Int J Clin Exp Med 2014;7(7):1721-1727
www.ijcem.com /ISSN:1940-5901/IJCEM0000669

Original Article

Antibacterial activity of leaf essential oil and its constituents from *Cinnamomum longepaniculatum*

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Received April 30, 2014; Accepted June 23, 2014; Epub July 15, 2014; Published July 30, 2014

Abstract: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella enteritidis* CMCC (B) 50041, were used in the antibacterial tests of *Cinnamomum longepaniculatum* leaf essential oil and its five chemical constituents. The effect of 1, 8-cineole on the ultrastructural structure of the bacteria (*S. aureus* and *E. coli*) was also investigated by transmission electron microscopy. The *C. longepaniculatum* leaf essential oil and the five chemical constituents showed variable levels of inhibition. Their MIC (minimum inhibitory concentration) and MBC (minimal bacteriocidal concentration) values were all in the range of 0.781 μL/mL~6.25 μL/mL and 0.781 μL/mL~12.5 μL/mL respectively except γ-terpinene. The MIC values of γ-terpinene against *E. coli* and *S. aureus* were all higher than 50 μL/mL, but the MIC and MBC values of γ-terpinene against *S. enteritidis* was only 3.125 μL/mL. Among them, α-terpineol possessed the best antibacterial activity. Under the transmission electron microscope, cell size of treated *E. coli* decreased, cell wall and cell membrane ruptured, and nucleoplasm was reduced and gathered onto the side. After the *S. aureus* was treated with 1, 8-cineole, the cell size and shape were damaged and nucleus cytoplasm was concentrated or reduced or agglomerated on the side. These results suggest that *C. longepaniculatum* leaf essential oil and its constituents have excellent antibacterial activities, the antibacterial mechanism of 1, 8-cineole against *E. coli* and *S. aureus* might attributable to its hydrophobicity.

Keywords: *Cinnamomum longepaniculatum*, essential oil, 1, 8-cineole, antimicrobial activity, transmitted electron microscopy

Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is an endemic tree in China. The antimicrobial activity of *Cinnamomum longepaniculatum* leaf essential oil has attracted great attention from many researchers. A series of studies have demonstrated the essential oil has strong antibacterial activity against *Escherichia coli*, *Bacillus subtilis, Staphylococcus aureus, Bacillus thuringiensis, Streptomyces microflavus, Diplococcus catarrhalis and Salmonella* [1, 2], and also exhibited inhibitory activity against plantpathogenic fungi and dermatophytes [3, 4].

In order to explore the potential usefulness of the *C. longepaniculatum* leaf essential oil, it is important to know their chemical constituents. Published reports indicate that the main constituents of *Cinnamomum longepaniculatum* leaf essential oil are 1, 8-cineole (58.55%), safrole (0.04%), γ-terpinene (0.37%), α-terpineo (15.43%), terpinene-4-alcohol (4.84%) and so on [5-9].

1, 8-cineole also known as eucalyptol or cajeputol is a terpene oxide and is a principal constituent of most *Eucalyptus* oil (75%), *C. longepaniculatum* leaf essential oil (8.55%), rosemary (40%), *Psidium* (40~60%) and many other essential oil [10-12]. It is often employed by the pharmaceutical industry in drug formulations, as a percutaneous penetration enhancer and for its decongestant and antitussive effects and in aromatherapy as a skin stimulant in the form of skin baths [13-16]. Also, it is considered useful for the treatment of bronchitis, sinusitis and rheumatism [17].

Table 1. MIC and MBC values of *C. longepaniculatum* leaf essential oil and its five important constituents against three species bacteria

Constituents	*E. coli* ATCC 25922		*S. enteritidis* CMCC (B) 50041		*S. aureus* ATCC 25923	
	MIC	MBC	MIC	MBC	MIC	MBC
essential oil	3.125	3.125	6.25	6.25	6.25	6.25
1, 8-cineole	3.125	3.125	6.25	6.25	6.25	6.25
α-terpineol	0.781	0.781	3.125	3.125	1.562	3.125
terpinene-4-alcohol	1.562	1.562	3.125	3.125	1.562	1.562
Safrole	12.5	12.5	12.5	12.5	12.5	12.5
γ-terpinene	>50	>50	3.125	3.125	>50	>50

MIC and MBC, μL/mL.

