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Clinical Observation

Mechanisms, clinically curative effects, and antifungal activities of cinnamon oil and pogostemon oil complex against three species of Candida

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Abstract

The anti-fungus mechanisms and curative effects of cinnamon oil and pogostemon oil complexes towards intestinal *Candida* infections were investigated. We measured the minimal inhibitory concentration (MIC) values of the complexes against *Candida* using proportionally-diluted test-tube medium, and examined the evolution of the morphology and structures of *Candida albicans* using scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM). We found that the average MIC values of the complexes against the fungi were 0.064 mg/mL (cinnamon oil), 0.032 mg/mL (pogostemon oil) for *Candida albicans*, 0.129 mg/mL (cinnamon oil), 0.064 mg/mL (pogostemon oil) for *Candida tropicalis*, and 0.129 mg/mL (cinnamon oil), 0.064 mg/mL (pogostemon oil), for *Candida krusei*. SEM examination over a 24-48 h period showed that the morphology of *Candida albicans* cells changed significantly. Irregular hollows appeared on the surfaces, inside organelles were destroyed and the cells burst after treatment. TEM examination over a 48 – 72 h period indicated that the cell walls were damaged, organelles were destroyed and most cytoplasms became empty bubbles. Sixty intestinal *Candida*-infected patients were treated with a capsule containing cinnamon and pogostemon oil. The curative ratio was 71.67% (43/60), and the improvement ratio was 28.33% (17/60), giving a total ratio of 100%. Thus, the cinnamon oil and pogostemon oil complexes had strong anti-fungus effects against *Candida albicans*, *Candida tropicalis*, and *Candida krusei*. They impacted the morphology and sub-micro structures of the fungus within 48 – 72 h, and eventually denatured and killed the cells. The complexes have also shown considerable curative effects to intestinal *Candida* infections.

Key words: Pogostemon oil and cinnamon oil complex; Candida; Antifungal activity; Curative effect

INTRODUCTION

Deep fungus infections have increased rapidly in recent years. Most fungus infections are caused by candida germ[1], mainly Candida albicans and to a lesser extent Candida tropicalis[2] and Candida krusei[3]. The death rate from these infections can reach 30%-60%[4-5]. Unfortunately, few anti-fungus medicines are available for treating fungus infections, not to mention that most of them have serious side effects. Because of this, it has been difficult to control deep fungus infection[6]. Fundamental research to develop effective Chinese anti-fungus medicines with low side effects has therefore attracted wide interest. To this end, we measured the

extracorporeal minimal inhibitory concentration (MIC) values of Chinese medicine complexes against three types of Candida (Candida albicans, Candida tropicalis, Candida krusei). We studied the morphology and sub-micro structure evolution of three types of candida using scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM). We also analyzed the data obtained from real cases of treatment of intestinal candida infection. This study was approved by the appropriate ethics committees and was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. All persons signed their informed consent prior to their inclusion in the study.

MATERIALS

Cinnamon oil and pogostemon oil complex capsules were provided by the Pharmaceutical Factory, Hebei Medical University (Shijiazhuang, China), lot no. 0201-03. Fluconazole capsules were provided by Shanghai Sunve Pharmaceutical Co. Ltd. (Shanghai, China), lot no. 0201016. Test strains were provided by the Fungus Room, Dermatology Department, the Second Hospital of Hebei Medical University (Shijiazhuang, China). These strains were isolated from the specimens of stools and sputums of fungus-infected patients. All strains were virulent (large amounts of mycelium and blastopore can be seen under the microscope), and were cultured and identified as candida (Candida albicans 30 strains, Candida tropicalis 20 strains, Candida krusei 20 strains). Standard Candida albicans, Candida tropicalis, and Candida krusei were provided by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China).

