

***Litsea cubeba* Essential Oil: Component Analysis, Anti-*Candida albicans* Activity and Mechanism Based on Molecular Docking**

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Abstract: The antifungal mechanism of plant essential oil has always been a concern in the agriculture and forestry science field. In this investigation, besides the evaluation of inhibitory activities of twenty-three essential oils against *Candida albicans* *in vitro*, identification and quantification of the chemical composition of *Litsea cubeba* essential oil by gas chromatography-mass spectrometry were investigated. Further development, we assessed the mechanism of *L. cubeba* essential oil against *C. albicans* by molecular docking. *Litsea cubeba* essential oil displayed the strongest inhibitory activity among these oils and the diameter of the circle against *C. albicans* was more than 50 mm. Maximum three components were identified with *trans*-citral (33.6%), *cis*-citral (30.3%), *d*-limonene (8.2%). Secretory aspartate protease (SAP5) and β -1,3-glucan synthase (β -1,3-GS) are two key enzyme proteins that inhibit the growth of *C. albicans*. Molecular docking studies reveal chemical binding forces of *cis*-citral, *trans*-citral and *d*-limonene to SAP5 are -21.76 kJ/mol, -22.18 kJ/mol and -24.27 kJ/mol, to β -1,3-GS are -23.01 kJ/mol, -25.52 kJ/mol and -23.85 kJ/mol, respectively. The most preferable binding mechanism was observed against SAP5 and β -1,3-GS due to hydrophobic interaction, as well as hydrogen bonding between citral molecules. The research results suggest the mechanism of chemical components in *L. cubeba* essential oil inhibits the growth of *C. albicans*, which provides a reference to the development and utilization of essential oil.

Key words: *L. cubeba* essential oil, *C. albicans*, molecular docking, chemical mechanism

1 Introduction

Essential oils are aromatic and volatile liquids that are extracted from plant tissues by steam distillation. They are composed of aliphatic compounds, aromatic compounds and terpenoids¹⁾. Most essential oils have certain inhibitory effects on microorganisms²⁾, so they are widely used in spices, food, chemical industry and other fields.

L. cubeba, is one of the unique aromatic plants in China, deciduous shrubs or small trees, with high appreciation value and application value. The *L. cubeba* essential oil generally refers to the essential oil distilled from *L. cubeba* fruit, with *trans*-citral, *cis*-citral, *d*-limonene other chemical components³⁾. The *L. cubeba* essential oil can against *V. parahaemolyticus*, *L. monocytogenes*, *L. plantarum*, and *H. anomala*⁴⁾, *Botrytis cinerea*, a fungus that causes the putrefaction of fruits and vegetables⁵⁾. Furthermore, *L.*

cubeba essential oil complex based on β -cyclodextrin can control the growth of *Penicillium italicum*, *Penicillium digitatum*, and *Geotrichum citri-aurantii* isolated from postharvest citrus⁶⁾. However, the antimicrobial mechanism of *L. cubeba* essential oil is mostly developed from the perspective of molecular biology and bioinformatics^{7,8)}, and not much from the chemical perspective.

Fungi are microorganisms that have long existed in nature and some fungi can become important pathogens when the body's immune system is reduced. For example, infections caused by *C. albicans* are prone to occur in severely immunocompromised patients due to cancer, AIDS, and organ trans-plantation⁹⁾. Therefore, screening antifungal chemical components from plant essential oils is a well-established idea.

Cell walls of fungi are mainly composed of polysaccha-

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rides, accounting for 90% of the dry weight. Inhibiting cell wall synthesis is an ideal target for screening antifungal components, for example, β -1,3-glucan synthase (β -1,3-GS) is the key enzyme for cell wall synthesis in *C. albicans*¹⁰. In addition, the toxicity of fungi is closely related to some enzymes of own. For example, the secretory aspartate protease (SAP) family is an important source of extracellular enzymes for *C. albicans*, some of which are associated with virulence, and SAP5 is a key enzyme for the inhibition of *C. albicans* infection¹¹.

