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# Antibacterial Activity and Mechanism of *Pogostemon cablin* Against Bacteria from Milk of Dairy Cows Suffering with Mastitis

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**Abstract:** Minimum Inhibitory Concentrations (MICs) of 3 extracts and oil from *Pogostemon cablin* against isolates from milk produced by dairy cow with mastitis were determined by Agar Dilution Method. Water extract had low activity; ethanol extract and ethanol-water extract had moderate effects having MICs ranging from 25.0-100.0, 12.5-100.0 mg $mL^{-1}$, respectively. Oil had strong activity against Gram-positive and Gram-negative isolates with MICs ranging from 0.2-8.2 mg $mL^{-1}$. Oil also, showed strong efficacy in mouse peritonitis model infected with *S. aureus* or *E. coli*. Their actions were dose-dependent. Obvious morphological changes were observed by TEM in oil-treated *E. coli* or *S. aureus* such as a remarkable electron-light region small electron-dense granules disturbed cytoplasmic membrane and abnormal cell division. In conclusion, antibacterial substance of *P. cablin* was oil which had broad-spectrum antibacterial activities so to make it a potential candidate as antibacterial agent to treat bovine mastitis.

**Key words:** *Pogostemon cablin*, essential oil, bovine mastitis, *in vitro* and *in vivo* antibacterial activity, antibacterial mechanism, China

## INTRODUCTION

Bovine mastitis is a major clinical problem because of the increasing presence of antibiotic-resistant bacteria and treatment failure (Kim *et al*., 2010) resulting in significant economic losses to the dairy industry (Chaneton *et al*., 2011). Owing to the deficiency of other effective antibacterial agents, intramammary infusion of antibiotics is the most common treatment method available for treating mastitis (Baskaran *et al*., 2009). Use of antibiotics against bacterial diseases may potentially lead to the emergence of antibiotic resistant strains of bacteria. Moreover, the use of antibiotics to treat bovine mastitis has been implicated as a common source of drug residues in milk. Approximately 90% of the residues detected in milk over a period of 5 years in Michigan, USA were originated from antibacterial therapy for mastitis (Erskine *et al*., 2003). To improve the safety of milk and dairy products there is a need for alternative approaches for controlling mastitis in dairy cows. Plant-based essential oils or organic extracts are well known to exhibit a wide range of biological activities but with low mammalian toxicity, less environmental effects and wide public acceptance (Paranagama *et al*., 2003).

*Pogostemon cablin* (*P. cablin*) originated in Malaysia and India was introduced into China for perfume and medicine and the Southern China's Guangdong province finally started culturing its own around the eleventh century. Up to date, *P. cablin* is widespread in Southern China including Guangdong (Guangzhou, Gaoyao, Zhanjiang) and Hainan provinces. The plant has been used as Chinese herbal medicine to remove dampness, relieve summer-heat, exterior syndrome, stop vomiting, stimulate appetite and treat common cold, nausea and diarrhea (Pharmacopeia Commission of PRC, 2004). And the oil is widely used in the cosmetic and oral hygiene industries to scent perfumes, flavor toothpaste, etc. (Zhao *et al*., 2005). Modern researches have repeatedly demonstrated various pharmacological activities of this oil including anti-emetic properties (Yang *et al*., 1999), trypanocidal activities (Kiuchi *et al*., 2004), anti-bacterial (Edwards-Jones *et al*., 2004; Liu *et al*., 2009), anti-fungal

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(Mo *et al*., 2004) and $Ca^{2+}$ antagonist activities (Ichikawa *et al*., 1989). To search for natural antibacterial substances for treating mastitis, researchers investigated the *in vitro* antibacterial activity of extracts of *P. cablin* against isolates from milk produced by dairy cow with mastitis *in vivo* antibacterial activity and potential antibacterial mechanism of essential oil from *P. cablin* which had broad-spectrum activities so to make it a potential candidate as antibacterial agent.

## MATERIALS AND METHODS

**Antibiotics, media and reagents:** Cefotaxime sodium was obtained from Sichuan Pharmaceutical, Inc. (China, Lot: 080301). Cefepime hydrochloride was obtained from Shandong Luoxin Pharmacy Stock Co. Ltd. (China, Lot: 0903238). Mueller-Hinton (MH) agar, yeast extract and tryptone were purchased from Oxoid Ltd. Mucin from porcine stomach (Type II) was obtained from Sigma. Polysorbate 80 were purchased from Chengdu Changzheng Huabo., Ltd. (Chengdu, China).

**Microorganisms:** The bacteria were isolated from 80 milk samples produced by dairy cow with clinical or subclinical mastitis from Chengdu, Mianyang and Meishan, Sichuan province, China in 2008. One hundred and twenty strains of isolates were identified by VITEK 32 Microbial Analytical System or Sequence Analysis of 16S rRNA (Table 1). Reference strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were provided by Sichuan