Clinical information about 100 voluntary, intestinal fungus-infected patients was obtained from the Gastroenterology Department, the Second Hospital of Hebei Medical University. Among these patients, 57 are males and the remaining 43 are females. They can be divided into groups: 4 between 21 and 30 years old, 16 between 31 and 40 years old, 28 between 41 and 50 years old, 36 between 51 and 60 years old, and 16 older than 60. The shortest course of treatment lasted a month, the longest was for three years, and the average treatment time was 3.6 months. All patients had a history of using two to three different kinds of antibiotics for more than half of a month without any improvement. The patients were randomly divided into two groups, one group contained 40 patients and was treated with fluconazole, whereas the other group contained 60 patients and was treated with the experimental complex.

METHODS

MIC measurement

The MIC values of the cinnamon oil and pogostemon oil complexes against three species of Candida fungus were determined. The medium make-up was prepared using the test-tube medium method. The complex was first dissolved in ethanol, it was then thoroughly mixed with high pressure sterilized glucose peptone agar medium at 50°C (liquid state), and the mixed liquid was diluted proportionally into A (cinnamon oil 2.06 mg/mL, pogostemon oil 1.03 mg/mL), B (cinnamon oil 1.03 mg/mL, pogostemon oil 0.515 mg/mL), C (cinnamon oil 0.515 mg/mL, pogostemon oil 0.257 mg/mL), D (cinnamon oil 0.257 mg/mL, pogostemon oil 0.129 mg/mL), E (cinnamon oil 0.129 mg/mL, pogostemon oil 0.064 mg/mL), F (cinnamon oil 0.064 mg/mL, pogostemon oil 0.032 mg/mL), or G (cinnamon oil 0.032 mg/mL, pogostemon oil 0.016 mg/mL). Fluconazole was proportionally diluted to a concentration of 2.06 mg/mL-0.032 mg/mL. The liquids were stored in test-tubes and placed on a slant.

To prepare the fungus strain inoculation, the appropriate amounts of activated fungus strains were added into 0.85% sodium chloride solution, and the concentration of fungus in the suspension liquid was adjusted to 1×10^5-1×10^6 CFU/mL by using a hemocyte counting board. A volume of 100 μL fungus suspension was inoculated into each test-tube medium. Two test-tubes were made for each concentration. In addition, 10% ethanol glucose peptone agar medium was used for comparison and glucose peptone agar medium was used for the blank control. All tubes were kept at a constant temperature of 36°C. The growth was examined and recorded daily and the final results were measured after 96 h.

The MIC value was determined by comparing the growth in each tube with the negative and positive tubes. The MIC terminal value was taken as the tube with the lowest drug concentration where the cultured substance no longer grew. The negative and positive comparison groups were established following the same procedures as described above.

Microscopy experiments

The cinnamon oil and pogostemon oil complex was dissolved in ethanol, and the liquid was then added into sterilized glucose peptone agar medium (4% glucose, 1% peptone) so that each milliliter of medium contained cinnamon oil 0.52 mg, pogostemon oil 0.25 mg, ethanol 0.1 mL, and 10% ethanol glucose peptone liquor medium (4% glucose, 1% peptone, 10% ethanol). Glucose peptone liquor medium (4% glucose,

1% peptone) was prepared as a blank. These liquids were added to sterilized tubes so that each tube contained 5 mL.

After activation for 48 h, C. albicans was added into 0.85% sodium chloride solution, and the concentration of the fungus in the suspension liquid was adjusted to 1×10^6-1×10^7 CFU/mL using a hemocyte counting board. The liquid was then added to the medium, along with the mixture of 10% ethanol glucose peptone liquor medium and glucose peptone liquor medium (100 μl/mL fungus). These liquids were then cultured in an incubator at a constant temperature of 36°C for 24, 48, and 72 h. Centrifugal experiments at a spin rate of 2500 r/min were carried out for 10 minutes to separate the fungus pieces from the clear liquid. SEM specimens were then solidified in 2.5% glutaraldehyde and TEM specimens were solidified in 4% glutaraldehyde. SEM and TEM samples were prepared using routine methods and were examined using Hitachi S-520 SEM and H-500 TEM instruments (Hitachi High-Technologies Corporation, Tokyo, Japan).