Molecular docking is a computational chemistry method that focuses on intermolecular interactions and predicts affinity based on intermolecular binding energy¹². The molecules of essential oils can be docked to key enzyme protein molecules of fungi, thereby elucidating the chemical mechanisms that essential oils inhibit fungi.

2 Materials and Methods

2.1 Materials

C. albicans strain number (ATCC0231) was purchased from Shanghai Conservation Biotechnology Center. The 23 essential oils were provided by Hunan Nominxin Bioengineering Co., Ltd.: *L. cubeba*, *Panax quiquefolium*, *Centella asiatica*, *Houttuynia cordata*, *Angelicae sinensis radix*, *Rosemary officinalis*, *Alpiniae oxyphyllae fructus*, *Panax ginseng*, *Schisandrae chinensis fructus*, *Zanthoxylum bungeanum Maxim*, *Cuminum cyminum*, *Forsythiae fuctus*, *Artemisia argyi Levl*, *Pogostemonis herba*, *Atractylodis rhizoma*, *Caryophylli flos*, *Pericarpium citri reticulatae*, *Magnoliae biondii immaturus rlos*, *Schizonepetae herba*, *Olibanum*, *Schizonepetae spica*, *Chuanxiong rhizoma* and *Acori tatarinowii rhizoma*. All of essential oils were prepared by steam distillation. Sabourauds dextrose agar (SDA) medium, Sabouraud liquid dextrose (SLD) medium were purchased from Suzhou Ha-bour biotech Co., Ltd (Jiangsu, China). Nystatin was obtained from Chengdu Alfa Biotechnology Co., Ltd (Sichuan, China). Sterile saline was purchased from Sichuan Kelun pharmaceutical Co., Ltd (Sichuan, China). All other conventional reagents were analytically pure.

2.2 Methods

2.2.1 Essential oils anti-*C. albicans* activity

Original powder of *C. albicans* strain was put into liquid Sabouraud medium, grown at 120 r/min and 28°C for 24~48 h by a thermostatic shaking incubator. An appropriate amount of *C. albicans* cells solution was transferred to a fresh SDA medium, incubated at 27°C for 24~48 h. To the mixture were added a loopful of this overnight growth and 1 mL of SLD medium under gently blowing several times by pipette gun and shaking well. After diluted with sterile

saline, the resulting solution was adjusted to 1×10^6 CFU/mL by Maier's turbidity tube.

In order to increase the solubility of essential oil, Dimethyl sulfoxide (DMSO) (1.0 mg/L) solution was used to prepare essential oil solutions, at the same time as a negative control. Essential oil and DMSO solution were mixed at a ratio of 95:5 as initial test solution, then diluted 10 and 100 times, respectively. Nystatin was used as a positive control for it is a drug to treat *C. albicans* infection¹³. Prepared Concentration: 50 mg/L, 100 mg/L and 200 mg/L.

C. albicans solution (1×10^6 CFU/mL) was evenly coated on SDA medium. Each medium plate was divided into four parts, each part made four holes evenly and vertically with a 100 μ L pipette tip. Test solution with different gradient concentrations and negative control solution 20 μ L were added into holes, respectively.

After the test solutions were diffused by standing for 20 min, the medium plate was sealed and incubated at 28°C for 24~48 h. The diameter of the inhibition circle was measured with a high-precision vernier caliper, repeated three times and took the average value.

According to "Performance Standards for Antimicrobial Susceptibility Testing" that was issued by the Clinical and Laboratory Standards Institute (CLSI): The diameter of the inhibition circle >20 mm is for high sensitivity, 10~20 mm for moderate sensitivity, 5~10 mm for low sensitivity, ≤ 5 mm for no inhibition effect.

2.2.2 Gas chromatography-mass spectrometry analysis of *L. cubeba* essential oil

The components analysis of *L. cubeba* essential oil was carried out using a GC-MS system (Intuvo 9000GC-QQQ-7000D, Agilent Technologies, Santa Clara, CA, USA), which composed of a gas chromatograph equipped with a DB-5MS column (20 m $\times$ 180 μ m, 0.18 μ m film thickness) and a mass spectrometer operated in electron ionization mode (70 eV).