Although the chemical constituents of leaf essential oil of *C. longepaniculatum* have been studied, the potential antimicrobial activity of its constituents has not yet been evaluated. In this study, the essential oils of leaves collected from *C. longepaniculatum* was extracted and their chemical compositions were analyzed, then the antibacterial activities of the essential oil and its chemical constituents to *S. aureus, E. coli* and *S. enteritidis* in vitro were investigated. The effect of 1, 8-cineole on the ultrastructural structure of bacteria was investigated to help understand the antibacterial mechanism.

Materials and methods

Essential oil distillation

Leaves of indigenous cinnamon (*C. longepaniculatum*) clone were collected from Sichuan experimental forest. The essential oil of *C. longepaniculatum* leaves was obtained with water distillation for 6 h.

GC analysis

GC analysis was performed using a Shimadzu model-14B equipped with a FID. The column used was 50 m long by 0.22 mm i. d. glass capillary coated with silica. GC was programmed from 60 to 220°C at a rate of 2°C/min. Identification of the major components of indigenous cinnamon leaf oils was confirmed by comparison with standards, as well as by spiking. The quantity of compounds was obtained by integrating the peak area of spectrograms.

Bacteria strains

Three bacterial strains used in this antibacterial study were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella enteritidis* CMCC (B) 50041, which were obtained from the Veterinary Pharmacology Lab, Sichuan Agricultural University, Ya'an, China.

Antibacterial activity test

The broth dilution method described in the National Committee for Clinical Laboratory Standards (NCCLS, 2000, 2008) was used to assess the antibacterial activities of the essential oil and its chemical constituents. The test samples were dissolved in 2% Tween-80 at a final concentration ranging from 50 μL/mL to 0.195 μL/mL. 2% Tween-80 was used as negative control. The antibacterial activities were examined after 24 hours of incubation at 37°C. MIC was defined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. MBC was estimated as the least concentration of the samples where no visible growth on agar subculture could be detected. The entire experiment was performed in triplicate and the results were averaged.

Time-kill curve study of essential oil

Time-kill curve studies were performed in duplicates with the drug and media for each sample at 3 different concentrations as follows: 1/2×MIC, 1×MIC, and 2×MIC. Tubes containing Mueller Hinton broth at various concentrations and with growth control group were seeded with a log-phase inoculum of roughly 5×10^5 CFU/mL to a final volume. Inoculated broths were incubated at 37°C. 0.1 mL of inoculated broths was sampled at different time intervals (0, 1, 2, 4, 8, 12, 24, and 36 h) from each tube

