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Probing the potential of apigenin liposomes in enhancing bacterial membrane perturbation and integrity loss

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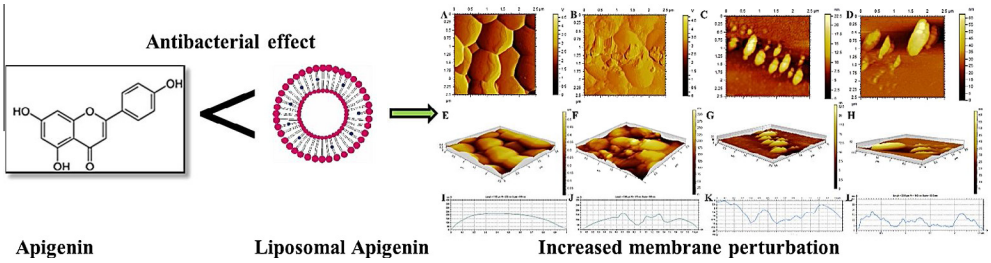
GRAPHICAL ABSTRACT



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ABSTRACT

Along with discovery of new antibacterial agents, it is important to develop novel drug delivery systems to effectively deliver drugs within bacterial cells for enhanced therapeutic activity. Liposomes have been extensively investigated as pharmaceutical carriers for improvement of therapeutic index of antimicrobial agents. The aim of this present study was to evaluate the antibacterial activity of free and liposomal formulation of apigenin, a plant based isoflavone and elucidate the mode of action. Distearoylphosphatidylcholine liposomes were prepared having nano-range particle size (104.3 ± 1.8 nm), narrow particle distribution (0.204) and high encapsulation efficiency of apigenin (89.9 ± 2.31%). Antibacterial activity of apigenin and efficacy of liposome-mediated apigenin delivery were determined from minimum inhibitory concentration values. Interaction studies using electron microscopy revealed adherence and fusion of liposomal apigenin with the bacteria causing membrane perturbation through reactive oxygen species generation which was evaluated by epi-fluorescence microscopy and fluorescence activated cell sorting. The interaction of apigenin liposomes with bacterial membrane increased intracellular drug concentration and thus, can be employed to deliver apigenin within cells to augment its antibacterial activity. Increased efficacy and hemocompatibility of this formulation paves way for future evaluation of underlying molecular mechanisms and *in vivo* testing for enhanced therapeutic effects.

1. Introduction

Recent advances in development of novel antibacterial chemotherapeutic agents in the last few decades, have considerably reduced the morbidity and mortality associated with bacterial infections, especially in developing countries. However, the recent emergence of drug-resistant bacterial strains has raised the alarming prospect of the recurrence of bacterial infections [1]. Thus, an opportunity to evaluate new agents for potential antibacterial activity and novel formulations to effectively deliver antibacterial payload in a wide variety of bacterial targets arises.

In recent years there has been widespread testing of numerous plant extracts for deciphering their pharmaceutical and therapeutic virtues. These phytochemical screening efforts have yielded

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numerous plant-based bioactive compounds with anti-infectious and preventive activity against different diseases. Isoflavones are a subclass of ubiquitous flavonoids currently generating increased interest as therapeutic agents owing to the presence of phenolic hydroxyl groups that have affinity for proteins which act as inhibitors of microbial enzymes as well as their biosynthetic pathways. There have been reports of isoflavones having a diverse spectrum of biological properties such as antimicrobial [2], antioxidant, estrogenic and anti-hemolytic activities [3]. In fact, rational design of therapeutic strategies utilizing phytochemical compounds can ultimately provide a new paradigm for next generation pharmaceutics.

Recently, a number of isoflavones such as Panduratin A, Isobavachalcone, Bartericin A, Kaempferol 3-O-L-l-(2″,4″-di-E-*p*-coumaroyl)-rhamnoside, Sepicanin A, Licochalcone A, were tested for their antibacterial activity against *Staphylococcus* sp. and *Enterococci* clinical isolates [4]. Although, therapeutically effective amounts of these isoflavones were found to inhibit bacteria the nature of the flavonoid ring system, substitution with prenyl groups and presence of phenolic hydroxyl groups play an important role in determining the efficacy of each of the isoflavones against various bacterial strains.

Trihydroxyflavone or apigenin is a naturally occurring bioflavonoid abundantly present in fruits and vegetables whose antibacterial activity against certain strains of Gram negative and Gram positive bacteria like *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa* has been reported recently [5].

Novel drug delivery systems including liposomes are currently being utilized for therapeutic efforts aiming treatment of pathogens that cause bacterial infections [6]. Liposomes are self-assembled, spherical vesicles primarily composed of phospholipids which have the capability to fuse with the bacterial plasma membrane and alter therapeutic index of a drug highlighting its utility in therapeutics applications [7]. Nano-formulations of antibacterial drugs provide certain useful advantages over their conventional counterparts, especially in their mechanisms of action [8]. Along with other possible modes of action, material-cell contact [9], internalization and release of encapsulated load could contribute to bacterial cell death [10]. Moreover, the ability of the nano drug delivery system to initiate point attachment often leads to enhanced lysis of bacterial cell membranes [11]. In fact *in vitro* studies reporting the increased efficacy of silver nanoparticles against *S. aureus*, *P. aeruginosa* and *Streptococcus pyrogens* emphasize the role of surface aggregation/attachment in increasing the antibacterial effect of antimicrobial agents [11–13].