Table 1: *In vitro* antibacterial activity of 3 extracts and essential oil from *P. calbin* against bacteria from milk produced by dairy cow with mastitis

| | | Water extract[a] | | | Ethanol extract[a] | | |
|---|---|---|---|---|---|---|---|
| Species of bacteria | N[0] | MIC | $MIC_{50}$ | $MIC_{90}$ | MIC | $MIC_{50}$ | $MIC_{90}$ |
| ATCC25923 | 1 | - | - | - | 100.0 | - | - |
| *S. aureus* | 8 | 100.0~* | - | 100.0~* | 50.0~100.0 | 100.0 | 100.00 |
| *Coagulase-negative Staphylococcus*[b] | 25 | 50.0~* | - | 100.0~* | 50.0~100.0 | 100.0 | 50.0~100.0 |
| *Kurthia gibsonii* | 9 | - | - | - | 50.0~100.0 | 100.0 | 50.0~100.0 |
| *Bacillus*[c] | 9 | 25.0~* | 100.0 | 100.0~* | 50.0~100.0 | 50.0 | 50.0~100.0 |
| *Macrococcus caseolyticus* | 6 | 25.0~* | - | 25.0~* | 50.0~* | 50.0 | 50.0~* |
| *Streptococcus*[d] | 4 | 50.0~"-" | - | 50.0~* | 50.0~100.0 | 100.0 | 50.0~100.0 |
| *Corynebacterium* | 3 | 12.5 | - | - | 25.0 | - | - |
| *Enterococcus faecalis* | 2 | - | - | - | 100.0 | - | - |
| ATCC25922 | 1 | - | - | - | - | - | - |
| *E. coli* | 17 | - | - | - | - | - | - |
| *Pseudomonad*[e] | 12 | 50.0~* | - | 50.0~* | 50.0~* | 50.0 | 50.0~* |
| Presumptive *Acinetobacter lwoffii* | 11 | 100.0~* | 100.0 | 100.0~* | 25.0~100.0 | 100.0 | 50.0~100.0 |
| *Enterobacter cloacae* | 6 | - | - | - | - | - | - |
| *Chrvseobacterium indologenes* | 6 | 100.0 | 100.0 | 100.0 | 50.0 | 50.0 | 50.0 |
| *Shigella flexneri* | 1 | 25.0 | - | - | 50.0 | - | - |
| *Proteus vulgaris* | 1 | 100.0 | - | - | 100.0 | - | - |

| | Ethanol-water extract[a] | | | Essential oil[a] | | |
|---|---|---|---|---|---|---|
| Species of bacteria | MIC | $MIC_{50}$ | $MIC_{90}$ | MIC | $MIC_{50}$ | $MIC_{90}$ |
| ATCC25923 | 50.0 | - | - | 0.5 | - | - |
| *S. aureus* | 25.0~50.0 | 50.0 | 25.0~50.0 | 0.5~1.0 | 0.5 | 0.5 |
| *Coagulase-negative Staphylococcus*[b] | 12.5~100.0 | 25.0~50.0 | 12.5~50.0 | 0.2~2.0 | 0.5 | 0.3~0.5 |
| *Kurthia gibsonii* | 25.0~50.0 | 50.0 | 25.0~50.0 | 0.5 | 0.5 | 0.5 |
| *Bacillus*[c] | 25.0~50.0 | 25.0 | 25.0~50.0 | 0.3~0.5 | 0.5 | 0.5 |
| *Macrococcus caseolyticus* | 25.0~* | 25.0 | 25.0~* | 0.3~0.5 | 0.3~0.5 | 0.3~0.5 |
| *Streptococcus*[d] | 25.0~50.0 | 25.0 | 25.0~50.0 | 0.5 | 0.5 | 0.5 |
| *Corynebacterium* | 12.5 | - | - | 0.3 | - | - |
| *Enterococcus faecalis* | 100.0 | - | - | 1.0 | - | - |
| ATCC25922 | - | - | - | 2.0 | - | - |
| *E. coli* | - | - | - | 0.3~8.2 | 2.0 | 1.0~4.1 |
| *Pseudomonad*[e] | 25.0~* | 25.0~50.0 | 25.0~"-" | 0.3~* | 0.3~2.0 | 0.3~* |
| Presumptive *Acinetobacter lwoffii* | 25.0~50.0 | 25.0 | 25.0~50.0 | 0.5~1.0 | 0.5 | 0.5 |
| *Enterobacter cloacae* | - | - | - | 4.1~8.2 | 4.1 | 4.1 |
| *Chrvseobacterium indologenes* | 25.0 | 25.0 | 25.0 | 0.3~1.0 | 0.5 | 0.5~1.0 |
| *Shigella flexneri* | 12.5 | | | 0.3 | | |
| *Proteus vulgaris* | 50.0 | | | 2.0 | | |

[a]Minimum Inhibitory Concentration (MIC) (MIC, $MIC_{50}$, $MIC_{90}$ values in mg $mL^{-1}$). "-" is no activity against corresponding isolates at maximum concentration and the maximum concentration of oil and other extracts were 32.7 and 100.0 mg $mL^{-1}$, respectively. [b]Twenty-five strains coagulase-negative Staphylococcus includes 5 strains *Staphylococcus auricularis*, 5 strains *Staphylococcus Saprophyticus*, 4 strains *Staephylococcus xylosus*, 3 strains *Staphylococcus* sp., 2 strains *Staphylococcus hominis*, 1 strain *Staphylococcus epidermidis*, 1 strain *Staphylococcus haemolyticus*, 1 strain *Staphylococcus warneri*, 1 strain *Staphylococcus cohnii*, 1 strain *Staphylococcus sciuri* and 1 strain *Staphylococcus nepalensis*. [c]Nine strains *Bacillus* includes 4 strains *Bacillus cereus*, 3 strains *Bacillus pumilus* and 2 strains *Bacillus flexus*. [d]Four strains *Streptococcus* includes 1 strain *Streptococcus agalactiae* (Group B),1 strain *Streptococcus uberis*, 1 strain *Streptococcus bovis* and 1 strain *Streptococcus mitis*. [e]Twelve strains *Pseudomonad* includes 6 strains *Pseudomonas aeruginosa*, 3 strains *Sphingomonas paucimobilis*, 2 strains *Pseudomonas fluorescens* and 1 strain *Aeromonas hydrophila*

