Nanoliposomes Containing Eucalyptus Citriodora Antibiotics for Specific Antimicrobial Activity

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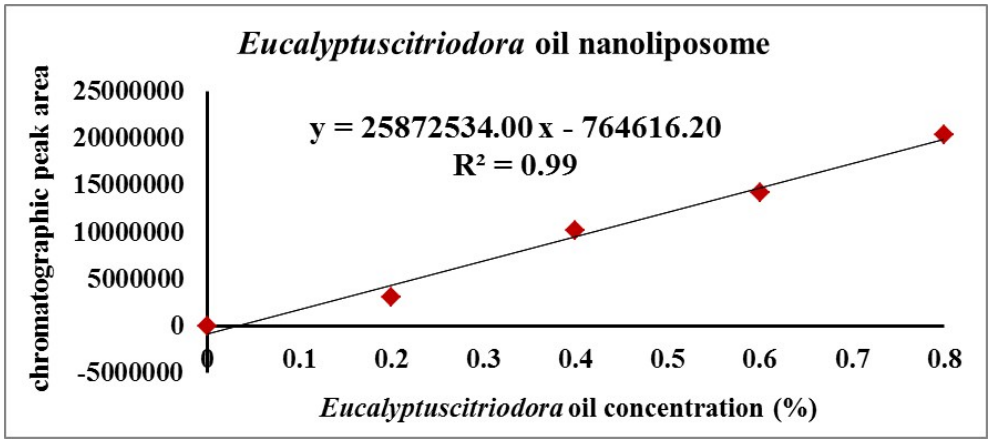
Experimental section

Preparation of liposomes: Soy lecithin (1 g) and cholesterol (0.2 g) were dissolved in chloroform (50 ml) in a 250 ml round-bottom flask and the organic solvent was removed by rotary evaporator until a thin film was formed on the walls, then dried in vacuum oven under the temperature of 30 °C. After 24 h, the lipid film was suspended in 50 ml of a phosphate buffer saline (PBS) which contained 0.05 g polyvinylpyrrolidone (PVP), and then treated by ultrasonic wave for 1 h. Subsequently, the sample was porphyrized into sonifier cell disrupter for 30 min, power 360 W. Then the porphyrized sample was centrifuged for 15 min at 6000 rpm, and the supernatant was kept. Finally, vesicular dispersions were filtered by 0.22 μm membrane and the filtrate was recovered. (Journal of Natural Pharmaceutical Products, 2012, 7, 117-122).

Preparation of liposome encapsulating *Eucalyptus Citriodora* oil: Soy lecithin (1 g), cholesterol (0.2 g) and *Eucalyptus Citriodora* oil (150 mg) were dissolved in chloroform (50 ml) in a 250 ml round-bottom flask and the organic solvent was removed by rotary evaporator until a thin film was formed on the walls, then dried in vacuum oven under the temperature of 30 °C. After 24 h, the lipid film was suspended in 50 ml of a phosphate buffer saline (PBS) which contained 0.05 g polyvinylpyrrolidone (PVP), and then treated by ultrasonic wave for 1 h. Subsequently, the sample was grinded in cell ultrafine grinding instrument for 30 min, power 360 W. Then the porphyrized sample was centrifuged for 15 min at 6000 rpm, and the supernatant was kept. Finally, vesicular dispersions were filtered by 0.22 μm membrane and the filtrate was recovered.

Determination of Encapsulation efficiency: *Eucalyptus Citriodora* oil was diluted with ethanol to obtain concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 (mg/ml) respectively, and analyzed by the gas chromatography-mass spectrometer (GC-MS). From this, the citronellal peak area - *Eucalyptus Citriodora* oil concentration standard curve was obtained. Subsequently, liposomes (25 ml) were spun in an ultracentrifuge at 4 °C and 30,000 rpm for 1h. The supernatant was removed and vesicles were dissolved in ethanol (25 ml), and then treated by ultrasonic wave for 3h. Finally, the mixture was centrifuged for 15 min at 8000 rpm. The

supernatant was kept and analyzed through GC-MS. The Citronellal peak area - *Eucalyptus Citriodora* oil concentration standard curve was used to calculate the amount of the *Eucalyptus Citriodora* oil.



