The Discovery of a Potential Antimicrobial Agent: the Novel Compound Natural Medicinal Plant Fermentation Extracts against Candida albicans

Mingzhu Song1,2,*, Xirui Wang3, Canquan Mao4 and Wei Yao1

1School of Medicine, Chengdu University, Chengdu, 610106, P. R. China
2National Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, 610041, P. R. China
3College of Tourism, Economics and Management, Chengdu University, Chengdu, 610106, P. R. China
4School of Life Science and Engineering, Southwest Jiaotong University, Chengdu
Email: *songmingzhu@cdu.edu.cn

Abstract. Natural medicinal plants and their extracts are important sources of antimicrobial drug development. In this study, we reported an ancient formula of Chinese folk medicine, the compound natural medicinal plant fermentation extracts (CNMPFE) for its antimicrobial effects. The effects and mechanisms of CNMPFE on C. albicans were studied by cell damage experiments including antimicrobial kinetics, fungal growth curve, alkaline phosphatase (AKP) activity, ultraviolet absorption, electric conductivity and the evaluation of cellular ultrastructure. The results showed that the minimal inhibitory concentration and minimum fungicidal concentration of CNMPFE against C. albicans were 75% (vol/vol) and 80% (vol/vol) respectively. The inhibition of CNMPFE for C. albicans was dose and time dependent, based on increasing of the AKP activities and the ultraviolet absorptions and the electric conductivities of the fungal solutions, it may exert its antifungal properties by disrupting the structure of cell wall and the cell membrane integrity and their permeability, subsequently resulting in cell death. Taken together, these findings suggest that CNMPFE may be a promising drug candidate for the treatment of fungal infections skin diseases.

1. Introduction
In recent years, the infections caused by fungi are becoming a topic concerned and attracting lots of scientists all over the word. Candida spp., filamentous Aspergillus spp. (e.g. A. fumigatus), yeast Cryptococcus neoformans and some other rare emerging fungi are the most common pathogens and cause disseminated fungal infections [1]. The main C. spp. included C. albicans, C. dublinsiensis, C. glabrata, C. krusei, C. parapsilosis, and C. tropicalis [2]. Among them, C. albicans is one of the common systemic pathogenic fungal strains which can live symbiotically with human in mouth, intestine and vagina. If there is no complete immunoreaction, the mucous membranes and skin of the host will be easily infected by the fungal or invaded by fungal disease. In fact, the death rate caused by some fungi was far more than those by Gram-negative bacteria which lead to sepsis. Hence, the fungal infection is an important area of human diseases and could not be neglected [3].

Currently, there were few types of drugs used for antifungal infection. Among them, the commonly used drugs are from polyenes, azoles, allylamines, fluoropyrimidines, amphotericin B, antimicrobial peptides and echinocandins [3]. However, many of the antifungal drugs have their own limitations. For example, amphotericin B has side effects or being very toxic [4]; peptide drugs always show a low
stability and are easily affected by their physiological environments [5]; azole and fluconazole will interact with each other to hinder the efficiency [6]. It is urgent to find novel antifungal drugs with more stability and less toxicities.

Natural medicinal plants have been used to treat diseases for thousands of years in eastern countries [7]. Medicinal plants and their extracts have many advantages in antifungal infection such as relatively low toxicity, multiple biological activities, cost-effective and so on [8], they are regarded as the important source of antimicrobial drugs [9]. Although western medicine practitioners often viewed them with skepticism, Chinese herbal medicine and plant-derived extracts/monomers have shown great potential in medical use. Currently there are many studies on the functions of medicinal plants including those extracts from Acorus calamus rhizome, Abutilon theophrasti and Hibiscus taiwanensis [10-12]. Reports have showed that Carthamus tinctorius and Lithospermum erythrorhizon contained alkaloids, flavonoids, saponins, phenolic, sterols and terpenoids, and they had many biological activities such as antimicrobial, anti-inflammatory, anti-tumor/anti-cancer and anti-ulcer functions [13-16].

