. The lipids in 0.4 DPPG/DPPE + DPPG molar ratio system were dissolved in a mixture of chloroform and methanol (2:1, v:v). The solvents were removed using a rotary evaporator at 45 °C until there was a thin film on the flask walls and no visible solvents remaining. The film was dried in a vacuum oven overnight. The PBS (0.01 M, pH 7.4) containing different concentrations of flavonoids (10%, 20%, 30% molar ratios for Raman determination and 10%, 15%, 20%, 25%, 30% molar ratios for DSC determination) was added to the dried lipid film. The negative control group contained the lipids suspended in PBS solution only. The positive control group contained PMB replacing the flavonoids.

The mixtures were sonicated for 20 min at 45 °C for dispersion and homogeneity to achieve the model liposome of high quality.

Raman spectroscopy

The FT-Raman spectra were obtained by a VERTEX70 instrument from Bruker Co. (Bremen, Germany). The test conditions were as follows: the laser line of 1064 nm with the power of 500 mW was used as excitation energy; samples were scanned from 50 to 3500 cm^{-1} at 4 cm^{-1} resolution with 2 cm^{-1} interval, with temperature maintained at 25 °C. The data analysis software Bruker OPUS 5.0 was used to analyze the spectra. The concentrations of antimicrobial substances were adjusted to 10%, 20% and 30% molar ratio. Before examining the interaction between the lipids and antimicrobial agents, the samples were equilibrated for 24 h.

Differential scanning calorimetry (DSC)

DSC measurements were performed using a Pyris Diamond DSC Perkin-Elmer Co. (Waltham, MA, USA) at the heating and cooling rate of 10 °C/min ranging from 25 to 100 °C. Temperature and enthalpy were calibrated with indium (T_m = 156.60 °C). Analysis of the thermograms was carried out by Pyris Software 5.0. The flavonoid, (+)-catechin hydrate, and PMB were selected for DSC measurement at 10%, 15%, 20%, 25%, 30% molar ratio. The samples (~5 mg) were sealed in hermetic aluminum cells with an empty hermetic cell acting as reference. The scanning cycle was repeated on three to four different aliquots to ensure stable thermograms and reproducible data.

Statistical analysis

All experiments were performed in triplicate. The data are presented as mean (n = 3) ± standard deviation for the results of intracellular proteins release and DSC. The statistical evaluation of the results was carried out by paired-samples T test in the statistical software SPSS, version 16.0 for Windows (SPSS Inc.). Differences between groups were considered significant when $P < 0.05$.

Results and discussion

Antibacterial activities

Determination of microbial inhibition concentration

The antibacterial properties of flavonoids were quantified initially by MIC values. The MIC_{90} values of kaempferol, hesperitin, (+)-catechin hydrate and PMB (positive control) were 185.6, 244.4, 5050 and 55.8 μg/mL, respectively. The *E. coli* ATCC 25922 strain was resistant to biochanin A (MIC_{90} > 10,000 μg/mL). According to the detected MIC_{90} values, the order of antimicrobial efficacy was PMB > kaempferol > hesperitin > (+)-catechin hydrate > biochanin A.

Analysis of bacterial proteins release

The results of protein leakage from cell membrane are shown in Fig. 1. It was observed that when flavonoids were added to the *E. coli* ATCC 25922 strain, leakage of bacterial protein to extracellular suspensions occurred over the assayed time period. The protein content in supernatant of the negative control sample was 1.4 μg/mL initially and 5.9 μg/mL after 4 h. In comparison, there was a significant ($P \leq 0.05$) increase of protein release in samples treated with flavonoids. The largest increase of protein content was observed for kaempferol from initial 20.5 μg/mL to 44 μg/mL after 4 h. Protein leakage was lowest for cells treated with biochanin A (5.4 μg/mL (0 h) and 16.2 μg/mL at 4 h). Values for hesperitin and (+)-catechin hydrate were 2.7–24.4 μg/mL and 8–32.5 μg/mL, respectively. The protein release data indicates that individual flavonoids damaged the cell membranes of *E. coli* ATCC 25922 to different extents, with the order kaempferol > hesperitin > (+)-catechin hydrate > biochanin A.

