© Med Sci Monit, 2011; 17(5): BR139-146
PMID: 21525802

Received: 2010.09.14
Accepted: 2010.10.31
Published: 2011.05.01

The inhibitory effect of *Zingiber corallinum* Hance essential oil on drug-resistant bacteria and evaluation of its acute toxicity

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Source of support: This work was supported by the Special Funds for Major State Basic Research Projects (2005CB522602), the Eleventh Five-Year Science and Technology Project of PLA (06G080), the Natural Science Foundation Project of CQ CSTC (2009BB5394) and Project Program of the Laboratory of Trauma, Burns and Combined Injury, 3rd Military Medical University (SKLZZ200908)

Summary

Background: The excessive and irregular use of antibiotics could result in the generation and diffusion of drug-resistant bacteria. The aim of this study was to investigate the inhibitory effect of *Zingiber corallinum* Hance essential oil (ZCHO) on drug-resistant bacteria, especially on drug-resistant *Acinetobacter baumannii*.

Material/Methods: Susceptibility testing was used to evaluate the effect of ZCHO on growth inhibition of drug-resistant bacteria by paper disk method. Mice orally administered with ZCHO were used to observe acute toxicity and to determine median lethal dose (LD_{50}) of ZCHO. Broth dilution method was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ZCHO on drug-resistant *Acinetobacter baumannii*.

Results: ZCHO exhibited an obvious inhibitory effect not only on gram-negative drug-resistant bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Acinetobacter baumannii*, but also on gram-positive drug-resistant bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. The ZCHO containing 79% terpinen-4-ol revealed better bacteriostatic effect than ZCHO with 34% terpinen-4-ol. The LD_{50} of ZCHO was 1790.427 mg/kg. The MIC and MBC of ZCHO on drug-resistant *Acinetobacter baumannii* were 1457.81 mg/L.

Conclusions: ZCHO has obvious bacteriostasis and bactericidal effects, especially against drug-resistant *Acinetobacter baumannii*. Therefore, ZCHO is a promising natural bioactive component with antibacterial effect and satisfactory safety due to its low toxicity.

key words: ***Zingiber corallinum* Hance essential oil • Terpinen-4-ol • LD_{50} • drug-resistant bacteria**

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=881760
Word count: 2787
Tables: 6
Figures: 2
References: 54
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Background

Antibiotics have played a vital role in controlling infectious diseases. Unfortunately, the excessive and irregular use of antibiotics can result in the generation and diffusion of drug-resistant bacteria [1,2]. Increasing difficulties in clinical therapy for infected patients have been observed due to the diminishing therapeutic effect of antibiotics. Currently, drug resistance to bacteria has become an important research focus. Previous studies confirmed that natural product drugs not only provided extensive antibacterial activities, but also avoided the generation of drug-resistant bacteria [3,4]. Therefore, natural product drugs have received tremendous attention.

Zingiber corallinum Hance, also referred to as Yin Jiang or Jab bangx hnaib diel, is a Chinese herbal plant in the ginger family and ginger group (Figure 1). It has anti-inflammatory, detoxifying and anti-bacterial functions [5,6]. As a traditional perennial herb native to Yunnan, Guangxi, Guangdong and Guizhou areas, *Zingiber corallinum* Hance has been used for catarrhal rhinitis, cough, lumbago and diarrhea with satisfactory therapeutic effects [7]. The bioactive components in *Zingiber corallinum* Hance are aetherolea and coral ginger essential oil, and *Zingiber corallinum* Hance essential oil (ZCHO) that has obvious antibacterial activity [8,9]. Through supercritical carbon dioxide extraction, the essential oil was isolated from *Zingiber corallinum* Hance and identified by gas chromatography-mass spectroscopy [10]. The major bioactive components among 60 compounds that were identified are terpinen-4-ol, sabinene, β-bisabolene and γ-terpinene. ZCHO has attracted extensive attention due to its antibiosis property. Previous reports described its inhibitory effect on fungi and some non-drug-resistant pathogenic bacteria [11]; however, there have been few reports on its role in controlling drug-resistant pathogenic bacteria.