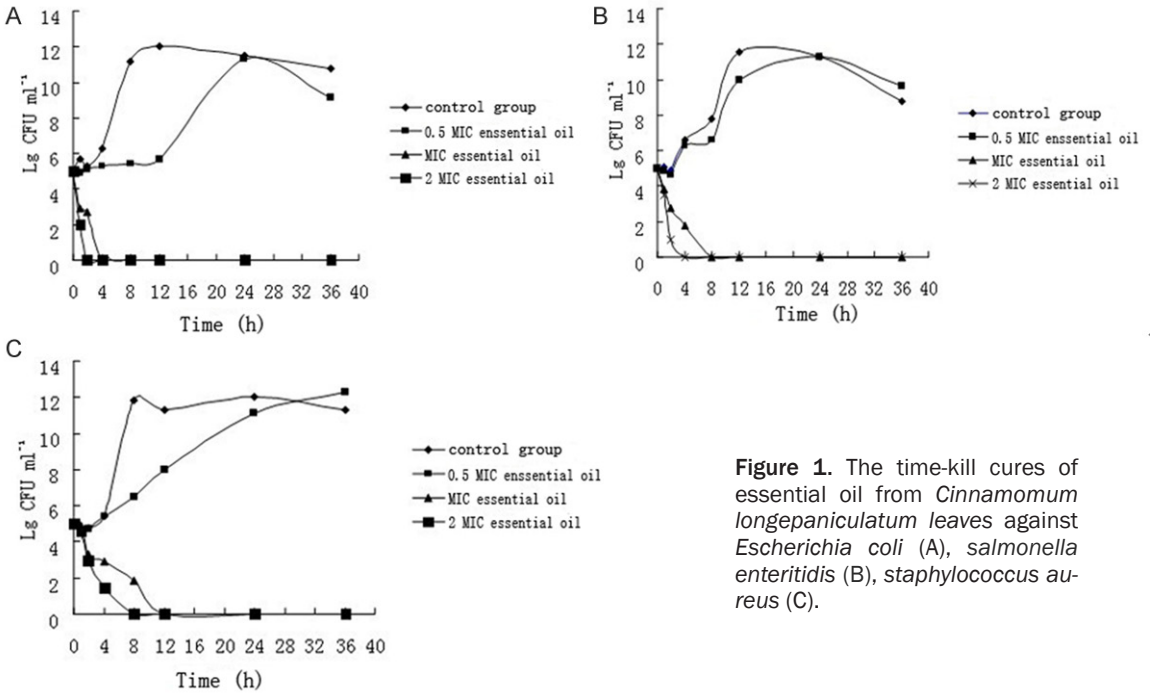

Figure 1. The time-kill cures of essential oil from *Cinnamomum longepaniculatum leaves* against *Escherichia coli* (A), *salmonella enteritidis* (B), *staphylococcus aureus* (C).

and was subjected to 10-fold serial dilution. Then, 0.1 mL of every dilution was spread on Mueller Hinton plates for colony counting and incubated at 37°C for 24 h. Only plates containing the count between 30~300 for each series of dilutions were counted. Finally, the time-kill curves were constructed for organism.

Transmission electron microscopy

In order to investigate the effect of 1, 8-cineole on the ultrastructure of bacteria, 100 mL of 10^6 CFU/mL *S. aureus* and *E. coli* in Mueller Hinton broth were exposed to MIC concentration of 1, 8-cineole and then were incubated at 37°C for 3 h in an incubator shaker. The control group was treated with solvent only. The bacterial suspensions were then centrifuged in sterile plastic centrifuge tubes at 8000 rpm for 10 min at 4°C. The supernatant was discarded and the pelleted cellular content was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate-buffer (pH=7.4) at 4°C overnight. Samples were then post-fixed for 2 h in 1% osmium tetroxide (OsO_4) dissolved in cacodylate-buffer at room temperature and washed in cacodylate-buffer three times for 15 min each. Samples were dehydrated in ethanol in a graded series of 40%, 60%, 75%, 80% and 95% dilutions two times for 15 min each. A final dehydration step was carried out for 1 h in 100% ethanol with changes every 30 min. Epoxy resin (Epon-618) was used to embed the post-fixed samples for 12 h~16 h at 45°C. Ultra-thin section of the embedded samples were prepared by LKB-II Ultra-cut instrument and double stained with uranyl acetate and lead citrate. Morphology of the bacterial cells was observed on a transmission electron microscope.

Results and discussion

Antibacterial activity of essential oils and its major compositions

The five compounds isolated from essential oil of *C. longepaniculatum* leaves were identified as: 1, 8-cineole, safrole, γ-terpinene, α-terpineol and terpinene-4-alcoho. The MIC and MBC values of *C. longepaniculatum* leaf essential oil and its five important constituents against three species bacteria (*E. coli*, *S. aureus* and *S. enteritidis*) are presented in **Table 1**. The *C. longepaniculatum* leaf essential oil had demonstrable antibacterial properties, and the five important constituents present in the oil also showed variable levels of inhibition. Their MIC and MBC values were in the range of 0.781 μL/mL~6.25 μL/mL and 0.781 μL/mL~12.5 μL/mL respectively except γ-terpinene.