Gas chromatography: GC of *Eucalyptus Citriodora* oil, liposomes and the liposomes incubated with *S. aureus* for 3 days were carried out respectively by using a Trare ultra ITQ1100 GC, (equipped with a HP-5 MS 0.25 mm × 30 mm × 0.25 µm film thickness capillary column). Nitrogen was used as carrier gas. Injection temperature was set at 250 °C and detector temperature at 280 °C. The initial temperature of the column was 60 °C for 1 min; then it was raised to 250 °C at 10°C/min, and keeping for 3 min (Food Chemistry, 2009, 112, 77-83).

Particle size analysis: The particle size was measured by the high concentration of laser particle size analyzer (BIC; BI-9000). The diameters of both liposomes in bulk solution with and without *Eucalyptus Citriodora* oil were characterized.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Eucalyptus Citriodora* Oil: *Eucalyptus Citriodora* oil was added into tubes containing NB to obtain concentrations of 0.1% vol/vol, 0.05% vol/vol, 0.025% vol/vol, 0.0125% vol/vol and 0.00625% vol/vol respectively. Subsequently, the tubes were inoculated with the freshly prepared bacterial suspension in order to maintain initial bacterial concentration 10^3-10^4 CFU/ml, and then incubated in a rotary shaker at 150 rpm and 37 °C for 24-48 h. The lowest concentration of *Eucalyptus Citriodora* oil showing growth inhibition (as seen visually) was considered as the minimum inhibitory concentration. The MBC was recorded as the lowest concentration of *Eucalyptus Citriodora* oil that showed no growth on Nutrient Agar (NA) plates after spot inoculation and incubation at 35 °C for 24-48 h. (Trop. J. Pharm. Res. 2011, 10, 335; Acta Biomater. 2008, 4, 707)

The antimicrobial activity of *Eucalyptus Citriodora* Oil: The antimicrobial activity of *Eucalyptus Citriodora* oil against the test *Escherichia coli* (ATCC 25922) and *Staphyloccocus aureus* (ATCC 25923) were also determined. Test strains were inoculated into PBS (phosphate buffered saline, 0.03 mol/L, pH 7.4) tubes containing 0.02% vol/vol *Eucalyptus*

Citriodora oil to obtain initial concentration of 10^5-10^6CFU/ml and cultured at 150 rpm and 37 °C. The bacterial suspension in sterile PBS without *Eucalyptus Citriodora* oil was also tested and served as negative control. Numbers of viable bacteria were enumerated at 0 h, 2 h, 4 h, 6h and 8 h by counting the number of bacterial colonies grown on the plate. (J. Antimicrob. Chemoth. 2006, 57, 573; Food Control. 2012, 25, 225)

The antimicrobial activity of liposome entrapping including *Eucalyptus Citriodora* oil: The antimicrobial activity of liposomes encapsulating Eucalyptus Citriodora oil against the test *Escherichia coli* (ATCC 25922) and *Staphyloccocu saureus* (ATCC 25923) were also determined in PBS (phosphate buffered saline, 0.03mol/L, pH 7.4). Test strains were inoculated into PBS tubes containing 20% vol/vol liposomes to obtain initial concentration of 10^5-10^6 CFU/ml and cultured at 150 rpm and 37 °C. Numbers of viable bacteria were enumerated at 0 h, 24 h, 48 h and 96 h by counting the number of bacterial colonies grown on the plate. As control, the bacterial suspensions in sterile PBS without liposomes were also tested.