The essential oil was diluted from 5 μ L to 1 mL by acetonitrile and the diluent (1 μ L) was injected into the GC column at 220°C with a split ratio of 10:1. For detection and analysis, the oven temperature was kept isothermally at 50°C for 2 min, then increased from 50°C to 80°C at a rate of 3°C/min and hold for 2 min, followed by an additional rise to 180°C at a rate of 5°C/min for staying 1 min. The next was increased to 230°C at a rate of 10°C/min and kept for 5 min, followed rise to 250°C at a rate of 20°C/min and maintained for 3 min. The flow rate of helium gas was 1 mL/min. The interface temperature, auxiliary heater temperature, ion source temperature and m/z scan range were 250°C, 280°C, 230°C and 40~400, respectively. The individual peak of essential oil was identified by comparing the components with those in literature, the mass spectra fragmentation patterns with those from the database (NIST17.L standard spectral library) and with those from Chemical Abstracts Service (CAS). The relative content of each component was calculated quantitatively according to

the peak area normalization method.

2.2.3 Molecular docking

As ligand molecules, *cis*-citral, *trans*-citral, *d*-limonene, and nystatin were modeled by the RDKit program, meanwhile, they were applied to optimize the lowest energy conformations with energy minimization modules, respectively. The structures are shown in Fig. 1. As receptor molecules, the 3D structure files of SAP5 (PDB ID: 2QZX) and β -1,3-GS (PDB ID: 4M80) were downloaded from the RSCB PDB database (<https://www.rcsb.org/>), shown in Fig. 2.

All water molecules and the original ligand in β -1,3-GS and SAP5 were removed by Auto Dock Tools 1.5.6 software, and polar hydrogen atoms were added and Gasteiger charges were calculated. *Cis*-citral, *trans*-citral, *d*-limonene, and nystatin were added with atomic charges to assign atomic types, and all the flexible bonds were rotatable by default.

Molecular docking was performed by Auto Dock Vina 1.1.2., after the active site and active pocket were determined according to the coordinates of the original ligand in

the crystal protein complex. The ligand and receptor were set to be flexible and rigid, respectively. The active center of SAP5 Gridbox was set to $x = 13.969$, $y = 38.141$, $z = 29.748$; size was set to $x = 60$, $y = 60$, $z = 60$; spaces = 1. The active center of β -1,3-GS Gridbox was set to $x = 1.41$, $y = 77.924$, $z = 6.971$; size was set to $x = 60$, $y = 60$, $z = 60$; spaces = 1. The search exhaustion precision was set to 32, the number mode was set to 5, other parameters defaulted, and the diagrammatic sketch of interaction was made using Ligplot+¹⁴⁾.

3 Results and Discussion

3.1 Screening results about essential oils anti-*C. albicans* activity

The screening results of inhibitory activity of essential oils on *C. albicans* are shown in Table 1. *L. cubeba* essential oil and *Cuminum cyminum* essential oil exhibit strong inhibitory activity on *C. albicans*, with both of the

