J Nat Med (2017) 71:357–366
DOI 10.1007/s11418-016-1065-2

ORIGINAL PAPER

Lupinifolin from *Derris reticulata* possesses bactericidal activity on *Staphylococcus aureus* by disrupting bacterial cell membrane

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Received: 5 August 2016 / Accepted: 8 December 2016 / Published online: 30 December 2016

Abstract In this study, lupinifolin, a prenylated flavonoid, was isolated from *Derris reticulata* stem, identified by NMR spectra and confirmed with mass spectrometry. Lupinifolin was freshly prepared by solubilizing in 0.1 N NaOH and immediately diluted in Müller–Hinton broth for antibacterial testing. The data showed that Gram-positive bacteria were more susceptible to lupinifolin than Gram-negative bacteria. Of four strains of Gram-positive bacteria tested, *Staphylococcus aureus* was the most susceptible. Using the two-fold microdilution method, it was found that lupinifolin possessed antimicrobial activity against *S. aureus* with minimum inhibitory concentration and minimum bactericidal concentration of 8 and 16 μg/ml, respectively, which is less potent than ampicillin. However, from the time–effect relationship, it was shown that lupinifolin had faster onset than ampicillin. The faster onset of lupinifolin was confirmed by scanning electron microscopy. To investigate the mechanism of action of lupinifolin, transmission electron microscopy (TEM) was performed to observe the ultrastructure of *S. aureus*. The TEM images showed that lupinifolin ruptured the bacterial cell membrane and cell wall. Due to its fast onset, it is suggested that the action of lupinifolin is likely to be the direct disruption of the cell membrane. This hypothesis was substantiated by the data from flow cytometry using $DiOC_2$ as an indicator. The result showed that the red/green ratio which indicated bacterial membrane integrity was significantly decreased, similar to the known protonophore carbonyl cyanide 3-chlorophenylhydrazone. It is concluded that lupinifolin inhibits the growth of *S. aureus* by damaging the bacterial cytoplasmic membrane.

Keywords *Derris reticulata* · Lupinifolin · *Staphylococcus aureus* · Antimicrobial · Cell membrane disruption

Introduction

It is widely known that the incidence of hospital-acquired infections (HAI) is continuously increasing, and that they are responsible for morbidity and mortality in hospitalized patients [1]. Recently, the Centers for Disease Control and Prevention (CDC) have reported that in 2014 HAIs were found in central line-associated bloodstream infections, catheter-associated urinary tract infections, certain surgical site infections and hospital-onset *Clostridium difficile* infections [2]. Similar to other species, many strains of *Staphylococcus aureus,* such as methicillin-resistant *S. aureus* (MRSA), are developing resistance to the available antibacterial agents, creating a serious problem in public health [2, 3]. Due to the increasing prevalence of failures in the treatment of infectious diseases, the identification and development of novel antibacterial compounds are urgently required. Flavonoids derived from natural plants have been

Electronic supplementary material The online version of this article (doi:10.1007/s11418-016-1065-2) contains supplementary material, which is available to authorized users.

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proved to have the potential to be new leads for antibacterial drug discovery [4, 5].

Lupinifolin is a prenylated flavonoid isolated from several medicinal plants, such as *Myriopteron extensum* [6], *Eriosema chinense* [7], *Albizia myriophylla* [8] and *Erythrina fusca* [9]. It is also reported to be a major compound in *Derris reticulata* [10]. There are several lines of evidence demonstrating that lupinifolin exerts antimicrobial activities, such as antiviral activity against herpes simplex virus type 1 [6], antimycobacterial activity against *Mycobacterium tuberculosis* [11] and antibacterial activity against *Bacillus cereus*, *Corynebacterium diphtheria* and *S. epidermidis* [6, 11]. Lupinifolin possesses very strong activity against *Streptococcus mutans* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 1 and 2 μg/ml, respectively [8]. It has been demonstrated to exhibit antidiarrheal activity on castor oil-induced intestinal fluid accumulation with significant recovery from Na^+,K^+ loss [7]. The same report showed antibacterial activity of lupinifolin against bacterial strains mainly implicated in diarrhea, such as *Bacillus cereus*. However, a mechanism underlying the antibiotic activity of lupinifolin has never been documented. In the present study, lupinifolin was isolated from *D. reticulata* stem and tested for antibacterial activities against four strains of Gram-positive and Gram-negative bacteria. Due to its highest susceptibility to lupinifolin, *S. aureus* was used to investigate the mechanism underlying this antibacterial activity. It is first reported here that lupinifolin purified from *D. reticulata* inhibits the growth of *S. aureus* by damaging the bacterial cell membrane.

Materials and methods

Plant collection and preparation

D. reticulata Craib. was collected from Prachinburi province, Thailand. Botanical identification was performed by Dr. Paul J. Grote, School of Biology, Suranaree University of Technology (SUT). A voucher specimen (Pharm-Chu-006) was deposited at School of Preclinical Sciences, SUT. The stems were cut into small pieces and dried at 50 °C in a hot-air oven. The dried stems were stored at room temperature until used for extraction.