Industrial Institute of Antibiotics Co., Ltd. All Strains were stored until use in refrigerator at -80°C in Luria-Bertani (LB) medium containing 10% glycerol.

**Laboratory animals:** Specific Pathogen Free (SPF) Kun-ming mice (body weight, 18-22 g) were purchased from Chengdu Institute of Biological Products (Chengdu, China) and acclimatized for 1 day prior to use. All animals were housed in cages (five animals of the same sex per cage) under constant temperature (25±2°C) and humidity (50±10%) free to access food and water throughout the study. All studies with animals were approved by the Animal Research Committee of the Institute of Medicinal Biotechnology (Beijing, China).

**Preparation of essential oil and 3 extracts from *P. cablin*:** *P. cablin* from Guangdong province, China was purchased from Beijing Tongren Drug Store and identified by Prof. Min Li at Chengdu University of Traditional Chinese Medicine. The essential oil was obtained by steam distillation and the density of oil was 0.98 mg $mL^{-1}$ (Pharmacopeia Commission of PRC, 2004). Water extract (or ethanol extract) was extracted as follows: *P. cablin* (150 g) was extracted with 1,200 mL of distilled water [or 75% (v/v) EtOH] for 2 h with the method of thermal circumfluence extraction. The heated decoction was filtered and the first filtrate was collected in a separate flask. The remnant was extracted repeatedly one more time. The first and second filtrates were combined and concentrated. While ethanol-water extract was extracted as follows: *P. cablin* (150 g) was first extracted with 1,200 mL 75% (v/v) ethanol for 2 h with the method of thermal circumfluence extraction then the remnant was extracted with 1,200 mL of distilled water for 2 h as the same method described above. The ethanol-water extract was obtained from the mixture of filtrates of ethanol extract and water extract followed by concentration. The concentration of three extracts was adjusted to 1.5 g $mL^{-1}$ with distilled water. The oil and 3 extracts were stored at -20°C until use. The concentrations of 3 extracts were calculated as follows:

$$\text{The concentration of extract} = \frac{\text{Weight of } P.\ cablin\ (\text{g})}{\text{Volume of extract (mL)}}$$

***In vitro* antibacterial activities of extracts and oil from *P. cablin*:** Minimum Inhibitory Concentrations (MICs) of 3 kinds of extracts and essential oil from *P. cablin* against the tested microorganisms were determined by the agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2008; Hayouni *et al*., 2008; Mimee *et al*., 2009; Yayli *et al*., 2010) with modification. The assay was carried out as follows. Water extract, ethanol extract and ethanol-water extract were diluted in distilled water and oil was dissolved with polysorbate 80 (final concentration 1%). Microbial strains were cultured overnight at 35°C. Test strains were suspended in physiological saline to give a final density of $1.5\times10^6$ CFU $mL^{-1}$ by VITEK DENSICHEK (BioMerieux, Inc.).

All tests were performed in MH agar. Geometric dilutions ranging from 1.6-100.0 mg $mL^{-1}$ of water extract, ethanol extract and ethanol-water extract and from 0.1-32.7 mg $mL^{-1}$ of essential oil were prepared in 90 mm plate (Kangjian Medical Apparatus Co., Ltd.) including one growth control (MH agar + distilled water or 1% polysorbate 80) and one sterility control (MH agar + distilled water + extract or MH agar + % polysorbate 80 + oil). Each bacterial suspension was inoculated on the plates by SAKUMA Anti-bacterial Determiner (Japan). The plates were incubated at 35°C for 24 h and MICs were then determined as the lowest concentration at which no bacteria growth was apparent in a given plate. The test was repeated three times. The data were expressed as the mean±SD. Statistical evaluation for differences among multiple extracts or bacterial species was carried out using one way Analysis of Variance (ANOVA) by SPSS for windows (SPSS 13.0; SPSS Inc., Chicago, IL, USA). The $p<0.05$ was considered significant statistically and $p<0.01$ was considered very significant statistically.