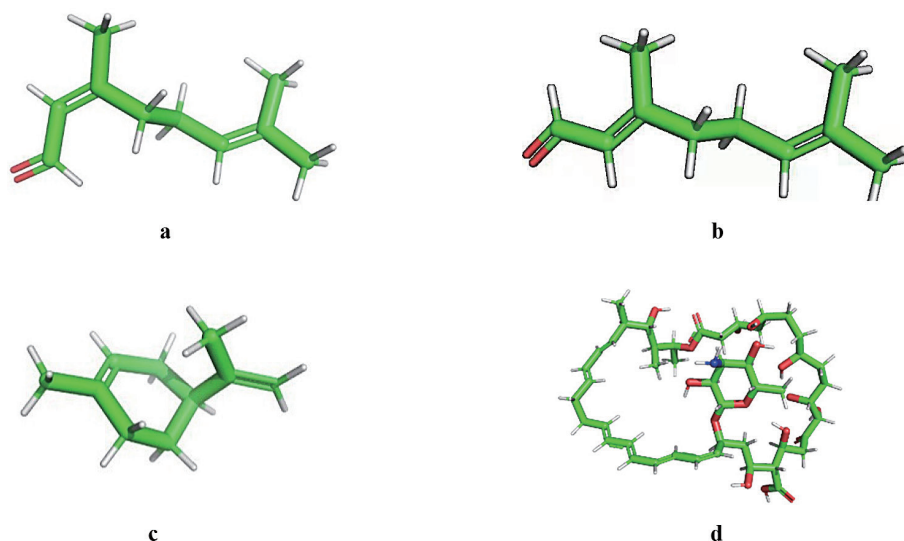


Fig. 1 Structure of (a) *cis*-citral, (b) *trans*-citral, (c) *d*-limonene, and (d) nystatin.

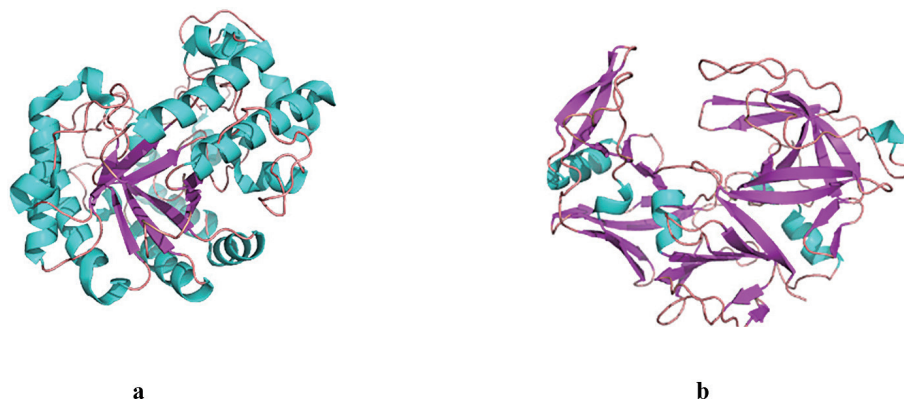


Fig. 2 Structure of (a) SAP5 and (b) β -1,3-GS.

Table 1 Results of plant essential oils antibacterial experiments (n = 3).