Purification of lupinifolin

Sixty grams of dried stems were extracted with 400 ml of hexane using a Soxhlet extractor. After washing twice with deionized water, the extract became turbid due to precipitation of lupinifolin. The hexane layer was collected and heated at 65 °C until the extract became clear, and was then left at room temperature overnight for crystallization. The purity of the yellow needle-shaped lupinifolin crystals was first analyzed by TLC. Dichloromethane:methanol (95:5) was used as the mobile phase and the composition of the extract was detected by UV light at 254 nm. Specific rotation was measured with a Bellingham & Stanley P 20 polarimeter (Tunbridge Wells, Kent, UK). Yellow crystallized lupinifolin was dissolved in 10 ml of chloroform (1.10 g/ml) and analyzed with a 20.0 cm polarimeter tube. The calculated specific rotation was −10.0°, which matched the published value [12]. Further identification was carried out with nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Identification of lupinifolin

NMR

The purified lupinifolin was confirmed by NMR spectra on a 500 MHz NMR spectrometer (Bruker, Avance III HD; Fällanden, Switzerland) with a CPP BBO 500 CryoProbe. Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane (TMS) was used as reference standard. The ^{1}H- and ^{13}C-NMR spectra were collected at frequencies of 500.366 and 125.83, respectively. They were consistent with the previously published data [12].

MS

The structure of lupinifolin was also confirmed by its mass spectrum. The yellow lupinifolin crystals were dissolved in methanol (containing 0.1% formic acid) and injected directly to the electrospray ionization (ESI) source of a Bruker micro-TOF-Q mass spectrometer (Bremen, Germany). The ESI source was used in positive mode, and the scan range of the mass detector was 50–1500 *m/z*. The expected value for detection of $[M+H]^+$ at *m/z* is 407.1853 ($C_{25}H_{27}O_5$).

Antibacterial assays

Disc diffusion

Bacteria used in this study were obtained from Thailand Institute of Scientific and Technological Research (TISTR). The antibacterial activities of lupinifolin were evaluated with Gram-positive bacteria *S. aureus* (TISTR 1466), *S. epidermidis* (TISTR 518), *B. subtilis* (TISTR 008) and *B. cereus* (TISTR 687), and Gram-negative bacteria *E. coli* (TISTR 780), *Pseudomonas aeruginosa* (TISTR 781), *Enterococcus aerogenes* (TISTR 1540), *Salmonella typhi* (TISTR 292) and *Proteus mirabilis* (TISTR 100). The screening of the antibacterial activity was done by the disc

diffusion method [13]. Bacterial suspensions were prepared by inoculating one loopful of a pure colony into Müller–Hinton broth (MHB), incubated overnight and diluted in 0.9% NaCl. Cell suspensions, with adjusted turbidity equivalent to that of a 0.5 McFarland standard ($\sim 10^8$ cfu/ml), were inoculated on Müller–Hinton agar (MHA) plates by swabbing over the entire agar surface. Lupinifolin (25, 50, 75 μg/disc) was impregnated on filter paper discs (Whatman No. 1, 6 mm diameter) and then placed on the previously inoculated agar plate. After 24 h of incubation at 37 °C, the antibacterial activity was determined by measuring the diameter of the inhibition zones formed around the disc. Ampicillin (10 μg) and 0.1 N NaOH (10 μl) were used as positive and vehicle controls, respectively.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A modified broth microdilution method according to Clinical and Laboratory Standard Institute Guidelines [14] was used to determine the MIC and MBC of lupinifolin [8]. Two-fold serial dilutions of lupinifolin were made in MHB using 96-well flat-bottom microtiter plates. A suspension of mid-logarithmic growth phase bacteria in MHB adjusted to 5×10^5 cfu/ml was added to each well. The final concentrations of lupinifolin ranged from 0.25 to 32 μg/ml. Ampicillin and 0.1 N NaOH (at the same volume as for lupinifolin) were used as positive and vehicle controls, respectively. The MIC was considered to be the lowest concentration of the agents showing no visible growth of microorganisms after incubation at 37 °C for 24 h. The MBC determination was carried out by sub-culturing 20 μl from the broth with no growth onto MHA plates after 24 h incubation at 37 °C. All tests were performed in triplicate independent experiments.

Time-course of inhibitory effect

Staphylococcus aureus cells at mid-logarithmic growth phase (1.8×10^8 cfu/ml: 100 ml) were incubated with lupinifolin at MIC in 250-ml flasks. The optical density was measured to compare the onset of inhibitory activity of lupinifolin to ampicillin for 24 h at 37 °C.

Scanning electron microscopy (SEM)

Staphylococcus aureus cells at mid-logarithmic growth phase (1.8×10^8 cfu/ml) were treated with either 8 μg/ml lupinifolin or 0.25 μg/ml ampicillin for 1, 3 or 6 h. After incubation in a 37 °C shaking incubator (200 rpm), the cells were spun down and MHB medium were removed. The cell pellets were spread on 0.1% gelatin-coated slides and air-dried for 15 min, and then fixed with 4% paraformaldehyde at 4 °C for 1 h. After fixation, the specimens were washed with phosphate buffer solution (PBS) twice and post-fixed with 1% osmium at 4 °C for 30 min. The samples were then washed twice with PBS at 4 °C for 10 min and dehydrated twice with serial graded concentrations of ethanol (50, 70, 80, 90 and 95%) at 4 °C for 7 min, followed by 100% ethanol. The samples were then dried to the critical point under CO_2 with a Leica EM CPD300 dryer (Vienna, Austria) and stained with gold ions in a pressure metallic chamber. Microscopy was performed with a JEOL JSM-6010LV scanning electron microscope (Tokyo, Japan).