The antimicrobial activity of *Eucalyptus Citriodora* oil and the one entrapped in nano liposome: The antimicrobial activity of *Eucalyptus Citriodora* oil and the one entrapped in nanoliposome was tesed by the spread plate method. Initially, the *Staphyloccocus aureus* (ATCC) was cultured for 48h. *Eucalyptus Citriodora* oil was diluted to 0.02% vol/vol, and liposomes containing *Eucalyptus Citriodora* oil were diluted to 20% vol/vol by PBS, after that, the agents were added to test tubes that containing the *S. aureus* (10^5CFU/ml). The antimicrobial activity of *Eucalyptus Citriodora* oil and the nanoliposome containing the *Eucalyptus Citriodora* oil against the *S. aureus* at 0 day, 0.5 day, 1 day, 2 day, 3 day and 4 day. 5 days later, more *S. aureus* (10^5CFU/ml) were added. The antimicrobial activity of *Eucalyptus Citriodora* oil and the nanoliposome containing the *Eucalyptus Citriodora* oil against the *S. aureus* at 5 day, 6 day, 7 day and 8 day. The repeated procedure was made 8 days later. The antimicrobial activity of *Eucalyptus Citriodora* oil and the nanoliposome containing the *Eucalyptus Citriodora* oil against the *S. aureus* at 9 day, 10 day, 11 day and 12 day. The experiments were repeated three times and the results were expressed as average values.

AFM Characterization: The surface structure of nano liposome was characterised by an atomic force microscope (AFM, Agilent 5500, Agilent Technologies, USA). The nano liposome was diluted with distilled water (ca.0.1 mg/ mL), placed on a mica surface, and then dried at room temperature before observation on the AFM. The imaging was processed with Agilent picoview version software.

DNA metabolism:

Observed DNA and RNA with Fluorescent staining method

We observed DNA and RNA with fluorescent staining method. Added *Eucalyptus Citriodora* oil into the bacterial suspension (10^8 CFU/ml) to obtain concentration of 0.02% vol/vol and cultured at 37 °C. After 28 h, an equal volume of diluted 4'6-diamidino-2- phenylindole (DAPI) (10 μg/ml, Roche Diagnostics GmbH，Germany) and bacteria sample were mixed, and then placed on a

micro slide and kept in the dark for10 min. The fluorescence of DAPI in cells was observed using inverted fluorescence microscope. As control, the bacteria without *Eucalyptus Citriodora* oil were observed (Leica TCS SP5) (Arch Microbiol, 2010, 192:893–898).

Quantification of DNA

Centrifuge the suspensions at 4000 rpm for 15 min at4 °C and the pellets were harvested. The pellets were washed three times with PBS and resuspended in the buffer containing 0.02% vol/vol *Eucalyptus Citriodora* oil to make up bacterial suspensions of 10^8 CFU/ml. 800 μLwere removed from the suspensions at 24 h and immediately added to three times the volume of diluted DAPI (10 μg/ml, Roche Diagnostics GmbH，Germany) and kept in the darkfor10 min. The fluorescence intensity of DNA was separately estimated using fluorescence spectrophotometer (Cary Eclpise, America) with the excitation wavelength of 364 nm. As control, the bacterial suspensions in sterile PBS without *Eucalyptus Citriodora* oil were tested (International Journal of Food Microbiology, 2004, 95: 147– 155; Food Control, 2014, 35:109-116).

ATP metabolism: The cellular ATP concentrations were measured according to the method described by Turgis et al. (Food Control, 2009, 20: 1073-1079). The working culture of E. coli or *S. aureus* containing approximately10^8 CFU/ml was centrifuged for 10 min at 8000 rpm and supernatant was removed. The cell pellets were washed three times and re-suspended in 0.03 M phosphate buffered saline (PBS, pH 7.4). Then, *Eucalyptus Citriodora* oil was added to obtain concentration of 0.05% vol/vol. Samples were maintained at 37 °C and 150 rpm for 30 min, centrifuged for 10 min at 8000 rpm, and then incubated in ice to prevent ATP loss. Finally, the cellular ATP concentrations of samples were measured using the CleanSenseTM Surface Hygiene Test Kit (LEYU Biotechnology, Shanghai, China). As control, the samples without Eucalyptus citriodora oil were tested (Talanta, 2009, 77: 1332-1336; International Journal of Pharmaceutics, 2012, 436: 851- 856).