Name of sample	The diameter of the bacteriostasis circle (mm)			Sensitivity
	The original concentration	10 times dilution	100 times dilution	
<i>Panax quiquefolium</i>	22.3 ± 3.0	12.6 ± 2.0	5.0 ± 0.0	High
<i>Centella asiatica</i>	9.4 ± 1.4	5.0 ± 0.0	5.0 ± 0.0	Low
<i>Angelica sinensis radix</i>	21.6 ± 5.5	15.9 ± 2.8	5.0 ± 0.0	High
<i>Houttuynia cordata</i>	21.5 ± 4.3	10.9 ± 0.7	5.0 ± 0.0	High
<i>Rosemary officinalis</i>	25.6 ± 7.2	9.0 ± 1.4	5.0 ± 0.0	High
<i>Alpiniae oxyphyllae fructus</i>	15.6 ± 1.9	8.2 ± 1.1	5.0 ± 0.0	Moderate
<i>Panax ginseng</i>	11.7 ± 0.9	5.0 ± 0.0	5.0 ± 0.0	Moderate
<i>Schisandrae chinensis fructus</i>	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	Not
<i>L. cubeba</i>	>50.0	11.6 ± 0.7	5.0 ± 0.0	High
<i>Zanthoxylum bungeanum Maxim</i>	19.6 ± 4.2	5.0 ± 0.0	5.0 ± 0.0	High
<i>Cuminum cyminum</i>	>50.0	5.0 ± 0.0	5.0 ± 0.0	High
<i>Forsythiae fuctus</i>	17.6 ± 1.6	9.7 ± 1.1	5.0 ± 0.0	Moderate
<i>Artemisia argyi Levl</i>	21.3 ± 4.3	5.0 ± 0.0	5.0 ± 0.0	High
<i>Pogostemonis herba</i>	26.2 ± 0.9	19.2 ± 0.5	5.0 ± 0.0	High
<i>Atractylodis rhizoma</i>	13.7 ± 1.3	10.3 ± 1.1	5.0 ± 0.0	Moderate
<i>Caryophylli flos</i>	33.6 ± 4.5	20.3 ± 1.4	5.0 ± 0.0	High
<i>Pericarpium citri reticulatae</i>	19.5 ± 2.6	5.0 ± 0.0	5.0 ± 0.0	High
<i>Magnoliae biondii immaturus rlos</i>	9.5 ± 0.6	5.0 ± 0.0	5.0 ± 0.0	Low
<i>Schizonepetae herba</i>	37.1 ± 4.4	5.0 ± 0.0	5.0 ± 0.0	High
<i>Olibanum</i>	20.1 ± 2.7	5.0 ± 0.0	5.0 ± 0.0	High
<i>Schizonepetae spica</i>	28.7 ± 1.9	5.0 ± 0.0	5.0 ± 0.0	High
<i>Chuanxiong rhizoma</i>	21.8 ± 3.3	16.2 ± 4.9	5.0 ± 0.0	High
<i>Acori tatarinowii rhizoma</i>	11.1 ± 0.2	5.0 ± 0.0	5.0 ± 0.0	Moderate

diameters of the inhibitory circle exceeding 50 mm. Nevertheless, the inhibitory activity of *Cuminum cyminum* essential oil decreased greatly after dilution. The diameters of the inhibitory circle for *Schizonepetae herba* essential oil and *Caryophylli flos* essential oil are over 30 mm, indicating that the anti-*C. albicans* effect is second only to *L. cubeba* essential oil and *Cuminum cyminum* essential oil. Furthermore, *Panax quiquefolium* essential oil, *Angelica sinensis radix* essential oil, *Houttuynia cordata* essential oil, *Rosemary officinalis* essential oil, *Artemisia argyi Levl* essential oil, *Pogostemonis herba* essential oil, *Schizonepetae spica* essential oil, *Olibanum* essential oil and *Chuanxiong rhizoma* essential oil exhibit moderate sensitivity against *C. albicans*. However, the diameters of the inhibitory circle for *Schisandrae chinensis fructus* essential oil only 5 cm in three repeated experiments are same as the negative group, indicating that it had no inhibitory effect on *C. albicans* in this study.

The diameters of the inhibitory circle of mycoplasma

solution (50 mg/L, 100 mg/L and 200 mg/L) were 14 mm, 21 mm and 24 mm, respectively, which belong to high sensitivity, indicating that the effect of a positive control group is in line with the expectation. The diameter of the inhibitory circle of dimethyl sulphoxide (DMSO) (1.0 mg/L) solution is less than 5 mm, which belonged to low sensitivity, indicating that the effect of a negative control group is not interference.

There are many reports on antibacterial studies of essential oils. But in this study, the inhibitory effects of a variety of plant essential oils on *C. albicans* (the representative strain of pathogenic fungi) were compared under the same experimental conditions as the first time. All the samples of essential oil used in our experiment were prepared by the same process from the same factory (Hunan Nominxin Bioengineering Co., Ltd.), which avoided errors from different sources. The results showed that the anti-*C. albicans* effect of *L. cubeba* essential oil is the best among 23 essential oils. *L. cubeba* oil is not only a material re-

source for perfume¹⁵⁾, but also an edible condiment¹⁶⁾. Therefore, this study provides significance for *L. cubeba* essential oil to expand the application and improve the value of essential oils.