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualize the change in morphology at the membrane and cell wall ultrastructure of *S. aureus* after treatment with lupinifolin. TEM preparations were made in accordance with the previously reported method with slight modifications [15]. The bacterial samples were prepared similar to the SEM method. After lupinifolin treatment for 12 h, cells were gently washed with 0.1 M PBS (pH 7.2), fixed with 2.5% glutaraldehyde in PBS and rinsed with PBS. Post-fixation was then carried out with 1% osmium tetroxide (Electron Microscopy Sciences: Hatfield, PA, USA) in 0.1 M PBS for 2 h at room temperature. After washing in the buffer, the samples were dehydrated using sequential exposure for acetone concentrations ranging from 20 to

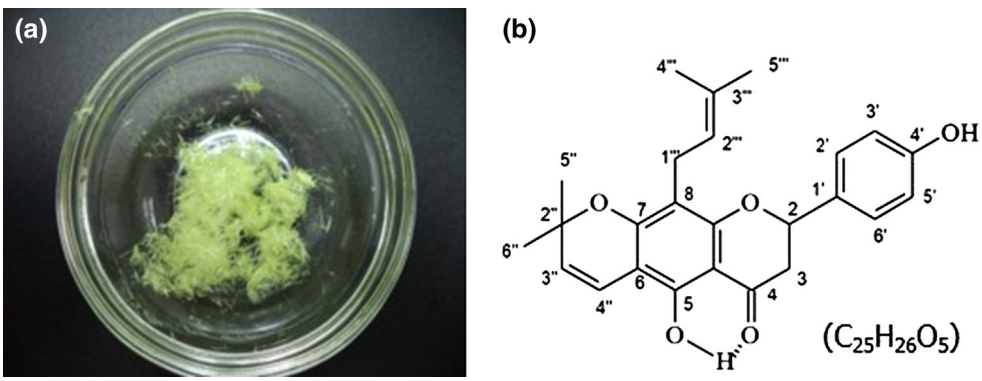

Fig. 1 Yellow needle-shaped crystals (**a**) and chemical structure (**b**) of lupinifolin extracted from *D. reticulata* stem

100%. Subsequently, infiltration and embedding were performed using Spurr's resin (EMS). Finally, the samples were sectioned using an ultramicrotome with a diamond knife and were mounted on copper grids. They were stained with 2% uranyl acetate and lead citrate. The samples were viewed with a JEM-1230 electron microscope (Tokyo, Japan). The morphology of bacterial cells was observed and compared to ampicillin-treated cells as positive control.

Flow cytometry analysis

In this study, flow cytometry was used to measure bacterial cell membrane integrity. Bacterial membrane potential was determined by using carbocyanine dye (3,3′-diethyloxacarbocyanine iodide; $DiOC_2$) according to the method previously described [16]. Bacterial cells at mid-logarithmic growth phase (1×10^6 cells/ml) were resuspended in PBS and treated with lupinifolin (8 μg/ml) and ampicillin (0.25 μg/ml). Then, 10 μl of 3 mM $DiOC_2$ was added to each tube and mixed. The samples were incubated at room temperature for 15 min and then the signal was examined. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 5 μg/ml) was used to produce a positive depolarized control. The analysis of the cells was performed using a flow cytometer (FACScan; BD Biosciences, San Jose, CA, USA) equipped with CellQuest software (BD Biosciences).

Hemolysis of rabbit red blood cells

To test the direct toxicity of lupinifolin on mammalian cell membranes, a hemolysis test was conducted using rabbit red blood cells (RBCs). RBCs (50% in PBS) were treated with lupinifolin at doses similar to those in the cytotoxicity test for 24 h in 96-well plates. After incubation, optical density at 600 nm was measured using a spectrophotometric microplate reader (Bio-Rad; Hercules, CA, USA).

Table 1 Comparison of ^{1}H- and ^{13}C-NMR spectra of the extracted compound and lupinifolin

Position	Yellow needle-shaped compound[a]		Lupinifolin[b]	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
4	196.68		196.84	
7	159.75		160.13	
8a	159.53		159.44	
5	157.50		156.48	
4′	156.56		156.09	
3‴	130.99		131.11	
1′	129.62		130.60	
2′/6′	127.56	7.32 (d, 8.4)	127.66	7.31 (d, 8.4)
3″	125.91	5.50 (d,10.0)	126.02	5.52 (d,10.1)
2‴	122.56	5.14 (dd, 7.2,7.2)	122.40	5.16 (dd,7.2,7.2)
4″	115.67	6.64 (d,10.0)	115.53	6.64 (d, 10.1)
3′/5′	115.67	6.87 (d,8.4)	115.53	6.89 (d, 8.4)
8	108.59		108.73	
6	102.70		102.79	
4a	102.69		102.61	
2	78.80	5.34 (dd,12.8,2.8)	78.47	5.33 (dd, 12.6,3.0)
2″	78.05		78.20	
$CDCl_3$	77.37			
$CDCl_3$	77.11			
$CDCl_3$	76.86			
3	43.23	3.04 (dd,17.6,12.8)	42.97	3.06 (dd, 17.1,12.6)
		2.80 (dd,17.6,3.0)		2.81 (dd,17.1, 3.0)
6″	28.39	1.45 (s)	28.25	1.46 (s)
5″	28.29	1.44 (s)	28.33	1.45 (s)
4‴	25.80	1.65 (s)	25.78	1.66 (s)
1‴	21.47	3.21 (d,7.2)	21.42	3.22 (d,7.2)
5‴	25.80	1.65 (s)	25.78	1.66 (s)
5-OH		12.24(s)		12.24 s

[a] Recorded in $CDCl_3$ at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR

[b] Recorded in $CDCl_3$ at 300 MHz for ^{1}H-NMR and 75.6 MHz for ^{13}C-NMR, cited in [12]