Clinical observations

Our patients all had various degrees of chronic diarrhea, shapeless and mucus stool 3-10 times a day, abdominal pain, and weight loss. Serious patients also had white festering tenesmus stools, low fever, nausea, and vomiting. Internal medicine diagnosis indicated that alimentary canal radiography, stool fungus culture and routine stool tests were all normal. The conditions of the patients did not improve or even became worse when treated using antibiotics or hormones. Test results also excluded the possibility of other intestinal diseases. Fungus microscopy examinations showed a large amount of fungus mycelium and blastopore (indicating a pathogenic state). Candida fungus was found to grow when cultured at 37°C.

During a 14-day course of treatment, patients took the medicines orally, three capsules each time, three times a day. For the 60 patient group, each capsule contained 18 mg cinnamon oil and 9 mg pogostemon oil. For the 40 patient group, each capsule contained 50 mg fluconazole. The curative effect was then evaluated. The patients were considered cured when the clinical symptoms completely disappeared and the results of fungus microscope examinations and fungus cultures were both negative in three separate tests performed on three consecutive days. The patients were considered improved when the clinical symptoms diminished significantly, the results of the fungus microscope examinations were negative or positive, and the results of the fungus cultures were positive. The patients were considered unaffected if the clinical symptoms remained or became worse, and the results of both fungus microscope examinations and fungus cultures remained positive. Statistical analysis was performed using the SPSS13.0 statistical software (IBM Corp., Armonk, NY, USA).

RESULTS

MIC values for Chinese medicine complex and the fluconazole capsules are compared in Table 1. The results indicate that the complex has strong anti-fungus activities against clinical fungus strains including the standard strains of Candida albicans, Candida tropicalis, and Candida krusei. Although fluconazole also has strong anti-fungus activities against Candida albicans and Candida tropicalis, its activity against Candida krusei is relatively low.

Results of the SEM analysis (×4500) are shown in Figures. 1(a) – 1(f). Figure. 1(a) shows control Candida albicans (blank) at 72 h without the treatment. It can be seen that the surfaces of the elliptic hypha and blastospore were smooth and full, and the ornamentations were clear. Figure. 1(b) shows the Candida albicans morphology at 24 h after the treatment using 10% ethanol. It can be seen that the blastospore was still oval in shape, and the surfaces were still smooth with clear ornamentation. Figure. 1(c) shows the Candida albicans morphology 24 h after the treatment using the complex medicine. Here the shapes of the blastospore cells became irregular, some organelles were damaged, and some cell walls were broken. Figure. 1(d) shows the morphology 48 h after the complex medicine treatment. At this stage, most cells were irregular and even hollow, many cell walls became detached and some were broken. Figure. 1(e) shows the Candida albicans hypha morphology obtained 72 h after the complex medicine treatment. It shows that most of the hypha cell walls became detached, and the contents either disappeared or were denatured. Figure. 1(f) shows the Candida albicans spore morphology 72 h after complex medicine treatment. It indicates that the walls of the blastospore partially disappeared or were perforated, the cytoplasm became hollow, and the cells were denatured and died.

The results of the TEM analysis (×12000) are shown in Figure. 1(g) – 1(j). Figure. 1(g) shows the morphology of Candida albicans after 72 h in the presence of the 10% ethanol control. It can be seen that the blastospores were round, and the cell walls, membranes, and organelles were still complete. Figure. 1(h) shows the Candida albicans morphology 24 h after the complex medicine treatment. Here we can see that the cells swelled, the cell shapes became irregular, and the organ-

elles atrophied. Most of the cytoplasm looked like empty bubbles and the cell walls became incomplete. Figure. 1(i) was obtained 48 h after the complex medicine treatment. At this stage, the cells were irregular in shape, the organelles completely disappeared, and some cell walls were broken. A total of 72 h after the complex treatment, Figure. 1(j), the organelles disappeared, most cytoplasm were reduced to empty bubbles, and some cell walls were broken. The effects of the Chinese complex and fluconazole are compared in Table 2.