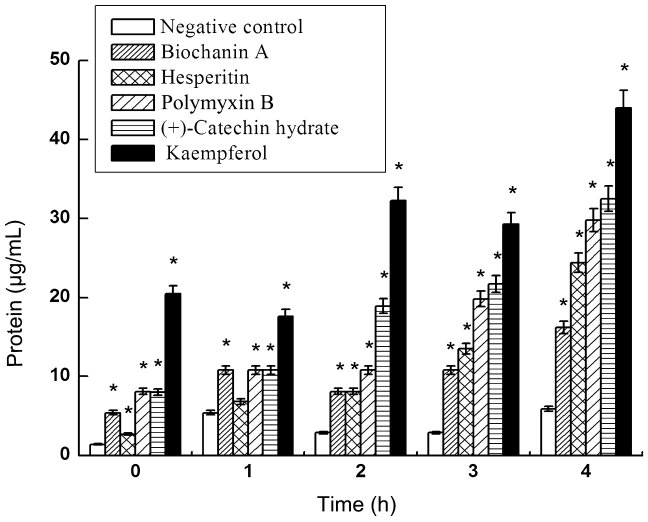

Fig. 1. The release of intracellular protein in *Escherichia coli*. Results are presented as mean (n = 3) ± standard deviations. Asterisk denotes a significant difference ($^*P < 0.05$). Flavonoids concentrations: MIC_{90} values. Determination temperature: room temperature (25 ± 2 °C).

The antimicrobial effect of flavonoids on membrane function has been previously evaluated by determining the leakage of intracellular components, such as sodium, potassium, reducing sugar, protein and intracellular enzyme. A study using galangin suggested that a large amount of K^+ was released from the cells when *Staphylococcus aureus* was incubated with flavonoids [5]. The leakage of nucleotides from bacterial cells and the change in membrane permeability were visible in the presence of chalcone in the cultures [24]. Cell damage by catechins was reported to the concentration of leaked proteins in the treated samples [25]. From protein leakage observed in this study we can similarly conclude that cell membrane was damaged.

Flavonoids have been reported to exert antimicrobial action by attacking cell membranes of bacteria. Extract from the root galangal was shown to cause a large burst of cell membranes of *S. aureus* resulting in the leakage of cytoplasm [26]. *E. coli* O157:H7 cells interacting with anthocyanins became partially disintegrated while cells were totally burst after incubation with organic acids [27]. Flavonoid compounds in the same subgroup (polymethoxylated flavone) were shown to display different activities of membrane disruption with tangeretin being more effective than nobiletin [28].

Transmission electron microscope (TEM) observation

Based on $MIC9_0$ values and intracellular protein release, TEM was used to compare damage to bacterial cells caused by kaempferol, illustrative of a high antimicrobial activity flavonoid and biochanin A, of low activity. The normal cell of *E. coli* ATCC 25922 is shown in Fig. 2A which displays typical Gram-negative structure with intact membrane and high density cytoplasm. The rough surface of cell membranes treated with PMB as positive control (Fig. 2B), indicates that the membrane has been disrupted having lost its regular and continuous structure. Significant changes on the morphology of cell membranes occurred when strains were treated with kaempferol (Fig. 2C). The most apparent phenomenon was plasmolysis involving the outflow of intracellular constituents, suggesting alteration in the fluidity and integrity of cell membranes. Biochanin A caused minor damage to membranes, however the inner membrane boundaries became unclear (Fig. 2D). The density of intracellular protoplasm of the treated cells was visibly lower than the normal one. The TEM images confirm that flavonoids