***In vivo* antibacterial activities of patchouli oil against isolates of *E. coli* and *S. aureus*:** *In vivo* efficacy of patchouli oil against mouse systemic infections was evaluated with one isolate of *S. aureus* or *E. coli*, respectively. Effective concentration levels of patchouli oil, Minimal Lethal Doses (MLDs) of tested *S. aureus* and *E. coli* were determined by using a preliminary test. The *in vivo* antibacterial activity experiment was carried out according to a described method with modification (Li *et al*., 2010). Mice were randomly divided into 6 parallel groups for each isolate and each group had 10 mice including 5 males and 5 females. There were one model group (drug negative to show the success of modeling), one control group (growth control), three experimental drug groups including high dose group (0.49 g/kg/day) mid dose group (0.25 g/kg/day) and low dose group (0.13 g/kg/day) and one antibiotic group (positive) in which cefepime was used in model infected with *S. aureus* and cefotaxime was in that infected with *E. coli*. Patchouli oil, antibiotic and physiological saline were administered intramuscularly for experimental drug groups, positive dug group model group and control

group once daily for 6 days, respectively. Except for the mice of control group other mice were infected intraperitoneally with Minimal Lethal Dose (MLD) of bacterial suspension in 5% mucin (Sigma) after the sixth administration of drugs or physiological saline. The MLDs of *S. aureus* and *E. coli* were $1.3\times10^8$, $1.5\times10^4$ CFU $g^{-1}$, respectively. Survivals in each group were recorded daily for 14 days. The test was repeated three times.

**Growth curves of oil-treated *E. coli* and *S. aureus*:** Inhibitory of the growth of *E. coli* ATCC25922 or *S. aureus* ATCC25923 after oil treatment was investigated. Patchouli oil was first emulsified with polysorbate 80 then diluted with Luria-Bertani broth to reach the final concentration of 1/2 MIC (1.02 mg $mL^{-1}$ for *E. coli*, 0.26 mg $mL^{-1}$ for *S. aureus*), 1/4 IC (0.51 mg $mL^{-1}$ for *E. coli*, 0.13 mg $mL^{-1}$ for *S. aureus*) and 1/8 MIC (0.26 mg $mL^{-1}$ for *E. coli*, 0.07 mg $mL^{-1}$ for *S. aureus*), respectively. The oil control group had the same concentration of oil but without corresponding bacterial suspension and the control group had bacterial suspension in LB broth containing 1% polysorbate 80 but without oil. All preparations were incubated with $1.5\times10^6$ CFU $mL^{-1}$ at 37°C with shaking at 150 rpm and 100 μL aliquots were then transferred to a 96 well microtitre plate at 2 h intervals. $OD_{600}$ values were monitored by using VARIOSKAN Flash (Thermo Scientific) until $OD_{600}$ value of each group reached stationary phase. The final $OD_{600}$ value at each time of each experimental group equaled to the mean of $OD_{600}$ value of experimental group minus that of oil control under the same condition. The growth curve of oil-treated *E. coli* or *S. aureus* was made using Microsoft Excel in which the growth time was defined as horizontal ordinate and the $OD_{600}$ value was defined as vertical coordinate. All analyses were carried out in triplicate.

**Ultrastructural changes of oil-treated *E. coli* and *S. aureus*:** After oil treatment, the ultrastructural changes of *E. coli* ATCC25922 or *S. aureus* ATCC25923 were observed by transmission electron microscopy. Tested bacterial inocula were prepared by inoculating followed by culturing overnight at 35°C, the logarithmic phase bacteria containing $1.5\times10^6$ CFU $mL^{-1}$ were inoculated in LB broth containing 1/2 MIC patchouli oil and cultured at 35°C, 150 rpm for 18 h. And logarithmic phase bacteria of control group were inoculated in LB broth. Cells were centrifuged at 1,000 g at 4°C for 10 min. The samples were fixed with PBS (pH 7.4) containing 0.5% glutaraldehyde for 30 min at 4°C. Subsequently, the cells were centrifuged at 15,000 g at 4°C for 10 min and the bacterial pellets were added to PBS (pH 7.4) containing 3% glutaraldehyde at 4°C for 1 h. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained with 1% uranyl acetate and dehydrated in a graded series of ethanol. The samples were cryofixed with a pressure of about 2100 bars and a reduction in temperature of 8°C $sec^{-1}$. The cryofixed cells were kept in liquid nitrogen and cryosubstituted in pure acetone containing 2% of osmium tetroxide and 0.1% of uranyl acetate for 72 h at -90°C. The temperature was gradually increased to 4°C (5°C $h^{-1}$). Samples were kept at this temperature for 2 h followed by 2 h at room temperature and were then washed twice with acetone. The fixed cells of the two microorganisms were then embedded in L R (London Resin Co., Ltd.) white resin. Ultra-thin sections were prepared and stained with 1% uranyl acetate and sodium citrate. Microscopy was performed with Tecnai G2 F20 at the Analytical and Testing Center, Sichuan University (Sichuan, China).

## RESULTS AND DISCUSSION

***In vitro* antibacterial activities of 3 extracts and essential oil from *P. cablin*:** *Pogostemon cablin* (Blanco) Benth. known as Guang-Huo-Xiang is a Chinese medical material traditionally used for the treatment of common cold, nausea and diarrhea (Wei and Shibamoto, 2007). It is a potentially useful source of antimicrobial compounds. Earlier reports showed that patchouli oil inhibited the growth of some bacterial strains in the genera *S. aureus*, *Pseudomonas aeruginosa*, dysentery bacterium (Liu *et al*., 2009) and plant pathogenic fungi (Mo *et al*., 2004). Water extract of *P. cablin* inhibited the growth of some bacterial strains in the genera *S. aureus*, Enterobacteriaceae (Liu *et al*., 1999) but no effect on *E. coli* (Luo, 2005). The results reported from different studies are difficult to compare, presumably because of different test methods, bacterial strains and sources of samples used. And there were not reports on antibacterial activity of *P. cablin* against isolates stains from milk cows with mastitis.

