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Research Paper

Chemical composition and antimicrobial activity of essential oil isolated from the cultured mycelia of *Ganoderma japonicum*☆

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Abstract

Objective: To explore a new natural antibiotic. **Methods:**The chemical composition of the essential oil from *Ganoderma japonicum* (*G. japonicum)* mycelia was analyzed by gas chromatography-mass spectrometry(GC-MS). The antimicrobial activity of the oil was evaluated against eighteen microorganisms, including bacteria, mildew and yeast by using a disc diffusion method. Furthermore, the minimum inhibitory concentrations(MIC) and the minimum bactericidal concentrations(MBC) of the essential oil against twelve clinical pathogens were determined. **Results:**The main components of the oil were nerolidol, decadienal, linalool and benzyl alcohol. The antimicrobial results indicated that the oil inhibited all the tested bacterium, especially *Methicillin-resistant Staphylococcus aureus* (MRSA) in which the antibacterial activity exhibited a MBC of 1.03 mg/ml. **Conclusion:** The essential oil of *G. japonicum* mycelium has significant inhibitory activity. It is a potential medicinal resource that can be used as a natural antibiotic.

Keywords: *Ganoderma japonicum*; essential oil; antimicrobial activity; clinical pathogens

INTRODUCTION

Ganoderma japonicum is a species of fungus found in China, belonging to the family of *Ganoderma P.Kars* that has been used by traditional Chinese medicine for the treatment of various diseases. Previously traditional Chinese medical research has proved that *G. japonicum* can play many significant pharmacological roles, such as modulating the immune system, having anti-tumor[1-3] and anti-aging[4,5] effects, reducing blood sugar[6]; improving cardiovascular function[7], and providing an effective therapy for respiratory diseases. Modern Chinese medicinal research has also proved that *G. japonicum* mycelium has effects that are similar to wild *G. japonicum* for the clinical treatment of respiratory diseases[8].

In order to explore the *G. japonicum* mycelium pharmacology, we analyzed the components of *G. japonicum* mycelium essential oil by GC-MS, and assayed its antibacterial activity by a disc agar diffusion assay and broth dilution method. The results showed that the essential oil of *G. japonicum* has multi-pharmacological effects and it also contains several bactericidal components, including(E)-nerolidol, linalool, and(2E,4E)-decadienal. The antibacterial experiments indicated that the oil has remarkable antibiotic activities against all the test microorganisms, especially the clinical pathogens. Our work provides a reliable experimental basis for the use of *G. japonicum* as a Chinese medicine in the treatment of respiratory diseases. Since antibiotics have become so widely used clinically, many microorganisms have developed antibiotic resistance, especially some clinical pathogens. Therefore, the search for new antibacterial drugs has become an important topic. Recently, some studies have shown that essential oils from certain plants and fungal mycelia have antibacterial activity, inhibiting the growth of drug-resistance bacteria[9,10]. For this reason, pharmacological research on the oils of medicinal fungi

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is important for the development of new potential antibiotics.

MATERIALS AND METHOD

Material and essential oil distillation

The strain of fungus used was obtained from China General Microbiological Culture Collection Center (CGMCC):*G. japonicum* CGMCC As5.69. The *G. japonicum* mycelia were collected, and then subjected to hydro-distillation in a Clevenger-type apparatus for 3 hours. The essential oil was dried by anhydrous sodium sulphate. We carried out a parallel experiment on sterilized mycelia but did not inoculate blank media as a control. The oil was stored at -10℃ in the dark prior to chemical analysis and microbiological testing.

GC-MS analysis and identification of compounds

The *G. japonicum* mycelia essential oil and the negative control were analyzed by GC-MS. The analyses were carried out by using three different fused silica capillary columns(30 m × 0.25 mm i.d.; film thickness 0.25 μm) of different polarities(DB-1, DB-5 and HP-Innowax). The oven temperature was programmed from 50 to 250℃ at a 3℃/min rate and held at this temperature for 10 min. Injector and interface temperatures were 220℃ and 250℃. Carrier gas was helium at 1.0 ml/min. Diluted samples(1.0 μl, 1/10 in ether) were injected manually and the split ratio was adjusted to 40:1. GC-MS analyses were performed with a Thermo Finnigan TRACE GC and coupled with a TRACE MS^{plus}(EI 70 Ev). The components were identified by comparison of the mass spectra with those of NIST 2.0 library data of the GC-MS system and Adams libraries spectra. The results were further confirmed by comparison of the compounds elution orders with the retention indices semi-polar phases reported in the literature[9]. Retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation. The relative amounts of individual components of the essential oil were expressed as percentages of the peak area relative to the total peak area.