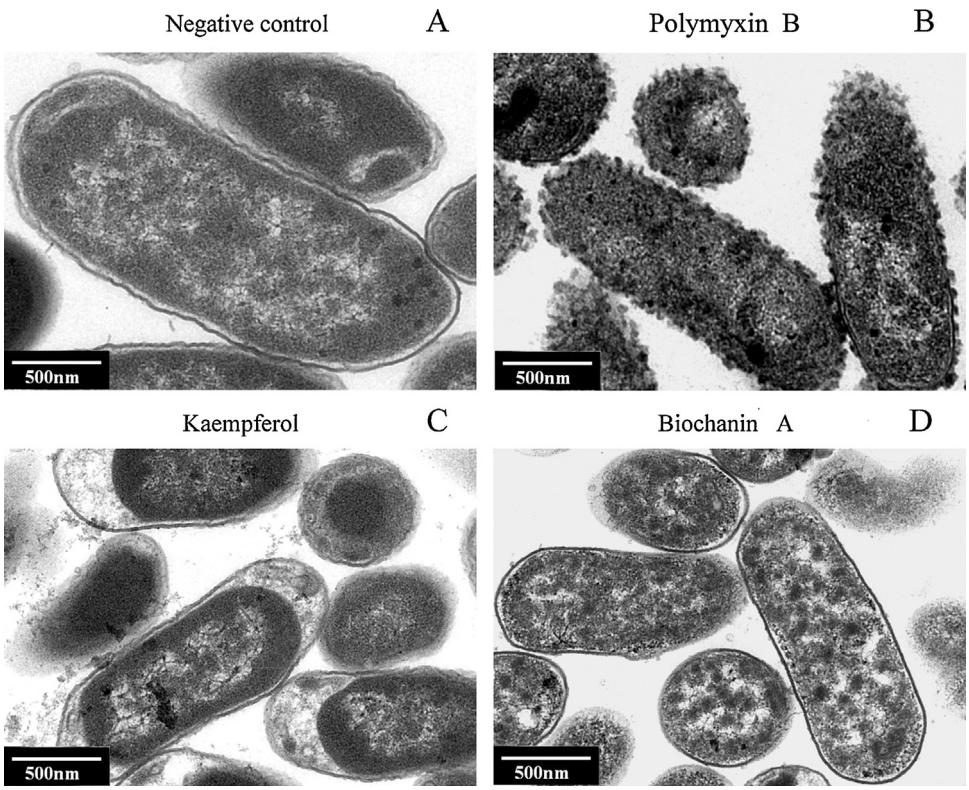

Fig. 2. Transmission electron microscope images of *Escherichia coli* cells (magnification 7800×).

exhibiting stronger antimicrobial effects cause greater degree of membrane damage than those with weaker antibacterial effects. The results are in accordance with protein release and microbial inhibition obtained for the selected flavonoids.

Research of liposome model

Raman spectroscopy analysis

Raman spectra were used to investigate flavonoid penetration into the lipid bilayer. The fluidity of liposomal membranes, reflected by the order of phospholipid molecule arrangement, was chosen as a key parameter. The peak intensity ratios I_{2934}/I_{2884}, I_{2848}/I_{2884}, I_{1095}/I_{1129}, I_{1095}/I_{1064} provide the order/disorder extent of the conformation of the alkyl chain of lipids. As shown in Fig. 3, the bands at 2848 and 2884 cm^{-1} were associated with the symmetric and asymmetric stretching vibration of C—H bond of the methylene groups while the absorption peak at 2934 cm^{-1} corresponded to the symmetric stretching mode of C—H bond of the terminal methyl group enhanced by Fermi resonance. The ratios of I_{1095}/I_{1129} and I_{1095}/I_{1064} represented the ratio of *gauche*/*trans* (disorder/order) conformations of the C—C bond vibration in the alkyl chains [15].

The results of Raman peak intensity ratios are shown in Table 1. All samples incubated with flavonoids at 10% molar ratio showed an increase in peak intensity ratios. The increase, followed by a decrease of intensity ratios, was likely due to the internal interaction of flavonoids before reaching the model membrane. This phenomenon has been reported in a study showing that chemical molecules accumulated which weakened the effects on phospholipid molecules [29]. However, changes occurred with an increase in flavonoid concentration. The intensity ratios of kaempferol, hesperitin and (+)-catechin hydrate treated samples decreased at 20%, 30%, 30% molar ratio, respectively, in contrast to biochanin A which a further increased at a constant rate. The changes of intensity ratios indicated the decrease of *gauche*/*trans* ratios that could be interpreted as a decrease in disorder/order ratios of alkyl chains and a decrease of the liposome fluidity. Flavonoids interaction with the head groups of the liposome weakening the C—H bond vibration and C—C bond vibration of the alkyl chains is the probable reason. The results are in agreement with a recent study that reported that helicid molecules interacted with the surface of liposomes increasing the lateral order of the membrane [16]. For samples treated with kaempferol (at 30% molar ratio) increased peak intensity ratios were observed (Table 1) with I_{2848}/I_{2884}, I_{1095}/I_{1129} values

Table 1
The Raman peak intensity ratios at different proportions of flavonoids/DPPE:DPPG and PMB/DPPE:DPPG systems.