Acinetobacter baumannii is an important pathogen for hospital onset of infections (HOI) [12,13]. Intensive care unit (ICU) patients are at increased risk of infection with multi-drug-resistant *A. baumannii* [14–16]. In the last decade non-fermentable sugar gram-negative bacilli have become a common nosocomial infection. Due to extensive use of broad-spectrum antibiotics, infection with multi-drug-resistant stato-bacillus has significantly increased. Recently, the infection of multi-drug-resistant *A. baumannii* has resulted in an explosive increase in prevalence in many places around the world, including Europe, North America, Argentina, Brazil, Taiwan, Hong Kong, Japan and Korea [17–22]. Therefore, infection with this bacterium could easily become epidemic in some locations due to the shortage of effective antibiotics.

Based on previous observations, we chose to further explore the inhibitory effect of ZCHO on common drug-resistant bacteria, especially on drug-resistant *A. baumannii*. The aim of the present study was to: (1) To investigate the bacteriostasis effect of ZCHO on common drug-resistant bacteria during clinical treatments; (2) To evaluate the acute toxicity of ZCHO for ensuring its safety in clinical practice; (3) To further examine its bacteriostasis and bactericidal effects on drug-resistant *A. baumannii*, an important nosocomial pathogen. We first used the paper disk method investigate the bacteriostasic effect of ZCHO on common drug-resistant bacteria, as well as to explore the major components of ZCHO for bacteriostasis effect. We also examined the acute toxicity of ZCHO by using modified Karber's method to determine median lethal dose (LD_{50}) in mice. Moreover, the broth dilution method was conducted to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ZCHO on drug-resistant *A. baumannii*. Therefore, this study will provide new experimental evidence to facilitate the development of effective antibacterial drugs for controlling common drug-resistant bacteria.

Figure 1. *Zingiber corallinum* Hance.

Material and Methods

Bacterial isolation

The clinical isolates from Daping Hospital, the Third Military Medical University, P. R. China, were used to examine the resistance to antibiotics (Table 1). The isolates included 3 gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus haemolyticus*) and 5 gram-negative bacteria (*Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii*). These bacteria were examined under a microscope and identified through physiological and biochemical methods [23,24]. The identified bacteria were stored at –80°C for future experiments.

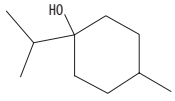
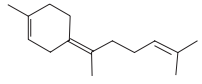
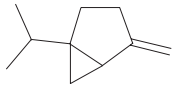
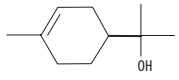
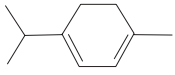
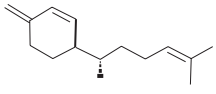
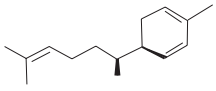
ZCHO

ZCHO was provided by the Chongqing Institute of Daily-used Chemical Industry, P. R. China. All batches complied with the International Organization for Standardization (ISO) 9001. The major bioactive component in ZCHO was determined by gas chromatography-mass spectrometry at Chongqing Institute of Daily-used Chemical Industry (Table 2). Two kinds of ZCHO are ZCHO standard essential oil with 34% terpinen-4-ol and concentrated ZCHO essential oil with 79% terpinen-4-ol.

Table 1. The types of bacteria with non-susceptive antibiotic spectrum.

Bacteria type	Gram type	Non-susceptive antibiotics
Escherichia coli	G–	penicillins • cephalosporins • aztreonam
Enterobacter cloacae	G–	amikacin • ampicillin • sulbactam • aztreonam • cephalosporins • ciprofloxacin • gentamycin • Levofloxacin • furantoin • piperacillin • tenebrimycin
Klebsiella pneumoniae	G–	penicillins • cephalosporins • aztreonam
Pseudomonas aeruginosa	G–	amikacin • cefotaxim • ceftizoxime • ceftriaxone • ciprofloxacin • gentamycin • levofloxacin • piperacill • ambramycin • bactrim • ticarcillin • tobramycin
Acinetobacter baumannii	G–	aztreonam • cephalosporins • gentamycin • piperacillin • ambramycin • bactrim • ticarcillin • tobramycin
Staphylococcus aureus	G+	penicillins • βlactam/β-lactam inhibitor complex • cephalosporins • carbapenem antibiotics
Staphylococcus epidermidis	G+	penicillins • βlactam/β-lactam inhibitor complex • cephalosporins • carbapenem antibiotics
Staphylococcus haemolyticus	G+	penicillins • βlactam/β-lactam inhibitor complex • cephalosporins • carbapenem antibiotics

Table 2. The major constituents of *Zingiber corallinum* Hance essential oil.