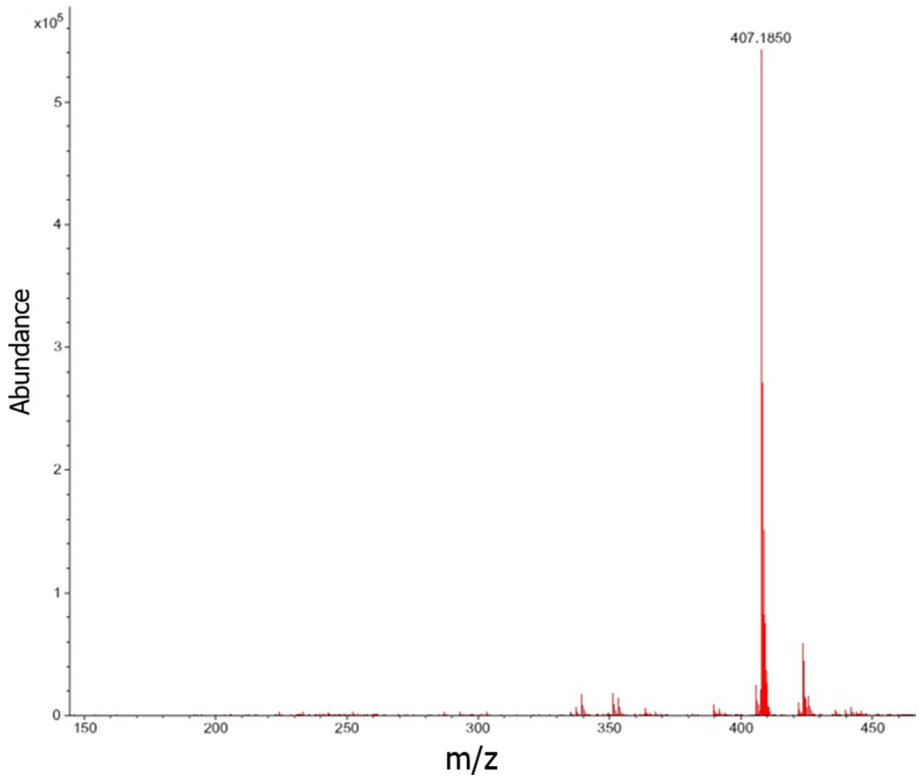

Fig. 2 Mass spectrum of lupinifolin

Cytotoxicity

MTT assay

HepG2 cells were used to determine the cytotoxicity of lupinifolin by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (2×10^5 cells/well) were seeded in triplicate into 96-well culture plates overnight. The medium was removed and replaced with fresh medium containing different concentrations of lupinifolin ranging from 5 to 100 μg/ml. After 24 h of incubation, the media were discarded and 20 μl of MTT solution (5 mg/ml in PBS) were added to each well followed by an incubation for 4 h at 37 °C with 5% CO_2. The MTT solution was then carefully removed. The insoluble purple formazan products formed in living cells were dissolved by 100 μl of dimethyl sulfoxide (DMSO). Absorbance was read at 570 nm using a microplate reader (Bio-Rad). Cell viability was expressed as a percentage after comparison with the control group which was assumed to have 100% viability.

Trypan blue exclusion assay

HepG2 cells (2×10^5 cells/well) were prepared similar to the experiment for MTT assay. After treatment with various concentrations of lupinifolin ranging from 0 to 100 μg/ml for 24 h, cells were harvested by digestion with 0.25% trypsin–EDTA solution at 37 °C for 5 min. The cell suspension was mixed with an equal volume of 0.4% (w/v) trypan blue. The number of viable (unstained) and dead (stained) cells were counted by hemacytometry under a light microscope. The results were calculated and expressed as a percentage of live cells compared to control.

Statistical analysis

Data were expressed as mean ± SD and the comparisons between different groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test, unless stated otherwise. A p value less than 0.05 was considered to show a statistically significant difference.

Results and discussion

In addition to the plant species mentioned earlier, lupinifolin has been found to be a constituent in at least ten more species, including *Citrus medica* [17], *C. limonia* [18], *Dorstenia mannii* [19], *Euchresta formosana* [20], *Tephrosia pumila* [21] and *Lonchocarpus guatamalensis* [22]. It also occurs in plants of the genus *Derris*, such as *D. trifoliate* [23], *D. scandens* [24] and *D. laxiflora* [25]. This indicates that sources of lupinifolin are readily available in nature. In the present study, hexane was used to extract lupinifolin from *D. reticulata* stem. The purified lupinifolin

Table 2 Antibacterial activity of lupinifolin

Microorganism	Diameter of inhibition zone (mm)			Ampicillin 10 μg
	Lupinifolin			
	25 μg	50 μg	75 μg	
Gram-positive				
Staphylococcus aureus	11 ± 0.6	15 ± 0.6	16 ± 0.7	37 ± 1.7
S. epidermidis	14 ± 0.5	18 ± 0.6	21 ± 0.6	52 ± 1.1
Bacillus cereus	10 ± 1.1	13 ± 0.6	25 ± 0.6	13 ± 0.6
B. subtilis	8 ± 0.1	11 ± 0.2	14 ± 0.5	28 ± 1.7
Gram-negative				
Escherichia coli	n.i.	n.i.	n.i.	20 ± 0.6
Enterobacter aerogenes	n.i.	n.i.	n.i.	n.i.
Salmonella typhi	n.i.	n.i.	n.i.	30 ± 0.6
Pseudomonas aeruginosa	n.i.	n.i.	n.i.	n.i.
Proteus mirabilis	n.i.	n.i.	n.i.	31 ± 1.1

Data are mean ± SD ($n = 3$)

n.i. no inhibition zone

Table 3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lupinifolin from *D. reticulata* against Gram-positive bacteria compared with ampicillin

Microorganism	Lupinifolin		Ampicillin	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Staphylococcus aureus	8	16	0.25	0.25

was obtained as yellow needle-shaped crystals (Fig. 1a) and its structural formula is depicted in Fig. 1b. The structure was identified by comparison of the NMR spectroscopic data (Table 1) with values in the previous report [12] and was confirmed by mass spectrometry. In the positive mode, we detected $[M+H]^+$ at *m/z* 407.1850, as shown in Fig. 2. This is in accordance with the monoisotopic mass of lupinifolin (406.1780). The purity of lupinifolin obtained from this study was more than 95%, based on the NMR spectrum.