Sample (mol%)	I_{2934}/I_{2884}	I_{2848}/I_{2884}	I_{1095}/I_{1129}	I_{1095}/I_{1064}
DPPE:DPPG	0.30	0.64	0.53	0.54
Kaempferol/DPPE:DPPG				
10	–	0.65	0.73	0.72
20	–	0.61	0.46	0.46
30	–	0.65	0.57	0.53
Hesperitin/DPPE:DPPG				
10	0.33	0.70	0.64	0.69
20	0.48	0.86	0.66	0.57
30	0.35	0.58	0.60	0.60
(+)-Catechin hydrate/DPPE:DPPG				
10	0.34	0.68	0.59	0.57
20	0.39	0.72	0.70	0.72
30	0.34	0.68	0.65	0.59
Biochanin A/DPPE:DPPG				
10	0.36	0.65	0.56	0.50
20	0.43	0.63	0.62	0.61
30	0.47	0.68	0.72	0.66
PMB/DPPE:DPPG				
10	0.33	0.65	0.62	0.64
20	0.27	0.60	0.50	0.47
30	0.31	0.59	0.48	0.45

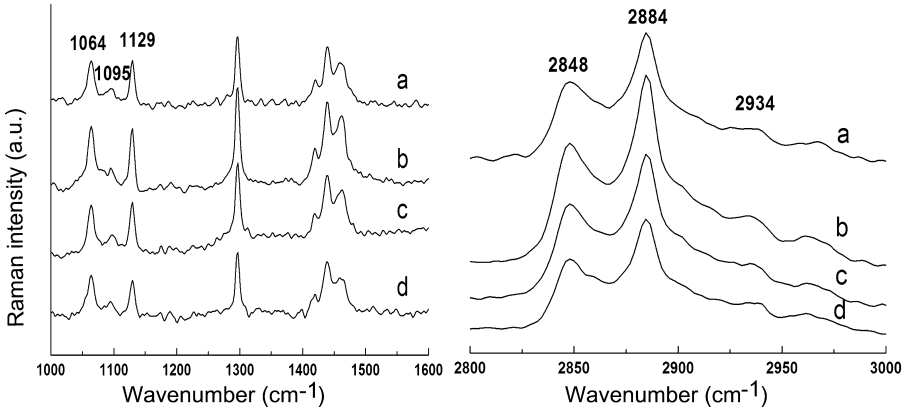

Fig. 3. Raman spectra of liposome mixtures at different concentrations of (+)-catechin hydrate (a: 0 mol%; b: 10 mol%; c: 20 mol%; d: 30 mol%).

higher than the negative control indicating vibration enhancement of C—H bond in the methylene groups and C—C bond in the alkyl chains. Meanwhile, the membrane disorder increased fluidity of the liposome indicating that kaempferol molecules penetrated the hydrophobic region of the lipid bilayer. The results are consistent with previous research reporting that the disorder/order ratio increased due to chemical interaction on alkyl chains of the liposome [15,30]. The peak band at 2934 cm^{-1} shifted in the presence of kaempferol, thus the I_{2934}/I_{2884} of the kaempferol group could not be measured. The liposome fluidity of the positive control (PMB) suggests that PMB located in the head group. Other studies have also reported that the antimicrobial mechanism of this polypeptide antibiotic was due to interaction with phosphate groups of the bacterial cell membrane [31–33].