Microbial strains

Eighteen organisms were selected for this study, including 12 bacteria, 3 yeasts and 3 mildews. Some strains were obtained from The China Center for Type Culture Collection(CCTCC). They were *Bacillus subtilis* (CCTCC AB92068), *Escherichia coli*(CCTCC AB90054), *Proteus vulgaris*(CCTCC AB91103), *Salmonella typhi*(CCTCC AB94010), and *Staphylococcus aureus*(CCTCC AB203010). Three yeast species were *Candida sp.* (CCTCC AY91001), *Hansenula anomala* AY92046, *Saccharomyces cerevisiae* (CCTCC AY92042), and the three mildew species were *Aspergillus niger*(CCTCC AF91004), *Mucor mucedo* (CCTCC AF93229), *Penicillium citrinum*(CCTCC AF93094).

In addition, we used seven bacteria which were isolated clinically: *Klebsiella pneumoniae, Citrobacter freundill, Enterobacter cloacae, Staphylococcus saprophyticus, Enterococcus faecalis, Methicillin-sensitive Staphylococcus aureus* and *Methicillin-resistant Staphylococcus aureus*(MRSA).

Screening of antimicrobial activity

The agar disc diffusion assay was employed for the determination of antimicrobial activity of the essential oil[11]. Briefly, a suspension of the test organism(2×10^8 CFU/ml) was spread on the solid media plates. Filter paper discs(6 mm in diameter) were individually impregnated with 15 μl of the diluted oil aliquots(200 mg/ml stock), then placed on the inoculated plates, for 2 h at 4℃. The plates were incubated at 37℃ for 24 h for bacteria, and at 30℃ for 48 h for yeast, using a spread restraint method for epiphytes at 30℃ for 48 h[12]. The diameters of the inhibition zones(DD) were measured in millimeters. Each test was carried out in triplicate, repeated three times, and the average was calculated for the inhibition zone diameters. A positive control was performed using the antibiotic, Levofloxacin(5 μg/disc).

Determination of minimum inhibitory concentration(MIC)

A broth microdilution method was used to determine the MIC and MBC[9,13] . All tests were performed in Mueller Hinton broth and Sabouraud Dextrose broth, both supplemented with ethanol at a final concentration of 0.5%(v/v) for both bacteria and yeasts, respectively. Serial doubling dilutions of the oils were prepared in a 96-well plate, ranging from 0.05 to 200.00 mg/ml. The final concentration of each strain was adjusted to 5×10^4 CFU/ml. Plates were incubated at 37℃ for 24 h for bacteria. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity.

Determination of minimum bactericidal concentration (MBC)

To determine MBC, broth was taken from each well and incubated in Mueller Hinton Agar at 37℃ for 24 h for bacteria. Levofloxacin served as a positive control in parallel experiments. The MBC was defined as the lowest concentration of the essential oil at which the incubated microorganism was completely killed[9,13].

Each test was carried out in triplicates and repeated three times. Modal values were selected.

RESULTS

Chemical composition of the essential oil

The yield of the essential oil from *G. japonicum* mycelia was 0.18%(w/w) based on the dry weight of the sample. Forty-three components were detected by GC-MS, and 16 of them were identified(***Fig. 1***).