Because of its nonpolar structure, lupinifolin is very soluble in organic solvents, but sparingly soluble in water. Estimated from Kow (octanol–water partition coefficient), the water solubility of lupinifolin at 25 °C is 0.009 mg/L [26]. When dissolved in alcohol or dimethyl sulfoxide, it precipitates after dilution in aqueous buffer. This problem was similar to that encountered by a group of researchers who studied the effect of curcumin on 4-hydroxy-2-nonenal protein [27]. To avoid precipitation in aqueous media, nonpolar chemicals can be dissolved in acidic or basic solutions before dilution. In the present study, lupinifolin was freshly prepared by solubilizing in 0.1 N NaOH and immediately diluted in Müller–Hinton broth (MHB) for antibacterial testing. With this method, lupinifolin remained soluble at all dilutions. The same volume of 0.1 N NaOH used in each experiment was also tested and found not to significantly affect the growth of bacterial cells compared to control.

Antibacterial activities of lupinifolin were screened in eight different bacterial species by the disc diffusion method. In agreement with the previous reports [6, 9], it

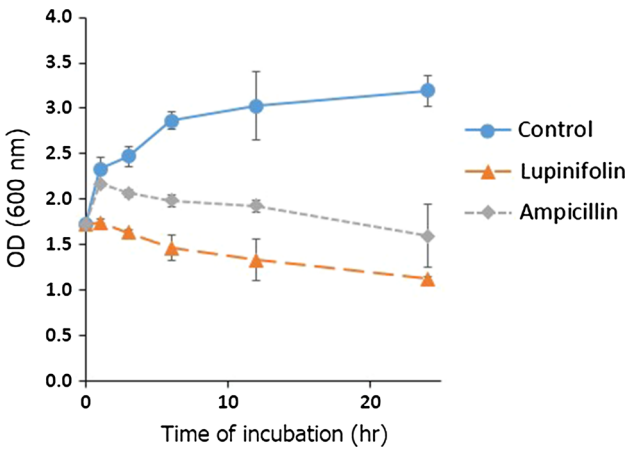

Fig. 3 Time-course effect of lupinifolin. *S. aureus* bacteria cells were treated with lupinifolin (8 μg/ml) and ampicillin (0.25 μg/ml) as described in "Materials and methods". The growth of bacteria was subsequently determined using spectrometry (600 nm) at 0, 1, 3, 6, 12 and 24 h of incubation. Values are expressed as mean ± SD ($n = 3$)