Kaempferol decreased the fluidity of model liposome at a lower concentration (20%) than hesperitin and (+)-catechin hydrate (30%), and the peak intensity ratios at 20% molar ratios were lower than the negative control (Table 1), indicating greater interaction with the model liposome. The peak intensity ratios of hesperitin and (+)-catechin hydrate treated samples began to decrease at the same concentration (30%), however, for hesperitin the I_{2848}/I_{2884} value was lower than negative control, while for (+)-catechin hydrate all the intensity ratios were slightly higher than the negative control. These results suggest that hesperitin decreases the fluidity of the membrane affected the membrane more than (+)-catechin hydrate. The ability of the selected flavonoids to interact with liposome was kaempferol > hesperitin > (+)-catechin hydrate > biochanin A which is in agreement with their antibacterial efficacy.

Differential scanning calorimetry analysis

DSC thermograms have been used to detect the transition thermodynamic parameters [(ΔH (transition enthalpy), T_m (transition temperature), T_{OS} (temperature at which the transition starts)] of flavonoid/lipid–water systems from the gel phase to the liquid crystalline phase during an increase in temperature [15].

Raman data suggested that (+)-catechin hydrate positioned on the surface of the liposome, however, detailed information (whether penetration occurred into the deeper region) could not be determined. DSC was used to confirm whether (+)-catechin hydrate would penetrate from the head group into the hydrophobic region of the liposome. Calorimetric parameters of the treated sample, (+)-catechin hydrate, and the positive control (PMB) are shown in Tables 2 and 3, respectively and thermograms in Fig. 4. As shown in Table 2, ΔH values decreased at low concentrations (<25%) followed by an increase at 25%. The ΔH decrease can be attributed to the internal action of (+)-catechin hydrate before interaction with the liposome. This phenomenon was also reported by a previous study [29] and supports Raman spectra data in this study. A sharp increase in ΔH was displayed at (+)-catechin hydrate/lipid 25% molar ratio, but decreased with increased concentration (30%). The sharp ΔH increase suggested the flavonoid was interacting with surface groups of the liposome membrane, with a sharp decrease indicating a deeper penetration of flavonoid into the internal hydrophobic moieties. For PMB, the variation trend of ΔH was not distinct. The T_m values in Tables 2 and 3 (main transition) decreased with an increase of catechin and PMB concentration as the polar head group of lipids is one of the target

Table 2
Calorimetric parameters observed in the heating and cooling process of (+)-catechin hydrate–DPPE/DPPG liposome.

(+)-Catechin hydrate/ DPPE:DPPG (mol%)	T_{OS}[a] (°C)	T_m[b] (°C)	ΔH[c] (J/g)
DPPE:DPPG	79.10 ± 0.01	81.49 ± 0.02	42.33 ± 0.05
10	78.60 ± 0.05	81.18 ± 0.02	42.84 ± 0.05
15	78.48 ± 0.03	81.23 ± 0.01	39.96 ± 0.03
20	78.34 ± 0.02	80.80 ± 0.03	35.76 ± 0.11
25	78.36 ± 0.01	80.69 ± 0.02	40.32 ± 0.04
30	77.82 ± 0.02	80.59 ± 0.04	30.44 ± 0.10

Results are presented as mean ($n = 3$) ± standard deviation from the experiments in triplicate.
[a] T_{OS}, temperature at which the transition starts.
[b] T_m, the maximum temperature of the calorimetric peak.
[c] ΔH, transition enthalpy.

Table 3
Calorimetric parameters observed in the heating and cooling process of PMB–DPPE/DPPG liposome.

PMB/DPPE:DPPG (mol%)	T_{OS}[a] (°C)	T_m[b] (°C)	ΔH[c] (J/g)
Pre-transition			
0	–	–	–
10	43.74 ± 0.04	46.86 ± 0.05	3.74 ± 0.14
15	43.65 ± 0.03	46.66 ± 0.04	3.85 ± 0.07
20	43.05 ± 0.06	45.73 ± 0.04	6.23 ± 0.04
25	45.04 ± 0.03	47.25 ± 0.03	4.01 ± 0.03
30	42.48 ± 0.01	45.50 ± 0.04	3.31 ± 0.09
Main transition			
0	79.10 ± 0.01	81.49 ± 0.02	42.33 ± 0.05
10	71.36 ± 0.01	74.52 ± 0.02	33.43 ± 0.08
15	71.03 ± 0.03	74.10 ± 0.01	31.66 ± 0.04
20	70.79 ± 0.02	74.19 ± 0.05	33.43 ± 0.06
25	72.23 ± 0.04	75.28 ± 0.03	32.55 ± 0.14
30	66.95 ± 0.02	72.43 ± 0.03	34.35 ± 0.12

Results are presented as mean ($n = 3$) ± standard deviation from the experiments in triplicate.
[a] T_{OS}, temperature at which the transition starts.
[b] T_m, the maximum temperature of the calorimetric peak.
[c] ΔH, transition enthalpy.