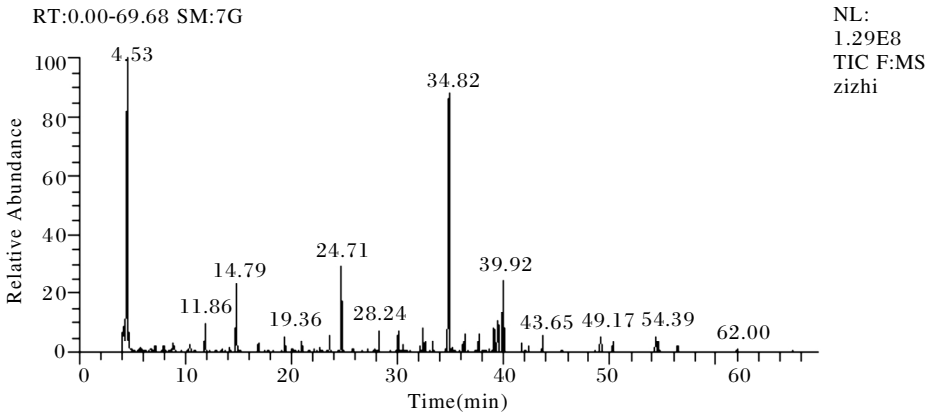

Fig. 1 GC-MS total ion count chromatograms for the essential oil of *G. japonicum* with n-alkanes in DB-5

As shown in ***Table 1***, the oil was composed of oxygenated sesquiterpene, oxygenated monoterpene and small amounts of aldehyde and fatty acid, including nerolidol (17.59%), linalool(4.49%), decadienal(6.18%), benzyl alcohol(2.24%), 9-octadecenal(1.56%), n-hexadecanoic acid(1.47%)and benzaldehyde(0.85%). No peaks were detected in the blank.

Table 1 **Chemical components of essential oil in *G. japonicum*(GC/MS)**

Compound	Composition(%)	R.I.
Oxygenated monoterpenes	**5.13**	
Linalool Oxide (trans-furanoid)	0.29	1069
Cis-naloloxide	0.28	1085
Linalool	4.49	1100
5,7-Octadien-2-ol,2,6-dimethyl	0.07	1193
Oxygenated sesquiterpenes	**17.59**	
(E)-Nerolidol	17.59	1562
Other	**14.09**	
Benzaldehyde	0.85	957
(EE)-2,4-Heptadienal	0.15	1010
Benzyl alcohol	2.24	1033
Benzene acetaldehyde	0.08	1043
(2E)-Nonen-l-al	0.1	1158
(2E,4E)-Nonadienal	0.34	1216
(Z)-2-Decenal	0.15	1262
2,4-Decadienal	0.97	1295
(2E,4E)-Decadienal	6.18	1320
n-Hexadecanoic acid	1.47	1966
9-Octadecenal	1.56	2134

R.I.: Retention Indix

Antimicrobial activity

The disc diameters of zone of inhibition(DDs), minimum inhibitory concentrations(MICs) and minimum bactericidal concentrations(MBCs) of *G. japonicum* essential oil for the microoganisms tested ware shown in ***Table 2***.

The antimicrobial DD test results showed that the *G. japonicum* mycelia essential oil has significant antibacterial activity against 18 microorganisms, including bacteria, mildew and yeast.

Furthermore, we determined MIC and MBC of the essential oil against 12 clinical pathogens. Levofloxacin served as a positive control in parallel experiments. The essential oil showed strong inhibitory activity against yeast and mildew, with inhibition zones of 25-34 mm, and the oil also showed inhibitory activity against bacteria such as *Staphylococcus aureus*, with inhibition zones of 27 mm. The lowest MIC and MBC observed for *G. japonicum* essential oils were each 1.03mg/ml. Our data demonstrated that the essential oil had a strong inhibitory effect on clinically important bacteria, such as *Staphylococcus saprophyticus* and *Enterobacter cloacae*, and especially MRSA, with an inhibition zone diameter of 26 mm, and MIC and MBC values of 1.03 mg/ml.

DISCUSSION

G. japonicum is a traditional Chinese medicine that has been applied clinically for a thousand years, and its efficiency and safety have been fully verified. Modern Chinese medicinal research and clinical applications showed that *G. japonicum* mycelium had similar effects to wild *G. japonicum* in the treatment of respiratory diseases[8].