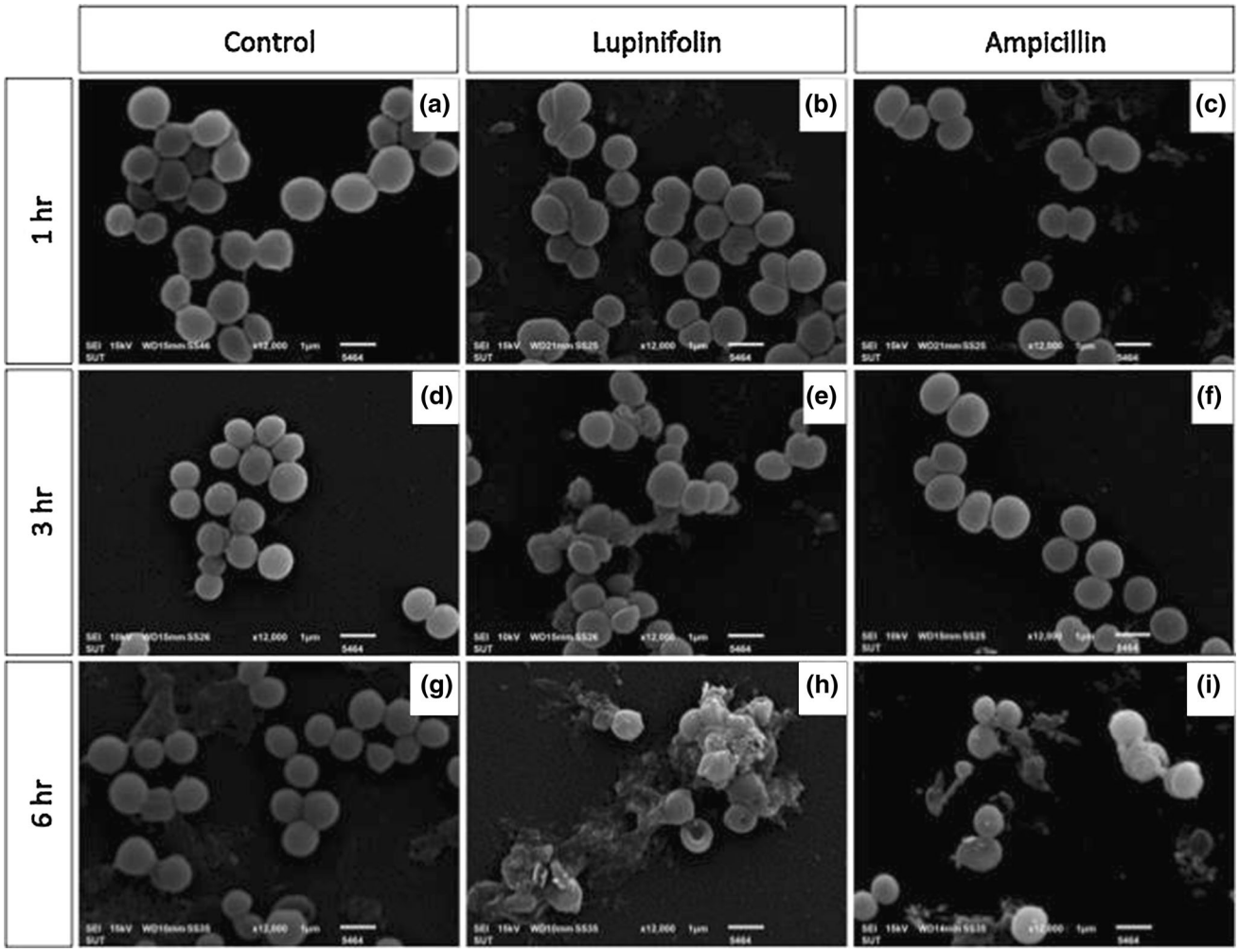

Fig. 4 Scanning electron micrographs of *S. aureus* treated with lupinifolin. Cells were treated as described in "Materials and methods". The action of lupinifolin (8 μg/ml) was faster than that of ampicillin (0.25 μg/ml). At 1 h, abnormal morphology could not be seen in any treated cells (**b**, **c**) compared to control (**a**). The damaged cells were observed after treatment for 3 h only by lupinifolin (**e**), but not ampicillin (**f**). At 6 h of incubation, cells treated with lupinifolin and ampicillin were destroyed, as shown in (**h**) and (**i**), respectively, compared to regular shape of control (**g**). Enlargement: *bar* = 1 μm, 12,000×

appeared that only Gram-positive bacteria were susceptible to lupinifolin (Table 2). Of four species of Gram-positive bacteria tested, *Staphylococcus* seemed to be the most sensitive, as revealed by the inhibition zone. Because *S. aureus* is the most highly opportunistic Gram-positive bacteria tested, the MIC and MBC of lupinifolin against this microbe was further evaluated using the microdilution method, and were found to be 8 and 16 μg/ml, respectively (Table 3).

It has been suggested that there are three principal direct mechanisms of action underlying the antibacterial activities of plant flavonoids [5]: (1) inhibition of nucleic synthesis, (2) inhibition of cytoplasmic membrane function, and (3) inhibition of energy metabolism. As shown by time-course effect curves (Fig. 3), lupinifolin evidently has a faster onset than ampicillin. It inhibited the growth of *S. aureus* within the first hour of incubation, whereas ampicillin was seen to affect bacterial growth later, at 3 hours. This observation was confirmed by the data from SEM (Fig. 4). SEM images showed some damage to bacterial morphology by lupinifolin and this effect occurred sooner than with ampicillin. The change in morphology of *S. aureus* was similar to that caused by ampicillin, suggesting that one of the targets of lupinifolin is the bacterial cell membrane or cell wall. To test our hypothesis, TEM analysis was conducted. The data showed that after incubation at MIC concentrations for 12 h, the morphology of bacteria treated with lupinifolin, as well as ampicillin, was changed compared to control (Fig. 5a–c and with higher magnification Fig. 5d–f); ruptured bacterial cell membranes and/or cell walls were observed. During cell division, cell wall synthesis is located between the daughter cells. As indicated by an arrow in Fig. 5f, the effect of ampicillin, an inhibitor of cell wall synthesis, was observed there, whereas the cell