To assess the antibacterial substance of traditional Chinese medicine *P. cablin*, the MICs of 3 extracts and oil of *P. cablin* cultivated in Guangdong province, China against 120 strains isolates were tested. The results showed that essential oil had the highest activity against 117 strains of clinical Gram-positive and gram-negative bacteria except for 3 strains of *Pseudomonad* with the MICs ranging from 0.2-8.2 mg $mL^{-1}$ (Table 1). Ethanol extract and ethanol-water extract had comparatively moderate antibacterial activities against a majority of Gram positive and negative bacteria with $MIC_{90}$ values of 50.0-100.0 and 12.5-50.0 mg $mL^{-1}$, respectively but no

Table 2: Comparative analysis on MICs of 2 extracts and essential oil from *P. cablin* (mean±SD)

| Species of bacteria | N[0] | Ethanol extract[a] | Ethanol-water extract[a] | Essential oil |
|---|---|---|---|---|
| *Staphylococcus aureus* | 8 | 92.9±18.9 | 42.9±12.2[B] | 0.6±0.2[BC] |
| **Coagulase-negative** | | | | |
| *Staphylococcus* | 25 | 84.6±23.5 | 39.9±22.6[B] | 0.6±0.3[BC] |
| *Kurthia gibsonii* | 9 | 87.5±23.1 | 40.6±12.9[B] | 0.5±0.0[BC] |
| *Macrococcus caseolyticus* | 6 | 60.0±22.4 | 25.0±0.00 | 0.3±0.1[bC] |
| *Streptococcus* | 4 | 87.5±25.0 | 31.3±12.5[b] | 0.5±0.0[bc] |
| *Bacillus* | 9 | 61.1±22.1 | 33.3±12.5[b] | 0.5±0.1[BC] |
| *Escherichia coli* | 17 | - | - | 3.1±2.2 |
| *Pseudomonad* | 12 | 62.5±23.1 | 37.5±13.4 | 1.7±2.5[BC] |
| **Presumptive** | | | | |
| Acinetobacter lwoffii | 11 | 77.5±29.9 | 37.5±13.2[b] | 0.6±0.2[BC] |
| *Enterobacter cloacae* | 6 | - | - | 4.8±1.7 |
| Chrvseobacterium indologenes | 6 | 50.0±0.00 | 25.0±0.00[B] | 0.6±0.3[BC] |

[a-]: No antibacterial activity against corresponding isolates. The antimicrobial activity of 3 kinds of extracts from *P. cablin* against the same species was analyzed by the pairwise comparison among which compared with ethanol extract, [b]p<0.05, [B]p<0.01; compared with ethanol-water extract, [c]p<0.05, [C]p<0.01

Table 3: *In vivo* efficacy of patchouli oil against *E. coli*

| Groups | No. of mice | Drug[a] | Dose (g/kg/day)[a] | No. of survival | Survive rate (%) |
|---|---|---|---|---|---|
| Control | 10 | - | - | 10 | 100.00 |
| Model | 10 | - | - | 0 | 0.00 |
| Antibiotic | 10 | Cefotaxime | 2.00 | 10 | 100.00 |
| High dose | 10 | Patchouli oil | 0.49 | 10 | 100.00 |
| Mid dose | 10 | Patchouli oil | 0.25 | 6 | 60.00 |
| Low dose | 10 | Patchouli oil | 0.13 | 4 | 40.00 |

Table 4: *In vivo* efficacy of patchouli oil against *S. aureus*

| Groups | No. of mice | Drug[a] | Dose (g/kg/day)[a] | No. of survival | Survive rate (%) |
|---|---|---|---|---|---|
| Control | 10 | - | - | 10 | 100.0 |
| Model | 10 | - | - | 0 | 0.0 |
| Antibiotic | 10 | Cefepime | 2.00 | 10 | 100.0 |
| High dose | 10 | Patchouli oil | 0.49 | 10 | 100.0 |
| Mid dose | 10 | Patchouli oil | 0.25 | 10 | 100.0 |
| Low dose | 10 | Patchouli oil | 0.13 | 10 | 100.0 |

[a-]: Indicates that the mice in the group had been given to saline

effect on *E. coli* and *Enterobacter cloacae* (Table 1). The antibacterial activity of ethanol-water extract against Staphylococcus, *Kurthia gibsonii*, Streptococcus and Presumptive *Acinetobacter lwoffii* was stronger than that of ethanol extract ($p<0.05$, $p<0.01$) (Table 2). Water extract obtained by traditional decoction showed the lowest antibacterial activity having no activity on the majority of tested bacteria without activity against *E. coli* as earlier study (Luo, 2005). Therefore, *in vitro* antibacterial substance of *Pogostemon cablin* cultivated in Guangdong, China was mainly its oil obtained by steam distillation which showed potent and broad-spectrum *in vitro* antibacterial activity against both gram-positive isolates (Staphylococcus, Streptococcus, *Kurthia gibsonii*, *Macrococcus caseolyticus*, *bacillus*, etc.) and Gram-negative isolates (*E. coli*, *Enterobacter cloacae*, Presumptive *Acinetobacter lwoffii*, Pseudomonad, *Chrvseobacterium indologenes*, *Proteus vulgaris* and *Shigella flexneri*). But the effect of the oil on gram-positive isolates was higher than that on gram-negative isolates. Furthermore, in the earlier study, resistance analyses of 120 isolates on penicillin, tetracycline, erythromycin, streptomycin, gentamicin, cephalothin, cefotaxime, ciprofloxacin were analyzed. The results showed that 91 strains of bacteria had resistance to 8 experimental antibiotics in which 64.8% (59/91) isolates showed multi-drug resistance. While patchouli oil had the same activity against resistant isolates compared to sensitive isolates. Therefore, patchouli oil may be a significant antibacterial agent in controlling resistant pathogenic infections, especially multi-drug resistant pathogenic infections.