3.2 Chemical compounds of *L. cubeba* essential oil

The total ionic flow diagram of the *L. cubeba* essential oil obtained by GC-MS is shown in Fig. 3, and the structures of the chemical compounds were determined by the NIST 17 database and the CAS system query. The relative contents of the main compounds of the *L. cubeba* essential oil were calculated by the area normalization method based on the total ion flow peak.

Trans-citral (geranial) and *cis*-citral (neral), combined content with about 63.9%, which are the two peaks of the largest area in the total ionic flow diagram, being 33.6% and 30.3% respectively. The other three compounds with higher content are *d*-limonene (8.2%), Cineole (3.8%) and Citronellal (3.1%). The information about these compounds is shown in Table 2.

Literature showed that the *L. cubeba* essential oil from Chinese Taiwan contained a large amount of *cis*-citral and *trans*-citral, accounting for 69.8% of the total essential oil⁴⁾, Limonene is found in roots, stems, leaves, buds, flowers and fruits of *L. cubeba*, and citronellal also exists in roots, stems and leaves¹⁷⁾. It is reported that cineole exists in *L. cubeba* essential oil¹⁸⁾. *Trans*-citral (geranial), *cis*-citral (neral), limonene, cineole and citronellal are determined in the essential oil of *L. cubeba* fruits from eight regions of China¹⁹⁾. The above reports do not conflict with the results of this study.

It was found anti-fungal effect on *Candida. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. stellatoidea*, *C. krusei*, *C. parapsilosis* and *Cryptococcus neoformans* by citral in a short period with solid medium diffusion technique²⁰⁾. A good inhibitory effect on *F. verticillioides* (a fungi) is exhibited by 75 ppm limonene²¹⁾.

It can be inferred that *cis*-citral, *trans*-citral, and *d*-limonene are the main active components against fungi. In this study, *cis*-citral, *trans*-citral, and *d*-limonene are key components against *C. albicans* in *L. cubeba* essential oil, so molecular docking was performed between these two components and key enzymes for the growth of *C. albicans*.

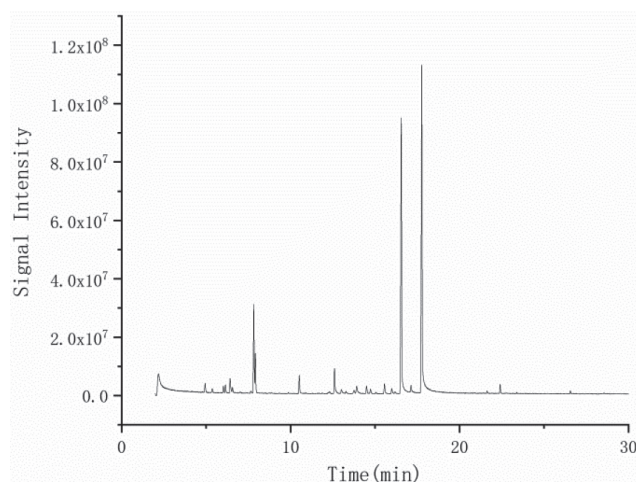


Fig. 3 Ionic flow diagram of *L. cubeba* essential oil.

3.3 Results of molecular docking

The binding energy value of molecule docking between *cis*-citral, *trans*-citral along with *d*-limonene and SAP5 as well as β -1,3-GS are shown in Table 3. The binding energies of the three compounds are -21.76 , -22.18 and -24.27 (kJ/mol) with SAP5, as well as -23.01 , -25.52 and -23.85 (kJ/mol) with β -1,3-GS, respectively.

According to the literature^{22, 23)}, the binding energies between protein receptors and small molecule ligands <0 kJ/mol indicate good molecular binding properties, and the higher the absolute value the more stable the conformation of the complex formed and the stronger the intermolecular binding. Therefore, we conclude that an effective docking between the receptors and the ligands is obtained in the present study.

As a control, the molecular docking binding energies between nystatin and SAP5 as well as β -1,3-GS are -29.29 and -38.91 kJ/mol, indicating the antifungal effect of synthetic antibiotics is still stronger than that of natural products, which can be verified in the results of 2.1.