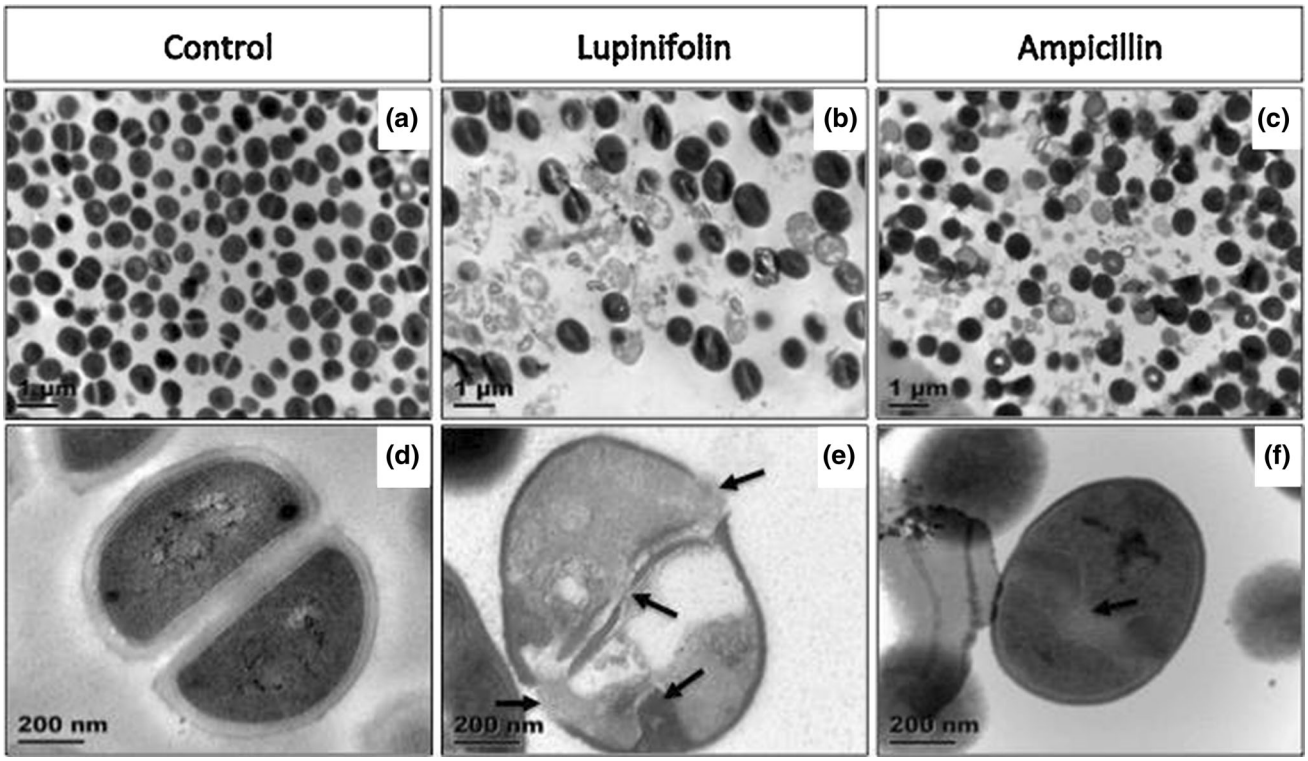

Fig. 5 Transmission electron micrographs of *S. aureus* treated with lupinifolin. **a**–**c** Overview of control and cells treated with lupinifolin (8 µg/ml) and ampicillin (0.25 µg/ml), respectively. Cell death and irregular shape of bacterial cells were seen in the treated groups, lupinifolin (**b**) and ampicillin (**c**). Damage to cell wall and cell membrane of dividing cells (indicated by *arrows*) were observed after 12 h of incubation with lupinifolin (**e**) and ampicillin (**f**), compared with control (**d**). Enlargement: *bar* = 1 µm, 10,000×; *bar* = 0.2 µm, 50,000×

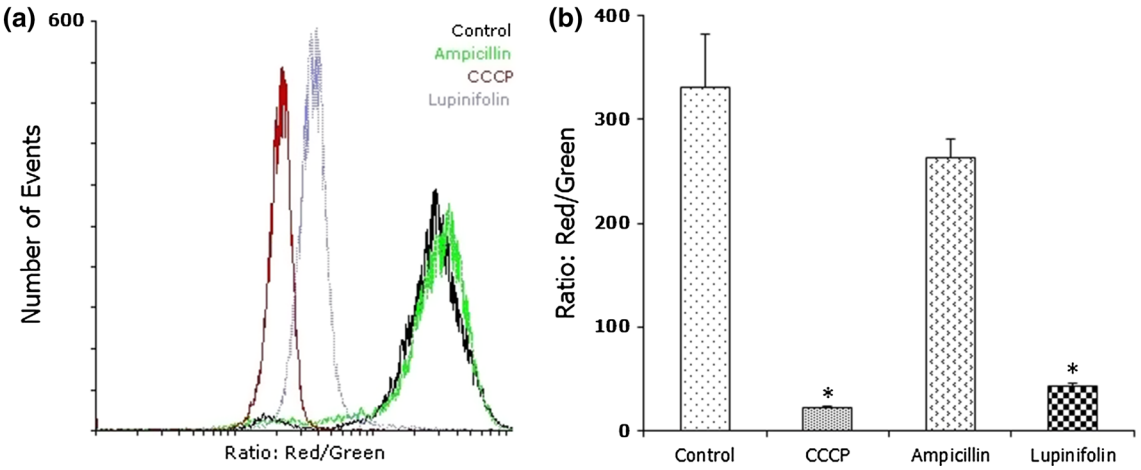

Fig. 6 Effect of lupinifolin on membrane potential. **a** After incubation of 30 µM $DiOC_2$ in the presence of 8 µg/ml of lupinifolin for 15 min, the red/green ratiometric histogram was shifted to the left similar to CCCP (5 µg/ml), a known protonophore. **b** Red/green ratios were calculated using population mean fluorescence intensities. It was found that lupinifolin and CCCP, but not ampicillin (0.25 µg/ml), significantly reduced the red/green ratio. $*p < 0.05$; statistically significant difference compared to control. Values are expressed as mean ± SD ($n = 3$) (color figure online)

wall damage caused by lupinifolin was seen around the cell, as indicated by arrows in Fig. 5e. In accordance with the fast onset of action, it is likely that the target of action of lupinifolin may be through disrupting the cell membrane, not interfering with cell wall synthesis as ampicillin does. This is because the inhibitory effect on cell wall synthesis needs more time than the direct interference effect on cell membrane structure. After damaging the cell