***In vivo* antibacterial activities of patchouli oil against *E. coli* and *S. aureus*:** Based on the strong activity *in vitro* of patchouli oil *in vivo* efficacy of patchouli oil from *P. cablin* cultivated in Guangdong, China against *S. aureus* or *E. coli* was firstly investigated with mouse systemic infection model in this study. Patchouli oil showed potent and broad-spectrum *in vivo* activity against both *E. coli* (Table 3) and *S. aureus* (Table 4). All mice infected with *S. aureus* or *E. coli* could survive if patchouli oil (0.49 g/kg/day) was administered intramuscularly for 6 days. The activity of patchouli oil against infection with *S. aureus* was stronger than that against *E. coli*. At different doses of high (0.49 g/kg/day), mid (0.26 g/kg/day) low (0.13 g/kg/day), the survival rates of mice infected with *S. aureus* or the survival rates of mice infected with *S. aureus* or *E. coli* were 100.0, 100.0, 60.0 and 40.0%, respectively. On the other hand, the survival rates of mice infected with *S. aureus* were all 100.0%. Its action against *E. coli* was dose-dependent. However, patchouli oil had higher *in vivo* anti *S. aureus* activity than that of anti *E. coli*. Furthermore, the tested *S. aureus* and *E. coli* were isolated from milk produced by dairy cow with clinical mastitis and resistant to antibiotics and it showed that patchouli oil had *in vivo* antibacterial actions against resistant bacteria. The *in vivo* effects of patchouli oil against bacteria were in accordance with its *in vitro* activities.

The result of preliminary test observed patchouli oil had weak therapeutical effect on mouse systemic infection model. But it had very good preventive effects on mouse systemic infection model. The reason of results may be slowly absorbed of patchouli oil. Patchouli oil showed the muscle stimulation and mucous membrane irritation on the animals at high concentration in the study. The use of patchouli oil at low concentration (1.5%) was free from side effects. However, owing to the methods of intramammary infusion and intramuscular injection used

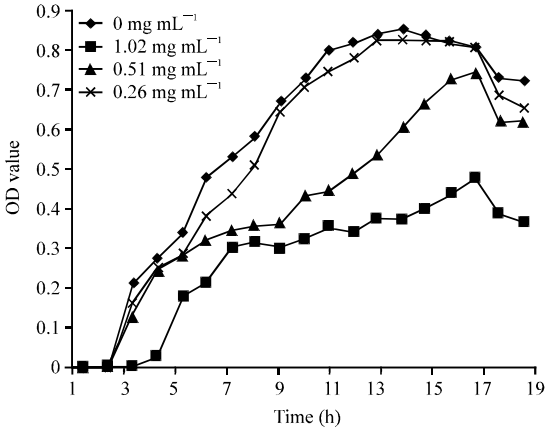


Fig. 1: Effect of different concentrations of patchouli oil on the growth of *E. coli* ATCC25922

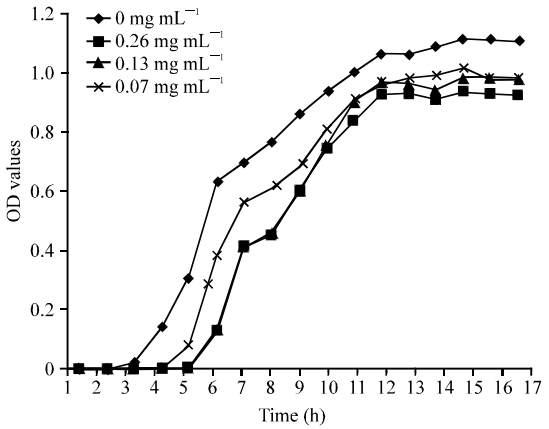


Fig. 2: Effect of different concentrations of patchouli oil on the growth of *S. aureus* ATCC25923

in treat mastitis of drugs, patchouli oil was administered intramuscularly. In fact, the subsequent researches showed that 1.5% patchouli oil was used in intramammary infusion and intramuscular injection, showed good therapeutical effect on the dairy cow clinical mastitis and low side effects at New Hope Demonstration Dairy Cow Farm (Hongya county, Sichuan province, China).

**Effect of patchouli oil on the growth of *E. coli* and *S. aureus*:** At different concentrations (1/2 MIC, 1/4 MIC, 1/8 MIC) of patchouli oil, the growth of *E. coli* and *S. aureus* could be inhibited. Their lag phase times of *E. coli* and *S. aureus* were distinctively prolonged at sub-MIC value of oil, the inhibitory actions were of concentration-dependent. The result was in accordance with its *in vivo* properties. Therefore, the essential oil from *P. cablin* may be a potential candidate as a therapeutic agent and can be further developed as a constituent for treating bovine mastitis.