Comparing the antibacterial activity of five important constituents of *C. longepaniculatum* leaf oil with *C. longepaniculatum* leaf oil, 1,

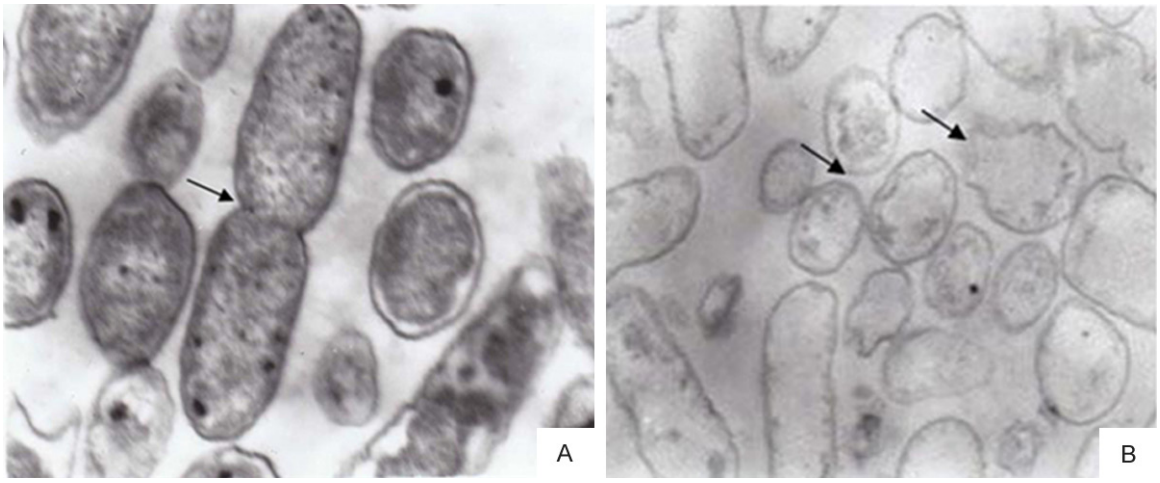

Figure 2. EM graph (×25000) of *E. coli* (non-treated) (A). Stationary phase cells on the micrograph showed a typical healthful bacilli-shape, even showed growth and cell division (arrows indicating); EM graph (×20000) of *E. coli* exposed to MIC of 1, 8-cineole (B). Cell deformation, breakage of cell wall and membrane, condensation of cellular material were observed from the damaged cells of *E. coli* (arrows indicating).

8-cineole and *C. longepaniculatum* leaf oil had the same antibacterial effect. Meanwhile, the inhibitory effect of safrole was less than *C. longepaniculatum* leaf oil, α-terpineol and erpinene-4-alcohol against *S. aureus, E. coli* and *S. enteritidis*. On the other hand, the inhibitory effect of *C. longepaniculatum* leaf oil was less than that of α-terpinene and erpinene-4-alcohol against *S. aureus, E. coli* and *S. enteritidis*. γ-terpinene had high specific bacteriostatic action. The MIC values of γ-terpinene against *E. coli* and *S. aureus* were all higher than 50 μL/mL, but the MIC and MBC values of γ-terpinene against *S. enteritidis* was only 3.125 μL/mL.

Among the five constituents, α-terpineol possessed the best antibacterial activity. So the inhibitory properties of *C. longepaniculatum* leaf oil against these three bacteria were not solely due to 1, 8-cineole. It might be that a synergistic effect derived from some constituents in *C. longepaniculatum* leaf oil endows it with such potent antibacterial activity.

There are several reports stating that other leaf oils (eg *C. zeylanicum* Bl.) exhibit antimicrobial activity. Hili and his co-workers [18] have assayed the antimicrobial activity of the *C. zeylanicum* leaf oil against three bacteria and four yeast strains. Their results demonstrated that the leaf oil completely inhibited the growth of *E. coli*, *S. aureus*, and *P. aeruginosa* at the level of 500 μg/mL. Another report found the MICs of *C. zeylanicum* against *E. coli*, and *S. aureus* were 0.05% and 0.04%, respectively [19]. Pitarokili and Sonboli have confirmed that essential oil from *Cinnamomum longepaniculatum* is an effective botanical bactericide [20, 21]. However, there are no reports on active constituents of essential oil from *Cinnamomum longepaniculatum*, and few commercial products are available because of the difficult quality control. Several researchers have reported that the antimicrobial activity of an essential oil is linked to its chemical composition [22, 23], the functional groups (alcohol, phenols, terpenes and ketones) of compounds found in plant materials (extracts/essential oil) are associated with their antimicrobial characteristics [24].