Cell lysis assay: *E. coli* and *S. saureus* were incubated at 37 °C for 24 h and 48 h respectively. Then, the suspensions were centrifuged at 4000 rpm for 15 min at 4 °C and the pellets were harvested. The pellets were washed three times and resuspended in 0.03 M phosphatebuffered saline (PBS, pH 7.4) to make up bacterial suspensions of 10^8 CFU/ml. Cell suspensions were incubated at 37 °C in the presence of *Eucalyptus Citriodora* oil at five different concentrations (0 MIC as control, 0.25 MIC, 0.5 MIC, MIC, 2 MIC). After 20 h, the suspensions were centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant were filtered by microporous membrane filter. The filtrate was determined by ultraviolet spectrophotometer (UV-1801, Beijing, China) at 260nm (Journal of Food Safety, 2013, 33(2): 197-208; Food Control, 2014, 42:2328).

The assay of immunoblotting: *S. aureus* was cultured to the logarithmic phase, and then the supernatant of bacterial cultures were collected after centrifugal separation. 20 μL supernatant of bacterial cultures was added to 5 μl 5×Loading Buffer and mixed. The mixtures were boiled for 8 minutes. After the treatment of SDS polyacrylamide gel (12%) electrophoresis, the protein was transferred to the PVDF (polyvinylidene fluoride) film by semi-dry transfer unit. Subsequently, the PVDF film was soaked in bovine serum albumin (5%) solution for 2h.

After then, the film was incubated with anti-α-toxin rabbit polyclonal antibody (1:6000 dilution) for 2h, and TBST (Tris Buffered Saline Tween) washed 2 times. In the next step, the film was incubated with goat anti-rabbit IgG –HRP (1:3000 dilution) for 2h. After the same washing as above, the coloration of protein was observed in the present of ECL as chromogenic agent. Three samples were numbered 1-3 and tested respectively. 10ng α-haemolysin standard substance (Sigma) was used as a control group.

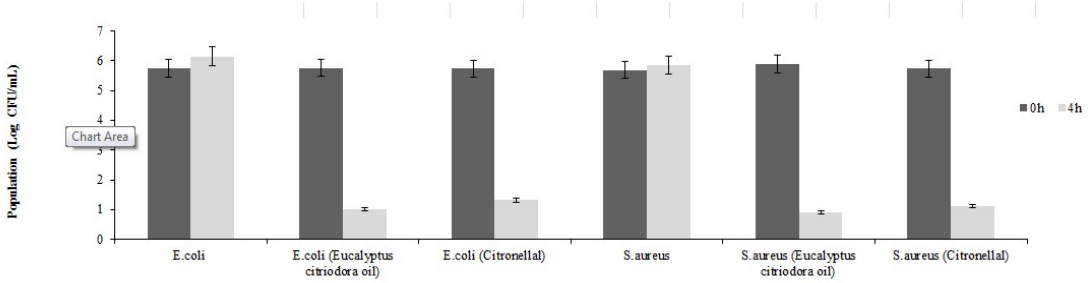


Figure S1. The negative control experiment of antimicrobial activity of *Eucalyptus Citriodora* oil (0.02% vol/vol) and citronellal for *E. coli* and *S. aureus*.

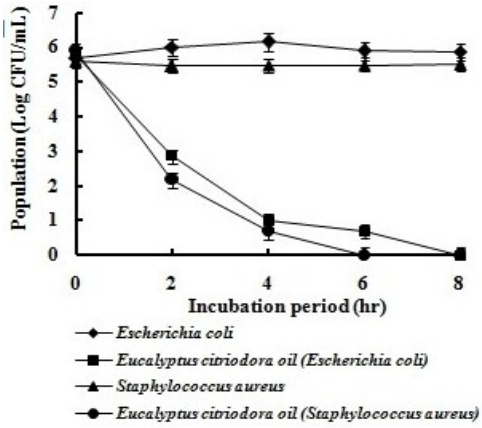


Figure S2. The bacterium population of *E. coli* and *S. aureus* before and after the treatment of *Eucalyptus Citriodora* oil.