The effect of patchouli oil on the growth of *E. coli* ATCC25922 was shown in Fig. 1 which showed an obvious concentration-dependent effect of patchouli oil on *E. coli*. At the 1/2 MIC (1.02 mg $mL^{-1}$) value for the condition, patchouli oil could obviously reduce the *E. coli* cell population compared with control sample. At the peak of log-phase bacteria, the OD value of untreated sample was about two times that of the oil-treated *E. coli* at the concentration of 1.02 mg $mL^{-1}$. The growth curve of *E. coli* at the concentration of 0.26 mg $mL^{-1}$ was almost in accordance with that of untreated group. Besides, patchouli oil could prolong the lag phase time of *E. coli* to 7 h at high concentration (1.02 mg $mL^{-1}$). However, the lag phase time of *E. coli* at lower concentration (0.51 or 0.26 mg $mL^{-1}$) was almost consistent with that of untreated *E. coli*. Effect of patchouli oil on the growth of *S. aureus* ATCC25923 was shown in Fig. 2. Besides the similar concentration-dependent effect on *S. aureus* to that found on *E. coli*, obvious differences were also observed in *S. aureus* after identical treatment. Compared with untreated group, *S. aureus* cell population only showed light decrease at different concentrations of patchouli oil. But patchouli oil could distinctly prolong all the lag phase time of *S. aureus* at high (0.26 mg $mL^{-1}$), mid (0.13 mg $mL^{-1}$), low (0.07 mg $mL^{-1}$) concentration to 11 h while the lag phase time of *S. aureus* oil-untreated was 6 h in control group.

**Morphological changes of *E. coli* and *S. aureus* after patchouli oil treatment:** TEM observations revealed some important ultrastructural changes in patchouli oil-treated cells of *E. coli* (Fig. 3) and *S. aureus* (Fig. 4) compared with untreated control. Figure 3 a showed the internal structure of untreated *E. coli* cell. It was clear that the cells showed unanimous electron density suggesting that the cells were in normal condition without environment disturbance. Significant morphological changes occurred in *E. coli* cells after the addition of patchouli oil. Figure 3a-c gave an overview of oil-treated cells while Fig. 3d-p shown separate single damaged cells of them. The cytoplasmic membrane showed several sites of disruption and appeared notably disturbed. A big gap existed between the cytoplasmic membrane and the cell wall of the oil-treated *E. coli* cells. Patchouli oil entered into the center of *E. coli* cell through damaged cytoplasmic membrane and the big gap. Further, patchouli oil could possibly destroy the cytoplasmic inclusion and DNA molecules of the cell. A remarkable electron-light region often appeared in the center of the *E. coli* cells treated with patchouli oil.

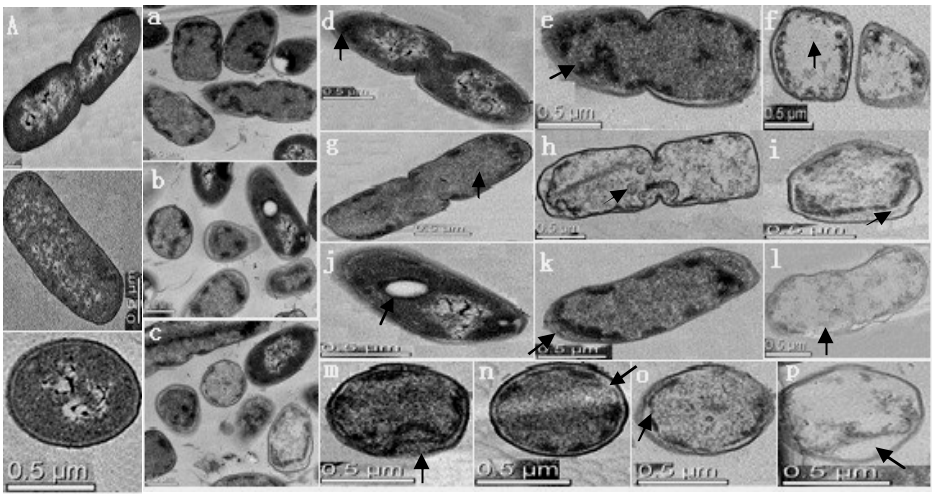

Fig. 3: Transmission electron micrographs of *E. coli* ATCC25922 treated with sub-MIC patchouli oil (1.02 mg $mL^{-1}$); a) Ultra-structure of the untreated *E. coli* cell; a-p) Ultrastructure changes of *E. coli* cell oil-treated; a-c) Overview of *E. coli* cell oil-treated; d, e, n and o) Damaged cytoplasm membrane (arrow); f and g) A remarkable electron-light region (arrow) in the center of the cell; h and j) Patchouli oil entered into the center of the cell through damaged membrane; i, n, o and p) A big gap between the cytoplasm membrane and the cell wall was formed (arrow). k-m) The cell wall was seriously damaged (arrow)