Chemical name	Molecular formula	Peak area(%)	Structure formula
terpinen-4-ol	$C_{10}H_{18}O$	>30.00	
β-bisabolene	$C_{15}H_{24}$	21.00	
sabinene	$C_{10}H_{16}$	10.55	
α-terpineol	$C_{10}H_{18}O$	2.88	
α-terpinene	$C_{10}H_{16}$	0.71	
β-Sesquiphellandrene	$C_{15}H_{24}$	0.57	
zingiberence	$C_{15}H_{24}$	0.19	

Preparation of bacterial fluid

According to NCCLS guidelines [25], the isolated bacteria were inoculated in Luria-Bertani broth (LB; Oxoid, Basingstoke, UK) and grown at 37°C overnight. The grown bacteria was adjusted to a 0.5 McFarland standard in broth according to turbidity on the turbidimeter (Marcy-I'Etoile, France), and a further dilution was made by adding 100 µL of bacteria in glass flasks containing 10 mL of Mueller Hinton (MH) broth. The final concentration of bacteria in the flasks was 5×10^5 CFU/mL. The bacterial isolates were cultured at 37°C in glass flasks with shaking of 150 revolutions per minute (rpm) for 240 min.

Experimental animals and grouping

Healthy C57 adult mice with body weight of 18–22 g were provided by the experimental animal center of Daping Hospital (SCXK(yu)2007017), at the Third Military Medical University. The mice were kept in a specific pathogen-free room with free access to standard laboratory food and water under the condition of controlled temperature, humidity and lighting (12-hour light-dark cycles). The mice were randomly divided into 9 groups, with 10 mice in each group, designated as 1 negative control group and 8 experimental groups treated with ZCHO at various concentrations.

Table 3. The diameters of bacteriostasis rings after *Zingiber corallinum* Hance essential oil treatment (mm, $\bar{x}\pm SD$).

Group	Gram-negative drug-resistant bacteria					Gram-positive drug-resistant bacteria		
	Escherichia coli	*Enterobacter cloacae*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*
1	31.00±10.77*,**	32.50±9.38*,**	24.71±6.18*,**	23.13±4.26*,**	7.30±0.67*,**	19.43±4.08*,#	15.00±3.74*,#	13.00±2.19*,#
2	14.40±3.85**	11.92±2.15**	12.64±2.63**	14.00±4.47**	0	10.86±5.76#	10.25±0.96#	4.83±3.78##
3	0	0	0	0	0	–	–	–
4	–	–	–	–	–	0	0	0

* $P<0.05$ *vs.* the group 2; ** $P<0.01$ *vs.* the group 3; # $P<0.05$; ## $P<0.01$ *vs.* the group 4.

All experiments were carried out according to the guidelines from the Third Military Medical University Animal Research Committee. These guidelines include minimizing the number of animals for experiments and minimizing the suffering for animals.

Concentration screening

The optimal concentration of ZCHO containing 79% terpinen-4-ol was screened from the range of 5–40%. The dilution ratio of concentration between adjacent groups was designed to be 0.769 according to modified Karber's method [26]. Polyoxyethylene sorbitan monolaurate (Tween-20) was added to all ZCHO dilutions in a final concentration of 1% (v/v) for enhancing the solubility of ZCHO. The stock ZCHO was diluted to a final concentration (v/v) of 6.5%, 8.4%, 11.0%, 14.3%, 18.6%, 24.1%, 31.4% and 40.0% using physiological saline.

Susceptibility test using paper disk

Susceptibility was determined by use of standard paper disks including ampicillin paper disk for gram-negative bacteria control, penicillin paper disk for gram-positive bacteria control and blank paper disk (Oxoid; Basingstoke, UK). The bacteria fluid was dipped with sterile swab paper to spread on Mueller Hinton agar (MHA) plates in duplicate. After drying for 5 minutes, the paper disks were tightly pasted on the surface of MHA plates. The distance between the centers of paper disks was more than 24 mm. The distance between the center of paper disks and the inner margin of plates was more than 15 mm. Finally, the plates with various concentrations were incubated at 35°C for 24 h.

Acute toxicity test

All mice were administered with ZCHO (0.01 ml/g) at various concentrations by oral gavage to determine median lethal dose (LD_{50}) [27]. During the 2-week observation period, the dead mice were subjected to immediate biopsy to analyze the acute toxicity of ZCHO. The tissues of lungs, livers, intestine and kidneys were fixed overnight in 4% paraformaldehyde solution. The samples were progressively dehydrated with ethanol and dimethylbenzene and then embedded with paraffin. Hematoxylin and eosin (HE) staining was used to examine histopathological changes in these tissues.