Through GC-MS analysis, we found that components of the *G. japonicum* oil with the pharmacological activity included(E)-nerolidol and linalool. Both components have been confirmed to have bacteriostatic and bactericidal activity, causing changes in cell membrane permeability and bacterial death[14]. In addition, linalool also has significant central nervous system properties, including causing sedation, acting as

Table 2 **Antimicrobial activities of *G. japonicum* mycelia essential oil**

Microorganisms	The essential oil of GJ			Levofloxacin		
	DD[a]	MIC[b]	MBC[b]	DD[c]	MIC[d]	MBC[d]
Reference strains						
Escherichia coli AB90054	24	2.05	2.05	30	2.44	2.44
Proteus vulgaris AB91103	20	4.11	4.11	34	0.61	0.61
Salmonella typhi AB94010	23	2.05	2.05	33	1.22	1.22
Staphylococcus aureus AB203010	27	1.03	1.03	31	0.61	0.61
Bacillus subtilis AB92068	25	2.05	2.05	32	NT	NT
Clinical isolated strains						
Methicillin-sensitive Staphylococcus aureus	24	2.05	2.05	32	0.61	1.22
Methicillin-resistant Staphylococcus aureus	26	1.03	1.03	11	9.75	9.75
Klcbsiella pneumoniae	21	4.11	8.22	31	0.31	4.88
Enterococcus faecali	22	4.11	4.11	26	9.77	9.77
Enterobacter cloacae	24	2.05	4.11	14	4.88	9.77
Staphylococcus saprophyticus	25	1.03	1.03	28	9.77	312.5
Citrobacter freundill	19	8.22	8.22	NT	NT	NT
Yeast						
Candida sp. AY91001	29	NT	NT	NT	NT	NT
Hansenula anomala AY92046	31	NT	NT	NT	NT	NT
Saccharomyces cerevisiae AY92042	25	NT	NT	NT	NT	NT
Mildew						
Aspergillus niger AF91004	32	NT	NT	NT	NT	NT
Mucor mucedo AF93229	34	NT	NT	NT	NT	NT
Penicillium citrinum AF93094	31	NT	NT	NT	NT	NT

DD(mm):disc diffusion; NT:not test; a:Tested at a concentration of 1.2 mg/disc. b:Values given as mg/ml. c:Tested at a concentration of 5.0 μg/disc. d:Values given as μg/ml.

a hypnotic, anticonvulsant, antispasmodic and regulating body temperature[15], all of which may be helpful in the cure of respiratory diseases.

Our antibacterial experiments showed that the oil had strong bacteriostatic and bactericidal effects against all of the 12 species tested. Linalool is known to have inhibitory activities against bacteria and fungi[16]. Carson and Riley have demonstrated the linalool activity against *Candida albicans, Escherichia coli* and *Staphylococcus aureus.*[17]. Conjugated unsaturated aldehydes like 2E, 4E-decadienal and(Z)-2-decenal, can inhibit the growth of bacteria and fungi[18]. Benzaldehyde can prevent the synthesis of cytoplasm, and has strong antifungal activity[19].

Most importantly, the essential oil of *G. japonicum* mycelium has significant inhibitory activity against isolates of disease-causing clinical pathogens, such as *Staphylococcus aureus, Escherichia coli*, *Salmonella typhi* and other resistant strains. These studies provide a theoretical basis for the use of *G. japonicum* in Chinese medicine in the treatment of infectious respiratory diseases.

According to the studies of Voss and Doebbeling, community-acquired or outpatient MRSA infections are increasing in both children and adults[20]. MRSA is responsible for worldwide outbreaks of nosocomial infections. However, the pharmaceutical arsenal available to control MRSA is very limited at this time[9,21,22]. The medicinal fungi, such as *G. japonicum,* can produce a variety of metabolites. We detected 43 components from it's essential oil by GC-MS, of which only 16 compounds are known. Given the clear antimicrobial activity of the essential oil of *G. japonicum*, it is necessary to conduct an in-depth analysis of it' s components and their antimicrobial activities, with a view to developing further potential medicinal resources.

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Reference

[1] Jiang JH, Slivova V, Harvey K, Valachovicova T, Sliva D. *Ganoderma lucidum* suppress growth of breast cancer cells through the inhibition of Akt/NF-kB signaling. *Nutr Cancer* 2004;49:209-16.

[2] Zhao HB, Lin SQ, Liu JH, Lin ZB. Polysaccharide extract isolated from *Ganoderma lucidum* protects rat cerebral cortical neurons from hypoxia/reoxygenation injury. *J Pharmacol Sci* 2004;95:294-8.