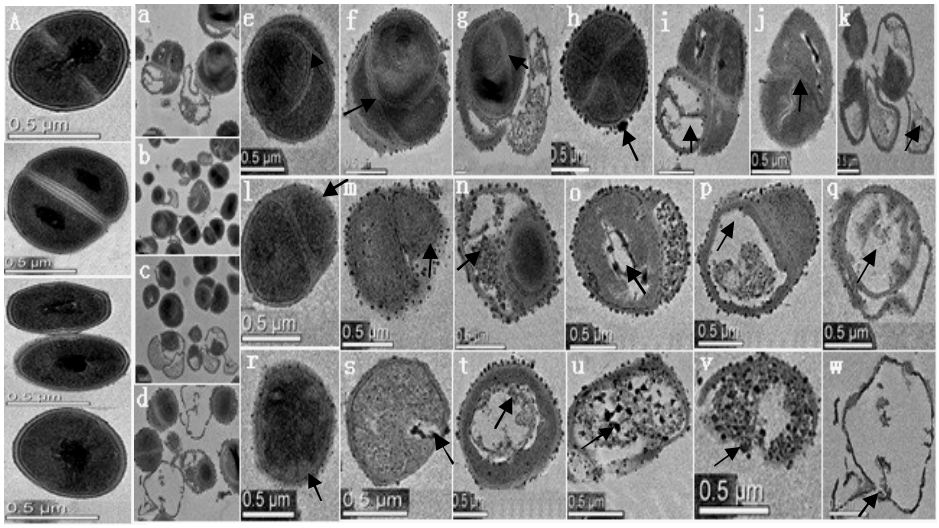

Fig. 4: Transmission electron micrographs of *S. aureus* ATCC25923 treated with sub-MIC patchouli oil (0.26 mg $mL^{-1}$); a) Internal structure of the untreated *S. aureus* cell; a-w) Ultrastructure changes of *S. aureus* cell oil-treated; a-d) Overview of *S. aureus* oil-treated cell; e-j) Abnormal division of *S. aureus*; m) Damaged cytoplasm membrane (arrow); u-w) Damaged cell wall (arrow); i, p, q, t and s) A remarkable electron-light region in the center of the cell; h, m, n, o, p and t) Electron-dense granule around the cell wall; u and v) A cell composed of a great amount of large electron-dense granule; k) Cell debris by abnormal division of the cell (arrow)

*S. aureus* is a typical gram-positive bacterium which has a thicker cell wall compared with *E. coli*, a typical Gram-negative bacterium. Besides the similar morphological changes between these two typical types of bacteria, For instance there was an electron-light region in the center of the treated cell and the cell

wall (Fig. 4u and v) and the cytoplasmic membrane (Fig. 4m) of the treated cell had been damaged to some degrees. Obvious differences were observed between *S. aureus* and *E. coli* after identical treatment. First, there were various cell division in *S. aureus* including quaternary fission, tertiary fission, binary fission and so on (Fig. 4e-j).

At the same time, an electron-light region occurred in the nuclear region of the *S. aureus* cell. From that researchers propose that patchouli oil may act on the DNA of the *S. aureus* cell and give rise to cell division abnormalities of *S. aureus*. Second, there were many electron-dense granules around the cell wall of *S. aureus* seen clearly in Fig. 4 h, m, n, o, p and t, the granules also, existed in the cytoplasm (Fig. 4u, v). But there were no electron-dense granules in the electron-light region. It was very interesting to observe that a large amount of electron-dense granules appeared to surround the electron-light region but not within the region. In contrast, no electron-dense granules were found in the control samples or in oil-treated *E. coli* cells.

It is suggested that the discrepancy in action between *S. aureus* and *E. coli* may result from different structure of the two typical bacteria. The structural diversity may contribute to dissimilar efficacy *in vivo* and *in vitro* of patchouli oil against both Gram-positive *S. aureus* and Gram-negative *E. coli*. It was reported that Gram-negative bacteria were more resistant to plant-based essential oils than Gram-negative bacteria (Smith-Palmer *et al*., 1998). According to the earlier antibacterial activity studies on the extracts of Traditional Chinese Medicines (TCM) (Dai *et al*., 2011), researchers found that Gram-positive isolates were more sensitive than Gram-negative isolates to extracts from TCM.

This study indicated that ethanol extract, ethanol-water extract and oil from *P. cablin* had higher antibacterial activities against Gram-positive isolates than those against Gram-negative isolates. It is also indicated that antibacterial substance of *P. cablin* may be liposoluble constituents. The hydrophilic cell wall structure of gram-negative bacteria is constituted of essentially a Lipopolysaccharide (LPS) that screens out the hydrophobic oil and avoids the accumulation of liposoluble constituents in the target cell membrane. This may be the reason that Gram-positive isolates were found to be more sensitive than Gram-negative bacteria. The presumption is in agreement with antibacterial properties of patchouli oil which had higher *in vitro* and *in vivo* antibacterial activity against Gram-positive *S. aureus* than against gram-negative *E. coli*.

## CONCLUSION

This study provides the adequate evidence indicating *in vitro* and *in vivo* antibacterial activities of patchouli oil from *P. cablin* against isolates from milk produced by dairy cow with mastitis. We suggest that essential oil from *P. cablin* was the antibacterial substance of *P. cablin* may be a potential candidate as a therapeutic agent and can be further developed as a constituent for antibacterial products, especially in treating bovine mastitis. Besides, the oil may be an antiseptic agent for preserving food.

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