The present study reports the evaluation of antibacterial properties of apigenin and its liposomal formulation against both Gram-positive and Gram-negative bacteria along with an objective to decipher the underlying basis of the antibacterial activity. Encapsulation of apigenin increases the interaction between drug and bacteria resulting in effective delivery of drug within bacterial cells for enhanced therapeutic activity. The results indicating enhanced activity of the liposomal formulation against both Gram-positive and Gram-negative bacteria suggest its potential as an effective new antibacterial chemotherapeutic agent. Increased efficacy and hemocompatibility of this formulation paves way for future evaluation of underlying molecular mechanisms and further testing at *in vivo* level for enhanced therapeutic effects.

2. Materials and methods

2.1. Materials

2.1.1. Chemical reagents

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), Apigenin, 2′,7′-dichlorofluorescein diacetate were purchased from Sigma Chemicals Co., USA. 4-2-hydroxyethyl-1-piperazineethanesulfonic acid was obtained from Sisco Research Laboratories Pvt. Ltd., India. Ammonium molybdate, H_2O_2 were obtained from Merck Millipore, Germany. The cation-adjusted Mueller Hinton II broth, Mueller Hinton II agar and phosphate buffered saline were procured from HiMedia Laboratories Pvt. Ltd., India. All other chemicals used were of the highest analytical grade available. The chemicals were used as obtained without further purification. Milli-Q water obtained from Milli-Q Integral 3 system (Merck Millipore, Germany) was used for all experiments.

2.1.2. Bacterial strains

E. coli [American Type Culture Collection] ATCC 27161, *S. aureus* ATCC 6538P, *Bacillus subtilis* ATCC 9524 and *P. aeruginosa* ATCC 27853 were obtained from the Central Drug Laboratory, India. Bacteria were preserved at −80 °C in cation-adjusted Mueller Hinton II broth containing glycerol at a final concentration of 20% (v/v) until use.

2.2. Methods

2.2.1. Preparation of large unilamellar vesicles (LUVs)

Liposomal formulation of apigenin was prepared according to the lipid film hydration method described elsewhere [14,15] with slight modifications. Briefly, appropriate volume of methanolic stock solutions of apigenin and high-transition temperature zwitterionic phospholipid distearoyl phosphatidylcholine was added in round-bottom flasks (to maintain a phospholipid to drug molar ratio of 2.5:1) and vortexed to form a homogeneous solution. The solvent was then evaporated and the obtained dry lipid and/or lipid-drug films obtained were hydrated with 2 ml of filtered 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffered saline (10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid and 150 mM NaCl, pH 7.0) and incubated at 65 °C (∼10 °C above the phase transition temperature of the phospholipid) for 1 h with intermittent severe vortexing to form multilamellar vesicles. The turbid multilamellar vesicle suspension was subjected to five freeze–thaw cycles by alternatively freezing in liquid nitrogen and thawing at 65 °C in a water bath and then sonicated using a probe sonicator (CPX130, 130 W, 20 kHz, Cole Parmer, USA) at 50% sonication strength for 20 min with a sequence of 59 s sonication and 10 s rest at 65 °C. The resulting optically clear unilamellar vesicles were left to stand at 65 °C for 1 h to anneal any structural defects, and then centrifuged (5430R, Eppendorf, Germany) at 8500*g* for 15 min to remove any titanium impurities from the probe and/or multilamellar vesicles or lipid aggregates. The liposome suspensions were further centrifuged (Sorvall UltraPro80, Kendro Laboratory Products, USA) at 100,000*g* for 1 h to remove free apigenin. The pellets were re-suspended in 4-2-hydroxye thyl-1-piperazineethanesulfonic acid buffered saline (pH 7.0) and used for subsequent experiments. All experiments were performed with freshly prepared liposomes.

2.2.2. Particle size distribution and zeta potential measurements

Particle size distribution and zeta (ζ) potential values of both neat and apigenin-loaded vesicles were measured using Zetasizer Nano ZS instrument (Malvern Instruments Ltd., U.K.) equipped with a 4 mW He–Ne laser (λ = 632.8 nm) at 25 °C. Liposome suspensions were adequately diluted with 4-2-hydroxyethyl-1-pipera zineethanesulfonic acid buffered saline (pH 7.0) before particle size analysis and ζ-potential experiments. The ζ-potential values obtained for each sample was the average of 15 measurements. Each experiment was performed in triplicate and the data obtained was analysed using the Zetasizer 6.01 software supplied with the instrument.