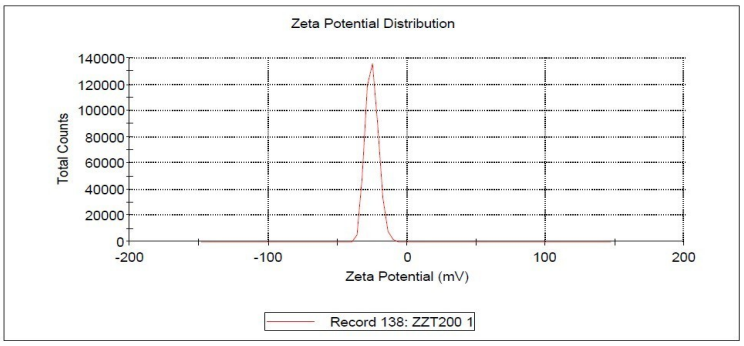


Figure S3. The surface potential of nanoliposome prepared containing *Eucalyptus Citriodora* oil with entrapment efficiency of 14.00%.

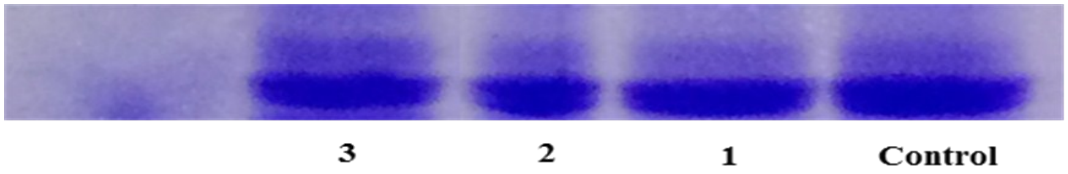


Figure S4. The immunoblotting of the protein extracted from bacterial culture S. aureus. The control was the α-haemolysin standard substance. Sample 1, 2, 3 are the different test samples extracted from *S. aureus* culture.

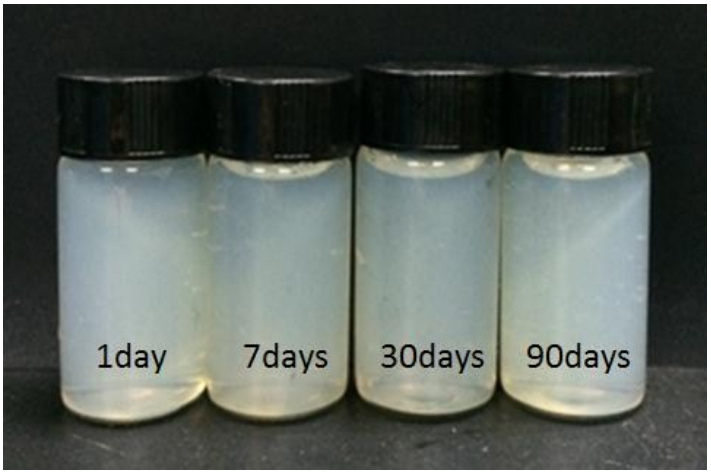


Figure S5 The emulsion preparation of nanoliposomes including *Eucalyptus citriodora* oil for 1 day, 7 days, 30 days and 90 days, respectively.

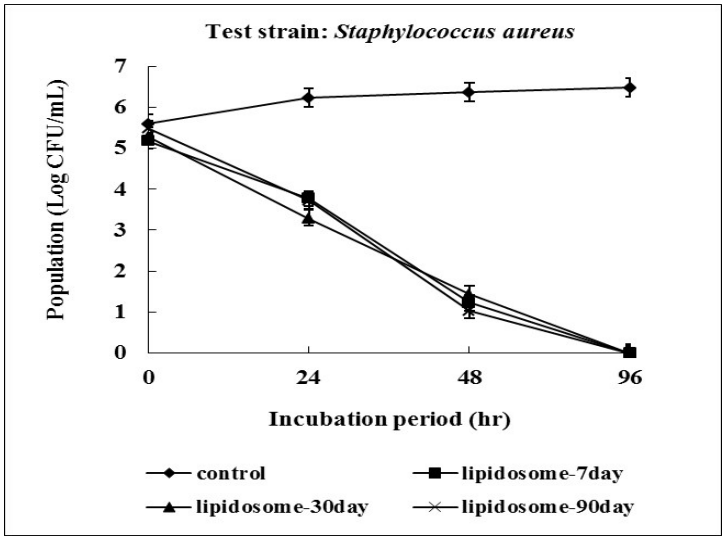


Figure S6. The antimicrobial activity of the nanoliposome containing *Eucalyptus Citriodora* oil to *S. aureus* bacterial with the agents for 7 days storage, 30 days storage and 90 days storage, respectively.