In comparison with those reports, it is evident from our results that the inhibitory activity of *C. longepaniculatum* leaf oil and its five important constituents on bacteria was better than that of *C. zeylanicum* leaf oil, Himalayan Lauraceae species and *Cinnamomum osmophloeum*, whose MIC values for bacterial strains were in the range of 3.90 μL/mL~31.25 μL/mL and 250 μL/mL~500 μL/mL [25, 26]. On the other hand, there was no discernible trend of inhibition reflected in the strain characteristics of bacterium: both Gram-negative and Gram-positive organisms were affected. Which supported by other researchers who reported that essential oil of *Cinnamomum longepaniculatum* and the compounds separated from essential oil were found to be active against Gram-

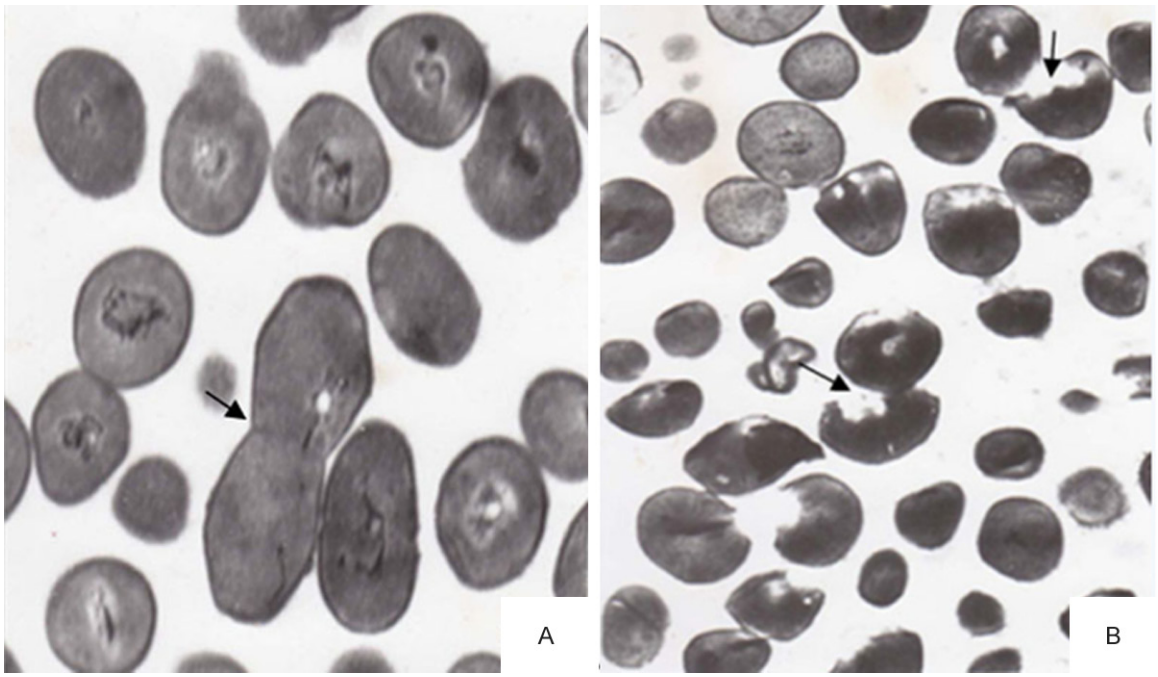

Figure 3. EM graph (×30000) of *S. Aureus* (non-treated) (A), cells showed a continuous thin smooth cell wall, cell membrane, nuclear material and cell division (arrows indicating); EM graph (×20000) of *S. Aureus* exposed to MIC of 1, 8-cineole (B). The cells became irregular, shriveled, leaking of the contents of the cells (arrows indicating).

positive bacteria, Gram-negative bacteria, fungi and some of which are food spoilage microorganisms [20, 21]. This is different from Taiwania (*Taiwania cryptomerioides* Hayata) heartwood essential oil, which had an inhibitory effect against Gram-positive bacteria only [27]. Furthermore, the results showed that the inhibitory activity of *C. longepaniculatum* leaf oil and its five important constituents on bacteria was a synergistic effect and associated with their antimicrobial characteristics of the functional groups (alcohol, phenols, terpenes and ketones) of compounds found in *C. longepaniculatum* essential oil.