As shown in Figs. 4a, 4b and 4c, the amino acid residues (Ile123, Asp32, Ser88, Ile30, Ile12, Gly220, Tyr84, Gly85 and Asp86) on SAP5 molecular are critical in docking with *cis*-citral molecular, the residues (Tyr84, Arg218, Asp32, Asp86, Gly220, Ile30, Ile123, Ala119 and Arg120) are key to docking with *trans*-citral molecular and the residues

Table 2 Main chemical compounds in *L. cubeba* essential oil.

Retention time (min)	Compound name	Molecular formula	CAS No.	Relative content (%)	RI calculated	RT literature
7.83	<i>d</i> -Limonene	$C_{10}H_{16}$	5989-27-5	8.2	1014	17)
7.92	Cineole	$C_{10}H_{18}O$	470-82-6	3.8	1016	18)
12.60	Citronellal	$C_{10}H_{18}O$	106-23-0	3.1	1018	19)
16.56	<i>Cis</i> -Citral	$C_{10}H_{16}O$	106-26-3	30.3	1028	4)
17.77	<i>Trans</i> -Citral	$C_{10}H_{16}O$	5392-40-5	33.6	1034	

Table 3 The binding energy of key compound between SAP5 as well as β -1,3-GS.

Compound	SAP5 (kJ/mol)	β -1,3-GS (kJ/mol)
<i>Cis</i> -Citral	− 21.76	− 23.01
<i>Trans</i> -Citral	− 22.18	− 25.52
<i>d</i> -limonene	− 24.27	− 23.85
Nystatin	− 29.29	− 38.91

(Thr13, Ile30, Gly220, Asp86, Tyr84, Asp32 and Ile123) are key to docking with *d*-limonene molecular. On the other hand, amino acid residues (Trp373, Tyr255, Phe258, Tyr29, Leu304 and Asn146) on β -1,3-GS molecular are critical in docking with *cis*-citral (Fig. 4d), the residues docking with *trans*-citral molecular are Phe258, Trp373, Ser292, Glu192, Asn146, Trp363, Glu27, Tyr29, His135, Leu304 and Tyr255 (Fig. 4e) and the residues docking with *d*-limonene molecular are Glu192, Phe258, Tyr255, Asn146 and Phe144 (Fig. 4f). It can be seen that the amino acids interacting with citral (*cis*- and *trans*-) and *d*-limonene are mainly aromatic amino acids and hydrophobic amino acids. The interaction of amino acids in the active pocket is mainly hydrophobic, and there is also hydrogen bonding: The hydrogen bond lengths between *cis*-citral and Gly85 as well as Asp86 on

SAP5 are 3.03 Å as well as 3.04 Å, between *trans*-citral and Arg120 on SAP5 is 3.15 Å, between *cis*-citral and Asn146 on β -1,3-GS is 3.03 Å, respectively.

SAP5 plays an important role in mycelium, adhesion to cells and infestation in the growth of *C. albicans*. β -1,3-GS is a key protease required for the synthesis of the cell wall in *C. albicans*. In this paper, citral (*cis*- and *trans*-) and *d*-limonene account for 75.1% of the detectable chemical components in *L. cubeba* essential oil, and the absolute values of binding energy between these two compounds and SAP5 along with β -1,3-GS molecules are >20.0 kJ/mol. It is reported that the binding energy between fluconazole (An antifungal drug) and SAP5 is 30.1 kJ/mol (− 7.2 cal/mol)²⁴, RRSKSH (A short peptide secreted by *Bacillus subtilis*) and β -1,3-GS is − 26.4 kJ/mol²⁵. We can conclude the inhibitory mechanism of *C. albicans* of *L. cubeba* essential oil is the result of the full combination of these three compounds with two key enzyme proteins.

However, it should also be noted: in this study, although it is well known that the higher compound content, the greater the impact on the activity, it cannot entirely exclude the possibility that the compounds with relatively low content could have strong activity. In addition, there may be synergistic enhancement between the active compounds of *L. cubeba* essential oil.