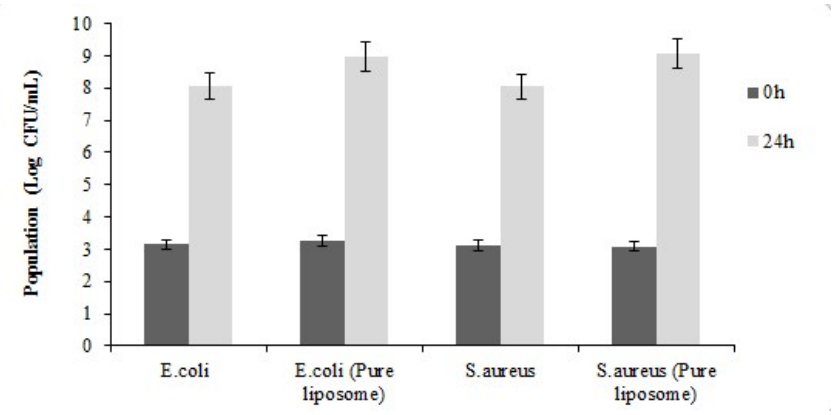


Figure S7. The negative control experiment of antimicrobial activity of pure liposome for *E. coli* and *S. aureus.*

Table S1. The MBC and MIC of *Eucalyptus Citriodora* oil.

Test strain	***Eucalyptus citriodora* Oil**	
	Minimum inhibitory concentration MIC(%)	**Minimum bactericidal concentration MBC (%)**
Escherichia coli	0.05	0.1
Staphylococcus aureus	0.05	0.1
Salmonella typhi	0.05	0.1
Klebsiella pneumonia	0.05	0.1
Pseudomonas aeruginosa	0.05	0.1
Bacillus subtilis	0.025	0.1
Bacillus pumilus	0.025	0.05

Table S2. The nanoliposome preparation with different surfactants.

nanoliposome	**surfactant（1.0mg/ml）**				
	PVP	PEG2000	PEG6000	PEG10000	PEG20000
Mean Diam	**63.9nm**	58.3nm	52.7nm	52.9nm	59.3nm
PDI	**0.178**	0.188	0.296	0.365	0.604
pH	**6.96**	7.02	7.14	7.13	7.19

Table S3. The nanoliposome preparation with different concentrations of PVP surfactant.

nanoliposome	PVP			
	0.5mg/ml	**1.0mg/ml**	1.5mg/ml	2.0mg/ml
Mean Diam	46.9nm	**63.9nm**	60.9nm	62.4nm
PDI	0.267	**0.178**	0.276	0.242
pH	6.61	**6.96**	7.2	7.16

Table S4. The nanoliposome preparation with fixed concentration of PVP but different ratio between the egg L-a-phosphatidylcholine: cholesterol.

nanoliposome	egg L-a-phosphatidylcholine: cholesterol(PVP 1.0mg/ml)			
	3:1	4:1	**5:1**	6:1
Mean Diam	65.6nm	59.9nm	**63.9nm**	63.2nm
PDI	0.359	0.212	**0.178**	0.238
pH	6.49	6.82	**6.96**	7.01

Table S5. The summary of basic physical properties of nanoliposome entrapped the *Eucalyptus Citriodora* oil with different amounts.

nanoliposome	***Eucalyptus citriodora* oil (mg/ml)**				
	2.0	3.0	**4.0**	5.0	6.0
Mean Diam (nm)	99.4	113.8	**120.5**	128.6	132.9
PDI	0.366	0.272	**0.146**	0.271	0.271
Turbidity (NTU)	612	665	**744**	851	891
pH	6.92	6.88	**6.88**	6.86	6.88
Zeta potential	-20.9	-25.2	**-25.8**	-24.5	-27.4
Entrapment efficiency (%)	7.89%	12.71%	**14.00%**	12.90%	10.89%