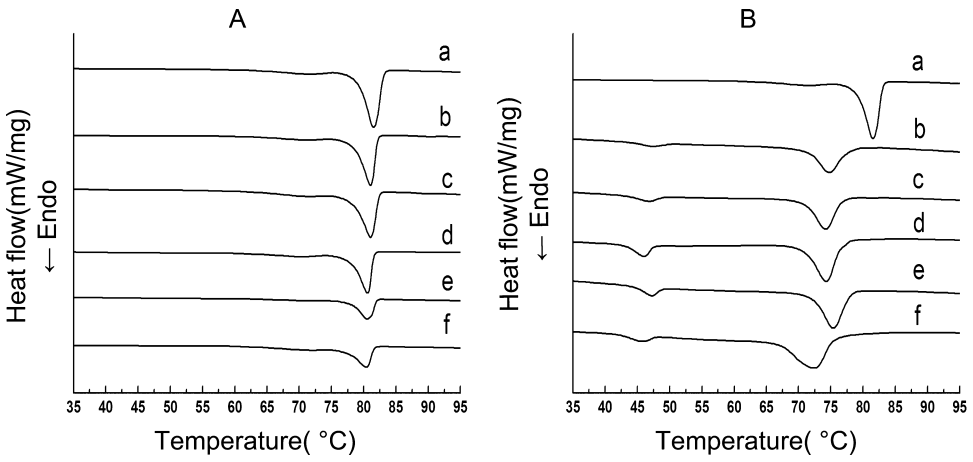

Fig. 4. DSC thermal response of liposome mixtures at different concentrations of (+)-catechin hydrate (A) and PMB (B) (a: 0 mol%; b: 10 mol%; c: 15 mol%; d: 20 mol%; e: 25 mol%; f: 30 mol%).

sites. A small T_m decrease indicates that chemical molecules poorly affect the hydrophobic region and mainly localize at the polar head level [14]. The scanning thermograms of PMB (Fig. 4B) differed from the (+)-catechin hydrate treated sample (Fig. 4A). After incubating with PMB, the thermograms displayed a pre-transition, the pure DPPE–DPPG/water system has been reported to undergo a single phase transition without pre-transition [9]. The emergence of the new phase indicates PMB-polar head of lipids interaction, in accordance with the reported mechanism of PMB inhibition (focusing on the phospholipids group) [14].

From DSC data we can infer that (+)-catechin hydrate at a relative high concentration affected the interface of water-bilayer and eventually penetrated the hydrophobic core supporting the results provided by Raman spectra analysis.

Conclusions

A model membrane confirmed by a polypeptide antibiotic (PMB) can be used to mimic the cell membrane of *E. coli* ATCC 25922. The acting site and the mode of action of flavonoids can be explained through changes of the liposome fluidity. Results in this study show good correlation between the antimicrobial properties and the membrane interaction activities of flavonoids (efficacy: kaempferol > hesperitin > (+)-catechin hydrate > biochanin A). The antimicrobial mechanism of bioactive flavonoids is suggested to be interaction with the hydrophilic region of phospholipids on cell membrane and eventual penetration of the hydrophobic core at increased flavonoids concentration. The results obtained from the model membrane used to mimic *E. coli* ATCC 25922 illustrate the antimicrobial affect of flavonoids against *E. coli* ATCC 25922. Gram-negative bacteria exhibit similar structure and components in their cell membranes suggesting that flavonoids may have widespread antimicrobial application. However, cell membrane is only one target of antimicrobial agents, and considering the complex antimicrobial mechanism of phytochemical components and individual differences of *E. coli* strains, further research is required for general conclusions.

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