Table 1 MIC anti-fungus activities against three species of Candida

Fungus strains (*Candida*)	Strain number	MIC distribution (mg/mL)							
		Cinnamon oil			Pogostemon oil			Fluconazole	
		MIC_{90}	MIC range	Average MIC	MIC_{90}	MIC range	Average MIC	MIC range	Average MIC
albicans (clinical)	20	0.064	0.064-0.515	0.064	0.032	0.032-0.064	0.032	0.064-0.129	0.064
albicans(standard)	2	0.064	0.064	0.064	0.032	0.032	0.032	0.064	0.064
tropicalis(clinical)	20	0.129	0.129-0.257	0.129	0.064	0.064-0.257	0.064	0.129-0.064	0.129
tropicalis(standard)	2	0.129	0.129	0.129	0.032	0.032	0.032	0.257	0.257
krusei (clinical)	20	0.129	0.129-0.257	0.129	0.257	0.064-0.257	0.257	1.03-2.06	2.06
krusei (standard)	2	0.129	0.129	0.129	0.129	0.129	0.129	2.06	2.06

Note: All values refer to statistical median.

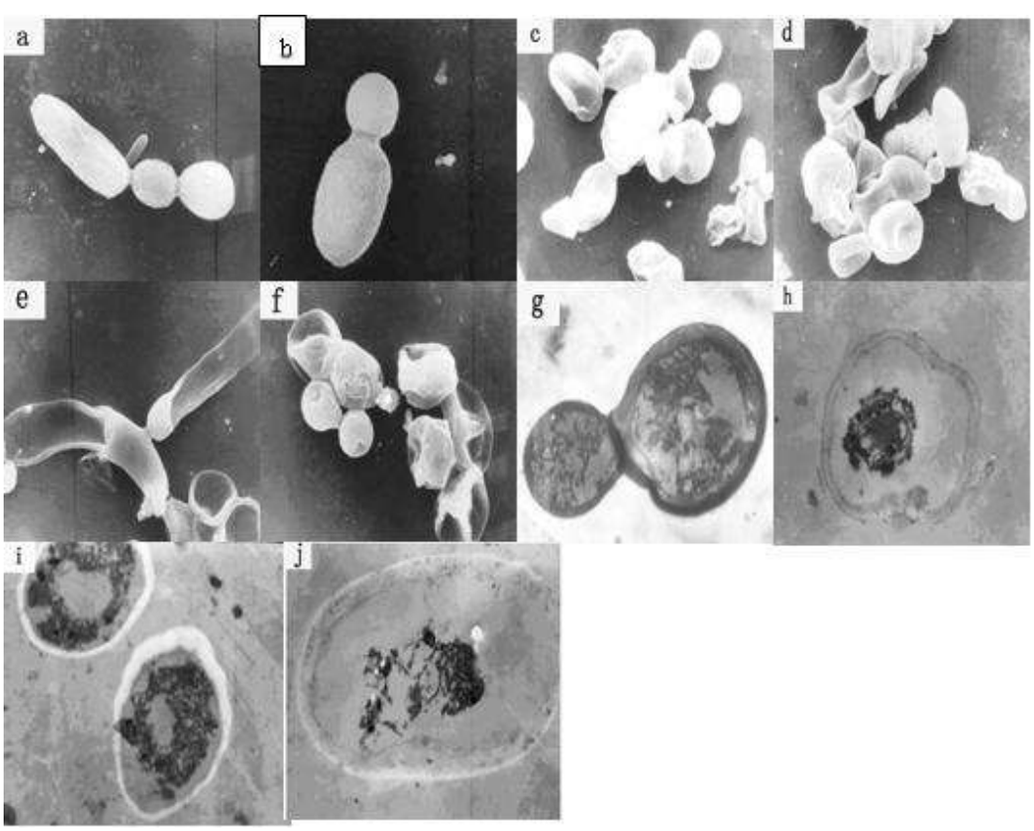

Figure 1 (a) Candida albicans without treatment (SEM×4500); (b): Candida albicans 24 h after ethanol treatment (SEM×4500); (c): Candida albicans 24 h after the complex treatment (SEM×4500); (d): Candida albicans 48 h after the complex treatment (SEM×4500); (e): Candida albicans hypha 72 h after the complex treatment (SEM×4500); (f): Candida albicans spores 72 h after the complex treatment (SEM×4500); (g): Candida albicans after ethanol treatment (TEM×12000); (h): Candida albicans 24 h after the complex treatment (TEM×12000); (i): Candida albicans 48 h after the complex treatment (TEM×12000); (j): Candida albicans 72 h after the complex treatment (TEM×12000).