[3] Sliva D. Cellular and physiological effects of *Ganoderma lucidum* (Reishi). *Mini Rev Med Chem* 2004;4:873-9.

[4] Guo F, Qi YJ, ZhangYJ, Wang Y. Antioxidation of mythic fungus broth on senile rat models induced by D-galactose. *Acta Academiae Medicine Militaris Tertiae*(in Chinese) .2005; 27:1564-8.

[5] Cao QZ, Lin ZB. Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptid. *Acta Pharmacol Sin* 2004;25:833-8.

[6] Cummings J, Macpherson JS, Meikle I, Smyth JF. Development of anthracenyl-amino conjugates as topoisomerase Ⅰ and

Ⅱ inhibitors that circumvent drug resistance. *Biochem Pharmacol* 1996;52:979-90.

[7] He XL.The Curative effect of Ganoderm *Ganoderma sinense* on treating poisoning patients by toxic mushrooms. *Acta Edulis Fungi*(in Chinese) .1999; 6: 47-8.

[8] Wang L, Wang YH, Zhang KC. A Study on the effect of 5 kinds of Chinese traditional medicines on submerged fermentation of *Ganoderma lucidum* and its fermented broth on chronic bronchitis. *Edible Fungi of China* 2004; 5: 39-40.

[9] Yu JQ, Lei JC, Yu H, Cai X, Zou GL. Chemical composition and antimicrobial activity of the essential oil of *Scutellaria barbata*. *Phytochemistry* 2004; 65: 881-4.

[10] Chorianopoulos NG, Lambert RJW, Skandamis PN, Evergetis ET, Haroutounian SA, Nychas GJE. A newly developed assay to study the minimum inhibitory concentration of *Satureja spinosa* essential oil. *J Appl Microbiol* .2006;100:778-86.

[11] Zhu SY, Yang Y, Yu HD, Ying Y, Zou GL. Chemical composition and antimicrobial activity of the essential oils of *Chrysanthemum indicum*. *J Ethnopharmacol* 2005; 96:151-8.

[12] Uzela A, Guvensena A, Cetinb E. Chemical composition and antimicrobial activity of the essential oils of *Anthemis xylopoda O. Schwarz* from Turkey. *J Ethnopharmacol* 2004;95:151-4.

[13] Chuang PH, Lee CW, Chou JY , Murugan M, Shieh BJ, Chen HM. Anti-fungal activity of crude extracts and essential oil of *Moringa oleifera Lam*. *Bioresource Technol*.2007;98:232-6

[14] Arruda DC, D′Alexandri FL, Katzin AM, Uliana SRB. Antileishmanial activity of the terpene nerolidol. *Antimicrob Agents Chemotherap* 2005;49:1679-87.

[15] Re L, Barocci S, Sonnino S, Mencarelli A, Vivani C, Paolucci G, Scarpantonio A, *et al*. Linalool modifies the nicotinic receptor ion channel kinetics at the mouse neuromuscular junction. *Pharmacol Res* 2000;42:177-81.

[16] Pattnaik S, Subramanyam VR, Bapaji M, Kole CR. Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios* 1997;89:39-46.

[17] Carson CF, Riley TV. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *J Appl Bacteriol* 1995;78:264-9.

[18] Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* 1999; 86:6985-90.

[19] Xu CK, Mo MH, Zhang LM, Zhang KQ. Soil volatile fungistasis and volatile fungistatic compounds. *Soil Biol Biochem* 2004; 36:1997-2004.

[20] Voss A, Doebbeling BN. The world wide prevalence of methicillin-resistant. *Staphylococcus aureus* Int *J Antimicrob* Agents 1995;5:101-6.

[21] Jiao M. The analysis of drug resistance and bacteria of acute respiratory tract infection in children. *Acta Universitatis Medicinalis Nanjing*(in Chinese) 2003;23:82-4.

[22] Li S, Xiao GY, Zhou GP, Cai JY, Huang YP, Li Q. The analysis of the drugs used by the patiants with MRSA infection. *Modem Hospital* (in Chinese) 2008; 8:19-21.