MIC and MBC determination

MICs were determined using serial dilution methods as previously described [28]. Tween-20 at a final concentration of 1% (v/v) was added in all dilutions to enhance the solubility of ZCHO. One milliliter of *A. baumannii* fluid was inoculated in each group. ZCHO was added to culture flasks at final concentrations of 10%, 5%, 2.5%, 1.25%, 0.625%, 0.3125%, 0.156% and 0.078% (v/v). One milliliter of *A. baumannii* fluid and 1 ml of LB were added to the positive control flask. In the blank control flask, 1 ml of *A. baumannii* fluid and 1 ml of Tween-80 were added. After mixing thoroughly, these flasks were shaken at 35°C, 150 rpm for 16 h. Since the tested samples with milliliter belong to emulsion. The dilution of multiple proportions still could not discriminate the growth of bacteria. Hence, the other inoculation of varying concentrations of cultures was used. Plates were incubated at 37°C and MICs were determined at 24 h. The MICs were defined as the lowest concentration of ZCHO that resulted in the inhibition of visible growth (colonies on a plate) under standard conditions.

MBCs determination was performed in duplicate using 100 µl of the above-mentioned *A. baumannii* fluid and MHA plates according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines [25]. The plates containing ZCHO at various concentrations were incubated at 37°C and MBCs were determined at 24 h. The MBCs were the lowest concentration of ZCHO that could kill 99.9% of the original inoculums in a given time. The number of colonies should be less than 5. All broth dilution tests were performed at least twice.

Statistical analysis

The data are presented as mean ± standard deviation (SD). Student's t test was used to calculate statistical difference between groups. The reforming Karber's method was used to calculate the LD50 and 95% confidence interval. The statistical level was set at 95%. All statistical calculations were performed using the SPSS13.0 software for Windows.

Results

ZCHO inhibits the growth of drug-resistant bacteria

ZCHO resulted in an obvious bacteriostasis ring for gram-negative drug-resistant bacteria such as *E. coli, E. cloacae, K. pneumoniae* and *A. baumannii,* as well as gram-positive drug-resistant bacteria including *S. aureus, S. epidermidis*

Table 4. The distribution of *Zingiber corallinum* Hance essential oil with various concentrations.

Group	Concentration (%)	Dosage (mg/kg)
Negative control	0	0
A	40.0	3732.00
B	31.4	2929.62
C	24.1	2248.53
D	18.6	1735.38
E	14.3	1334.19
F	11.0	1026.30
G	8.4	783.72
H	6.5	606.45

ring in the group treated with ZCHO with 79% terpinen-4-ol was larger ($P<0.05$) (Table 3).

The acute toxicity of ZCHO

The mortality of mice was increased due to the increase of ZCHO concentration in experimental groups. Their mortality reached 100% when ZCHO concentration was 40%; however, all mice in the negative control group survived (Tables 4 and 5).

The mice in the groups treated with ZCHO at the dosages of 3732.00, 2929.62 and 2248.53 mg/kg were lethargic, with tachypnea, laryngeal stridor, and decreased vigor. Instability of gait, muscle trembling and ataxia were also observed within 15 min after the administration of ZCHO, and these mice finally became comatose and died. Nearly 33% of mice died within 4 h, while others died within 3 days after the administration of ZCHO. The mice treated with ZCHO at the dosages of 1735.38, 1334.19 and 1026.30 mg/kg showed tachypnea and laryngeal stridor at the same time. Some of them recovered within 12 h and others in these groups died within 8–48 h. In general, all animals survived after the fourth day. The mice in groups treated with ZCHO at the low dosage and the control groups survived without abnormal symptoms (Table 6).

Table 5. The mortality frequency distribution of mice treated with *Zingiber corallinum* Hance essential oil with various dosages (n=10).

Group	Dosage (mg/kg)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A	3732.00	6	3	1	0	0	0	0
B	2929.62	6	1	0	0	0	0	0
C	2248.53	5	3	0	0	0	0	0
D	1735.38	4	1	0	0	0	0	0
E	1334.19	1	0	0	0	0	0	0
F	1026.30	2	0	0	0	0	0	0
G	783.72	1	0	0	0	0	0	0
H	606.45	0	0	0	0	0	0	0

Table 6. The mortality of mice in acute toxicity test (n=10).