Table 2 Comparison of the effects of the complex and fluconazole

Effect	Cases (percentage)	The complex group (60 cases)			Cases (percentage)	Fluconazole group (40 cases)		
		albicans	tropicalis	krusei		albicans	tropicalis	krusei
Cured	52 (86.67)	31	16	5	33 (82.50)	21	12	0
Improved	8 (13.33)	5	2	1	5 (12.50)	4	1	0
Unaffected	0 (0.00)	0	0	0	2 (5.0)	0	0	2
total	60 (100.0)	36	18	6	38 (95.00)	25	13	2

DISCUSSION

Cinnamon oil, which consists mainly of cinnamaldehyde (CA)[7-8], is the main constituent of two traditional Chinese medicines, cassia twig and Chinese cinnamon. It has many functions including relieving fever, inducing sweat and dispersing and easing pain, in addition to anticancer and antivirus properties [9-10]. Cinnamon oil also has strong antimicrobial action for a variety of pathogenic fungi[11-13] including three species of Candida (Candida albicans, Candida tropicalis, Candida krusei) [14]. Cinnamon oil has been shown to protect mice from gastric stress ulcers and increase the secretion of bile in rats[15]. Other in vivo studies indicate that cinnamon oil has very good curative effects in mice infected with Candida albicans[16-17]. Pogostemon oil is extracted from pogostemon cablin, labiatae. It is mainly composed of patchouli alcohol and patchouli ketone[18]. Its functions include eliminating turbid pathogens, reducing vomiting, relieving sunstroke[19], and inducing appetite, and it has antifungal activities against many dermatophytes and conditional pathogenic fungi[20]. The main antimicrobial component is patchoulenon[21]. The work reported herein describes the in vitro antifungus experiments of the complex of cinnamon oil and pogostemon oil against candidas fungus (Candida albicans, Candida tropicalis, Candida krusei). The results indicated that at the MICs (0.032 mg/mL - 0.129 mg/mL), the capsules have considerably strong effects against the three species of Candida, including Candida krusei that is relatively resistant to fluconazole. SEM analysis showed that after the complex acted on C. albicans for 48-72 h, the organelles were destroyed, and some cell walls were broken. This suggests that the medicine penetrated through the cell walls and membranes to damage them, which in turn affected the synthesis of mannan and chitin-glucan[22-23]. Furthermore, the broken cell walls can also penetrate the cell membranes, causing mechanical damage. In turn, the exchange of substances between the inner and outer parts of the cells becomes more difficult. The cell metabolism and the activity of some enzymes are also affected, thereby interfering with the synthesis of nucleic acids and proteins. These processes cause the organelles and cell nucleus to be further damaged and the contents to escape from the cells, eventually resulting in a cell burst and then death [24-26].Our work also shows that the capsules containing the complex can quickly kill the Candida spores and hypha within 72 h. Presently, western medicines are still the main choice to treat Candida infections. However, more and more C. albicans and other Candida strains are becoming resistant to western medicines[27]. Development of Chinese anti-fungus medicines has therefore become increasingly important. Our studies have applied capsules of complex of cinnamon oil and pogostemon oil to treat 60 cases of intestinal Candida infections. The results showed a curative ratio of 71.67% (43/60), and a total effective ratio of 100% . Ma Yao-hui *et al*[22]. established animal models of mouse to study infection by gastric Candida albicans. After gavaging the mice with cinnamon oil, and harvesting the mouse gastric tissue for fungal microscopy and histopathological examination, both of the outcomes demonstrated that cinnamon oil can kill mouse gastric Candida albicans. This further supports our results that the use of cinnamon oil in the treatment of deep candida infection may provide a valid treatment option. Most importantly, China is very rich in cinnamon and patchouli thus making the development of new anti-fungus agents economical.

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