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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.