Group	Dosage (mg/kg)	Mortality time point (h)	Mortality number	Mortality (%)	Survival (%)
Negative control	0	–	0	0	100
A	3732.00	1	10	100	0
B	2929.62	3	7	70	30
C	2248.53	2	8	80	20
D	1735.38	8	5	50	50
E	1334.19	11	1	10	90
F	1026.30	0.5	2	20	80
G	783.72	12	1	10	90
H	606.45	–	0	0	100

and *S. haemolyticus* ($P<0.05$). However, its inhibitory effect on *P. aeruginosa* was not obvious and no bacteriostasis ring in the group treated with ZCHO containing 34% terpinen-4-ol was observed. Compared with the treatment of ZCHO containing 34% terpinen-4-ol, the diameter of bacteriostasis

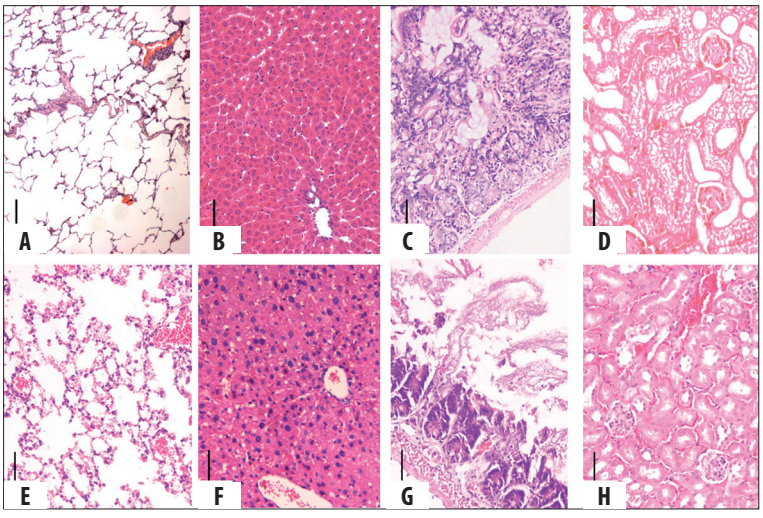


Figure 2. Pathological changes of different tissues from mice administered with *Zingiber corallinum* Hance oil. (**A–D**) representative tissues in the control group, (**A**) lung tissue, (**B**) liver tissue, (**C**) intestinal tract tissue, (**D**) kidney tissue. (**E–H**) representative tissues in experimental groups, (**E**) lung tissue, (**F**) liver tissue, (**G**) intestinal tract tissue, (**H**) kidney tissue. (HE staining, ×100).

LD_{50} and 95% confidence interval

The LD_{50} of ZCHO was 1790.427 mg/kg. The 95% confidence interval was between 1629.018 and 1951.836 mg/kg.

The pathological analysis

Through histopathological examination, toxicity lesions in mice treated with ZCHO at high dosage of 2248.53 mg/kg were mainly observed in lung, liver, intestine and kidney tissues. The alveolar space of dead mice had obvious pink fluid and hemorrhage. The alignment of hepatocytes exhibited a slight derangement, with fuzziness of the hepatic cord, sporadic punctiform and lamellar necrosis. A large number of intestinal epithelial cells had degeneration and necrosis. The integrity of villi was destroyed (Figure 2); however, no damage to other organs was observed.

MIC of ZCHO on drug-resistant *Acinetobacter baumannii*

Multiple proportion microdilution assays of ZCHO showed that the bacteria fluid in flasks at the concentrations of 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313% and 0.156% was clear, but the bacteria fluid in flasks at the concentration of 0.078% was cloudy. Since the density of ZCHO was 0.933 g/mL, the MIC for drug-resistant *A. baumannii* was 1457.81 mg/L.

MBC of ZCHO on drug-resistant *Acinetobacter baumannii*

Multiple proportion microdilution assays of ZCHO showed that 10% ZCHO did not result in colony formation. Sporadic colonies less than 3 in number were occasionally observed in the plates with ZCHO at the concentrations of 5%, 2.5%, 1.25%, 0.625%, 0.313% and 0.156%; however, more than 3 colonies were observed in the plates containing ZCHO at the concentration of 0.078%. Therefore, the MBC of ZCHO on drug-resistant *A. baumannii* was 1457.81 mg/L.