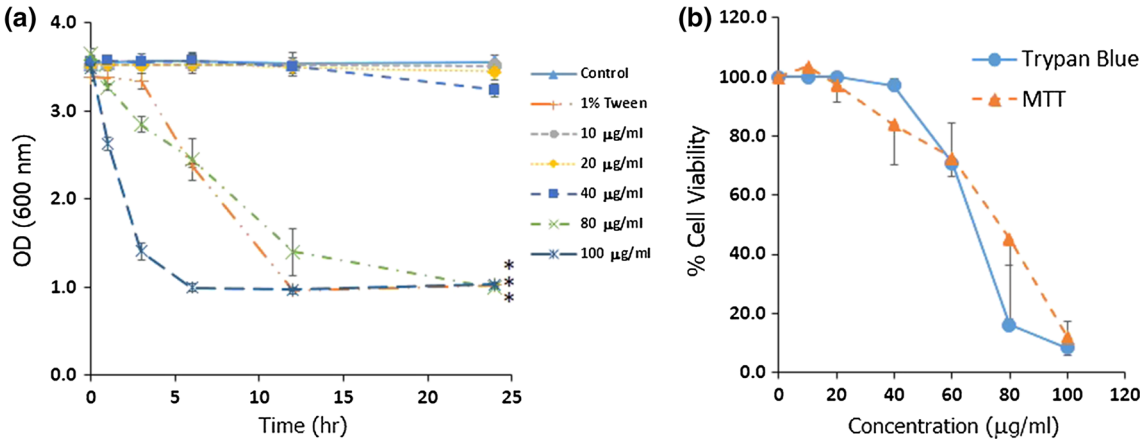

Fig. 7 Cytotoxic effect of lupinifolin. **a** Hemolytic effect of lupinifolin on rabbit red blood cells (RBCs). Two-way ANOVA followed by Student–Newman–Keuls test was used to analyze the data. It was found that lupinofolin at concentration of ≤40 µg/ml did not significantly affect the RBC after 24 h of incubation, whereas at concentrations of 80 µg/ml and higher caused RBC lysis in the first hour of incubation. **b** Effect of lupinifolin on % cell viability of HepG2 cells measured by MTT and trypan blue assays. The calculated IC_{50}s were 78.3 ± 5.6 and 66.7 ± 13.3 µg/ml, respectively. Values are expressed as mean ± SD ($n = 3$). *$p < 0.05$ statistically significant difference compared to control

membrane, which acts as a barrier for most molecules, bacteria degrade the cell's permeability control, resulting in an increase in intracellular pressure and subsequently destruction of the cell wall.

To corroborate this postulated mechanism, the fluorescent probe $DiOC_2$ was used to measure bacterial membrane potentials. When exposed to bacterial cells, molecules of $DiOC_2$ enter cells and reside either in the membrane or the cytoplasm. In normal cells, $DiOC_2$ emits green fluorescence at 530 nm, but the fluorescence shifts toward red at 576 nm as the dye molecules self-associate at the higher cytosolic concentrations caused by large membrane potentials. With higher cytosolic concentration, the ratio of fluorescent light emitted at $\lambda_{576}/\lambda_{530}$ (red/green ratio) increases. Conversely, when the bacterial membrane potential is dissipated by eliminating the proton gradient with proton ionophores such as CCCP, $DiOC_2$ cannot accumulate inside the cell and the red/green ratio consequently decreases. For several Gram-positive bacteria, including *S. aureus*, the $DiOC_2$ red/green ratio has been shown to vary with the magnitude of proton gradient [28]. In the present study, the red/green fluorescence ratio of bacterial cells treated with CCCP and lupinifolin, but not ampicillin, dropped dramatically (Fig. 6a). Figure 6b shows a significant decrease in red/green ratio after only 15 min of treatment with CCCP and lupinifolin ($p < 0.05$) compared to control, which indicated that the membrane potential dissipated rapidly. Antibiotics, including ampicillin, that do not target the bacterial membrane have been shown to decrease the potential over a longer period of exposure [16]. The rapid action of lupinifolin strongly suggests that the dissipation of membrane potential is due to its direct effect on the bacterial cell membrane.

CCCP is widely known as a protonophore whose structure is an aromatic compound with a negative charge. It collapses cell membrane potential by transporting protons across the membrane when it attaches to the molecule. Several flavonoids have aromatic structures with hydroxyl groups which are able to dissociate and produce negatively-charged molecules similar to CCCP. It is possible that the flavonoid lupinifolin, which also has an aromatic structure with a side-chain hydroxyl group, acts as an ionophore that moves protons and/or positive-charged molecules across lipid bilayers similar to CCCP.

To test the toxicity of lupinifolin against mammalian cell membranes, we measured the hemolysis of rabbit red blood cells (RBCs). It was found that in concentrations up to 40 µg/ml, lupinifolin did not significantly disrupt RBC membranes after 24 h of exposure (Fig. 7a). However, at concentrations ≥80 µg/ml, lupinifolin produced a strong hemolysis effect in the first hour of incubation. The toxicity of lupinifolin in mammalian cells was further studied using HepG2 cells. Similarly, we founded that lupinifolin at MIC and MBC did not affect HepG2 cell viability (Fig. 7b). The IC_{50}s of lupinifolin on cell viability measured by MTT and trypan blue exclusion assays were 78.3 ± 5.6 and 66.7 ± 13.3 µg/ml, respectively.

From our in vitro experimental data, the margin of safety of lupinifolin seemed to be narrow; however, in vivo safety data of lupinifolin extracted from the same plant, *D. reticulata*, have been reported [10]. Oral administration of lupinifolin in mice at high dose (5 g/kg body weight) showed no acute toxicity. No animal died after 14 days of drug administration. In the same study, a subacute toxicity study was performed in Wistar rats for 28 days. The results

showed that lupinifolin did not affect body weight, food consumption or animals' health.

In conclusion, the mechanism of action underlying the antibacterial activity of lupinifolin against Gram-positive bacteria is first reported here. The results obtained from this study provide direct evidence to support the hypothesis that lupinifolin inhibits bacterial growth by damaging the cytoplasmic membrane. The data suggested that lupinifolin may have the potential to be used as antibacterial agent. However, its in vivo efficacy needs further investigation.

Acknowledgements We thank Dr. Paul J. Grote for verification of plant botanical classification.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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