Because the main constituent of *C. longepaniculatum* leaf oil is 1, 8-cineole and it has strong antibacterial activity, the effect of 1, 8-cineole on the ultrastructural structure of the bacteria was further tested to help understand the antibacterial mechanism.

Time-kill curves

Time-kill curves of *C. longepaniculatum* leaf essential oil were shown in **Figure 1**. At 0.5×MIC concentration of *C. longepaniculatum* leaf essential oil, the time-kill curves were basically the same to the control group, which had the integral growth cycle (lag phase, logarithmic phase, stationary phase and death phase). But at 1×MIC and 2×MIC concentration of *C. longepaniculatum* leaf essential oil, three kinds of bacteria directly enter into decline phase without adjustment phase, logarithmic phase and stable phase. All the bacterial cells of *E. coli*, *S. enteritidis* and *S. aureus* were killed at 1×MIC in 4 h, 8 h, 12 h and 2×MIC in 2 h, 4 h, 8 h respectively. The rate of killing increased by increasing the concentration of *C. longepaniculatum* leaf essential oil. Time-kill curves of *C. longepaniculatum* leaf essential oil showed a concentration-dependent effect. This will be a more rational basis for determining optimal dosage for antimicrobial treatment regimens.

Ultrastructural changes of bacteria

The *E. coli* and *S. aureus* cells treated with 3.125 μL/mL and 6.25 μL/mL respectively of the purified 1, 8-cineole at 37°C for 3 h were observed on a transmission electron microscope (**Figures 2** and **3**). Non-treated *E. coli* cells showed a typical healthy bacilli-shape, even showed cell growth and cell division (**Figure 2A**), and non-treated *S. aureus* cells

showed a continuous thin smooth cell wall, cell membrane, nuclear material and cell division (**Figure 3A**). But the treated cells were severely damaged and destructed in the surface and inside structure. Under the transmission electron microscope, the cell size of treated *E. coli* decreased, cell wall and cell membrane ruptured, nucleoplasm reduced and gathered onto the side (**Figure 2B**). After the *S. aureus* was treated, the cell size and shape were damaged and nucleus cytoplasm was concentrated or reduced or agglomerated on the side (**Figure 3B**).

Antimicrobial mechanisms of natural compounds found in herbs or spices have been discussed [28]. Thymol and carvacrol have been reported to possess inhibitory effects against the growth of enteric bacteria (E. coli O157: H7 and Salmonella typhimurium) at a similar concentration as shown above. They were proposed to have prominent outer membrane disintegration and increased the permeability of ATP through cytoplasmic membrane.

In comparison with those reports, our results indicated that the 1, 8-cineole has prominent outer membrane disintegration and nucleus cytoplasm concentration or reduction. The results are comparable to the action of nisin, which acts on the cytoplasmic membrane by inducing pores and cytoplasmic leakage [29, 30].

In conclusion, *C. longepaniculatum* leaf essential oil and its constituents have excellent antibacterial activities and α-terpineol, one of the major compounds in the leaf essential oils, possessed the strongest antibacterial activities compared with the other components. And the antibacterial mechanism of 1, 8-cineole was realized through producing alterations on the structure of *E. coli*, *S. enteritidis* and *S. aureus* directly. This shows *C. longepaniculatum* leaves have the potential to be used for medical purposes. In this study, we investigated the antibacterial activities and antibacterial mechanism of major components from *C. longepaniculatum* leaf essential oil against *E. coli*, *S. aureus and S. enteritidis*, which has not been previously reported.

Acknowledgements

The Project Supported by Scientific Research Fund of Sichuan Provincial Education Department (No. 14TD0031), and The Sichuan Youth Science and Technology Innovation Research Team for Resources Utilization of Cinnamomum longepaniculatum (2011JTD0035).

Disclosure of conflict of interest

None.

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