Discussion

In the present study, clinical isolates were used to explore the role of ZCHO in inhibiting drug-resistant bacteria, and the acute toxicity of ZCHO was also evaluated in mice. The results revealed that ZCHO had significant bacteriostasis and bactericidal effects with a low toxicity, especially for drug-resistant *A. baumannii*. Terpinen-4-ol, a major antibacterial component, made a critical contribution to the treatment of infectious diseases, with satisfactory safety due to its excellent antibacterial capability and low toxicity [4].

Currently, although HOI has attracted tremendous attention, and multiple strategies in management and monitoring of HOI have been significantly improved, the incidence of HOI continues to increase year by year [29,30]. According to statistical analysis of clinical isolates from infected patients, 13% of infections were mixed. In addition, among infections from a single bacterium variant, gram-positive and gram-negative infections were 65% and 25%, respectively. Fungi caused 9.5% of infections [31,32]. Increasing evidence also suggests that the infection caused by gram-negative bacteria was the principal factor for morbidity and mortality [33–36]. These bacteria invade the human body through ventilation and surgical equipment, and invasive cannula such as deep vein, intra-urethral and gastric canal. Therefore, HOI incidence reached 9.1% in clinical patients receiving treatments using invasive equipment for chronic or deterioration status [37]. Some patients also suffered from multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF), the leading cause of death in ICUs [38–41]. Since the prophylaxis and treatment of HOI mainly depends on antibiotics, the extensive and irregular use of antibiotics can result in the increase and diffusion of drug-resistant bacteria, which attenuates therapeutic effects of antibiotics [42]. Hence, the screening of novel and effective anti-bacterial drugs to drug-resistant bacteria is one of the most compelling medical research topics. Natural product drugs such as ZCHO, tea tree essential oil and others have gained extensive attention. These drugs have not only demonstrated robust anti-microbial activity, but also exhibited low possibility of drug resistance.

In clinical practice, ZCHO has been extensively used as an antibacterial and antimycotic drug for dermatological diseases including tinea corporis, tinea cruris and beatle acne [43]. Previous studies only reported the inhibitory effect of ZCHO on fungi and non-drug resistant bacteria, except for penicillin-fast *S. aureus* [44]. However, our results indicate that ZCHO exhibits a clear inhibitory effect on both

gram-negative and gram-positive drug-resistant bacteria such as *E. coli*, *K. pneumoniae*, *E. cloacae*, *S. aureus*, *S. epidermidis* and *S. haemolyticus*.

In addition, *A. baumannii* is widely distributed in water, soil, skin and hospital environmental surfaces as the major pathogenic bacteria in hospital-acquired infections [45]. This multi-drug-resistant stato-bacillus may spread throughout an entire city, or between districts and nations. It has also become a dangerous pathogenic bacterium in war wound-related infections. Soldiers infected with *A. baumannii* have attracted extensive attention in the Iraq and Afghanistan wars [46–48]. Our results also demonstrated that ZCHO played an important role in bacteriostasis and sterilization on multi-drug resistant *A. baumannii*.

The possible bacteriostasis and bactericidal mechanisms of ZCHO are described as follows. First, ZCHO may damage the plasma membrane to enhance its permeability. The successful infiltration of ZCHO to microorganisms can result in disruption of organelle and nucleus [49,50]. Second, bacterial enzymatic systems may also be impaired by ZCHO [51]. Third, interaction between ZCHO and plasma membrane can interrupt respiratory chain activity and energy production [52]. The intrinsic resistance of *P. aeruginosa* may result from the lack of pharmacological targets or failure of binding to active sites [53]; therefore, low sensitivity of ZCHO on *P. aeruginosa* may be due to its intrinsic resistance. In addition, tea tree essential oil also contains monoterpene alcohol and terpinen-4-ol for executing anti-microbial activity [54].

Conclusions

ZCHO is a low toxicity drug with obvious bacteriostasis and bactericidal effects, especially for drug-resistant *A. baumannii*. Terpinen-4-ol is the major antibacterial component of ZCHO. Therefore, ZCHO is a promising natural bioactive drug with satisfactory safety and antibacterial effect, especially for the bacteriostasis on drug-resistant *A. baumannii*. Moreover, due to technological improvement in the isolation and purification of bioactive components in Chinese herbal medicines, more novel and effective formulations containing ZCHO will be developed, which will further increase the antibacterial capability due to its high purity and low toxicity. Therefore, ZCHO should have promising clinical application value in the future.

Acknowledgments

The authors thank Ms. Li Cui for her technical assistance.

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