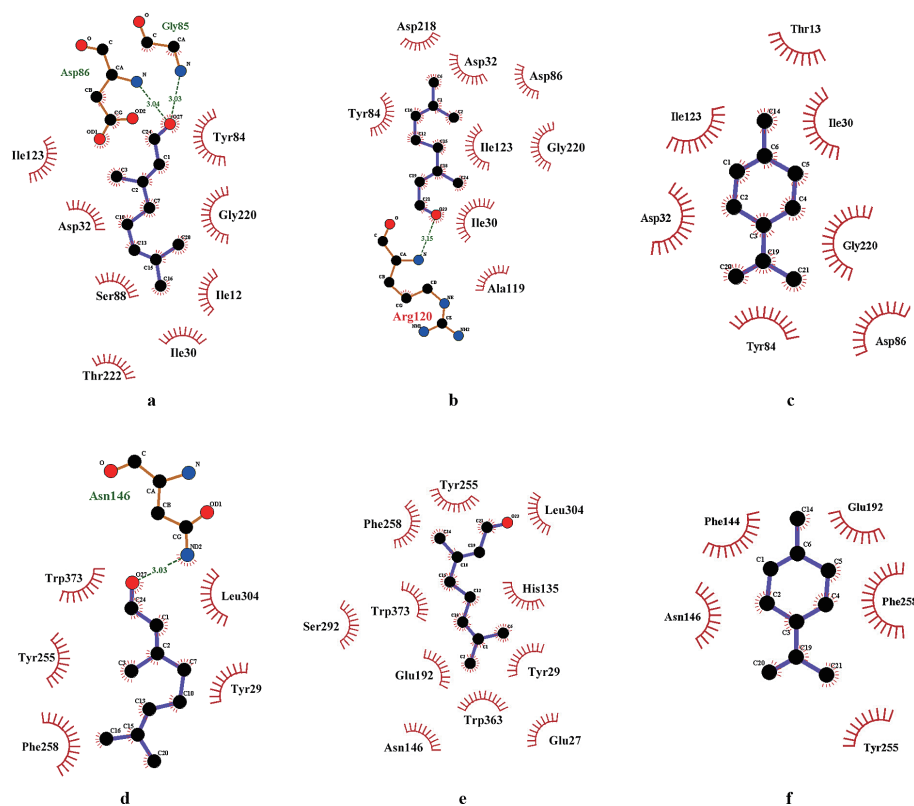


Fig. 4 Diagrammatic sketch of interaction between (a) SAP5 and *cis*-citral, (b) SAP5 and *trans*-citral, (c) SAP5 and *d*-limonene, (d) β -1,3-GS and *cis*-citral, (e) β -1,3-GS and *trans*-citral, and (f) β -1,3-GS and *d*-limonene.

4 Conclusions

In summary, we report the mechanism of *L. cubeba* essential oil against *C. albicans*. We demonstrate that *L. cubeba* essential oil exhibit the strongest inhibitory activity among these oils among twenty-three essential oils. In addition, *trans*-citral, *cis*-citral and *d*-limonene are identified as the three main components of *L. cubeba* essential oil, the content is 33.6%, 30.3% and 8.2%, respectively. Through a series of molecular docking carried out with SAP5 and β -1,3-GS, we suggest the mechanism of chemical components in *L. cubeba* essential oil inhibits the growth of *C. albicans*, which provides a reference to the development and utilization of essential oil. Although the inhibitory effect on *C. albicans* is not as good as antibiotics, *L. cubeba* essential oil is a better choice under the promotion of alternative antibiotic products by the national policy of China.

Authors' Contributions

Hong Li: Investigation, Data curation and Writing-original draft. Yiwen Kong: Formal analysis and Writing-original draft. Wei Hu: Revision of manuscript. Sheng Zhang: Conceptualization, Writing-review and editing. Wei Wang: Investigation, Validation. Min Yang: Investigation, Formal analysis. Yicheng Luo: Investigation, Methodology.

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