The compound natural medicinal plant fermentation extracts (CNMPFE) was a complex ethanolic extract of Chinese natural and folk medicinal plants with multi-bioactive components. It is originated from an effective formula and has been used clinically in China for hundreds of years during war and peaceful time to prevent inflammation and to promote wound healing. It is cost-effective, and the delivery of CNMPFE by fabrication for topical use is considered to be safer and more acceptable. In 2015, CNMPFE was approved to be the hospital preparation by China Yunnan Food and Drug Administration (CFDA) for prevention of wound infection and festering, cell necrosis, dry gangrene and blood circulation obstacles. In addition, it was found in local application that CNMPFE could be curative for skin lesions; however, the underlying mechanism for the effects of CNMPFE was not clearly understood. In the present study, we quantified the inhibition of CNMPFE for C. albicans was dose and time dependent, the primary mechanisms were correlated to the damage and disruption of cell wall and cell membrane, which was confirmed by the marked increasing of the AKP activities, ultraviolet absorptions and electric conductivities of the fungal solutions and further confirmed by the scanning electronic microscope evaluation. Current work laid the foundation for the development of new antifungal products derived from natural folk medicinal plants.

2. Materials and Methods

2.1. Preparation of CNMPFE

CNMPFE was developed by the Institute of Yunnan Folk Medicine Co., Ltd. and produced by Yunnan Puer Danzhou Pharmaceutical Co., Ltd. (Yunnan Province, P. R. China). Briefly, dry samples of the medicinal plants including Carthamus tinctorius, Lithospermum erythrorhizon, Solanum indicum, and Cymbopogon distans (20.0, 30.0, 10.0 and 20.0 g, respectively) were milled, mixed and extracted with 90% alcohol at room temperature for 30 d, then the extracts were collected and the residues were again extracted with 90% alcohol for 15 d. The total ethanol extracts were mixed and the solution was diluted with double distilled water to 1 L (the final alcohol content was adjusted to 40-50%) and stored at 4°C. CNMPFE was an invent patent authorized by the State Intellectual Property Office of P. R. China in 2013 (No. 201110393033.3), in addition, it was approved to be disinfectant product in 2011 (China Yunnan Hygienic Disinfection Certificate [2011] No. 0004) and Hospital Preparation in 2015 (China Yunnan FDA (No. Z20150009)]. The raw medicinal plants were identified by Mr. Zhiwen Wu, Manager of the Quality Control Department of the Company.

2.2. Preparation of C. Albicans Suspensions

C. albicans strain (CCTCC AY 206001) was obtained from the China Center for Type Culture Collection (CCTCC) (Wuhan, Hubei Province, China). For preparation, the culture of C. albicans was picked from the 4°C stock and re-inoculated in potato dextrose agar (PDA) media for incubation at 30°C. The suspensions of C. albicans were thus prepared from overnight cultures and diluted the cells to concentrations of about 10^6 CFU/mL as standard fungal suspension; they were stored at 4°C for further use [17].
2.3. CNMPFE time Dependant Inhibition Assay
100 µL CNMPFE was added into the C. albicans suspensions (10^4 CFU/mL) with sterile distilled water as control. At each treatment time point (2, 5, 10 and 20 min), 500 µL of the above mixed suspensions was immediately transferred into PBS (15µM, pH 7.2) respectively, then a 100 µL aliquot was removed and serially diluted (10-fold) in saline water and plated on PDA plates. Colony numbers were determined after incubation at 30°C for 48 h. The fungal inhibition percentage of CNMPFE X was calculated using the following equation. (A: the average number of colonies in the control group, B: the average number of colonies in the CNMPFE group)

\[ X = \frac{(A-B)}{A} \times 100\% \]  

(1)

2.4. Determination of MIC and MFC for CNMPFE
CNMPFE was diluted with PDA broth medium to concentrations of 75%, 80%, 85%, 90%, 95% and 100% (by vol.) while alcohol (37.5%, 40%, 42.5%, 45%, 42.5% and 50%, by vol.) were set as the solvent control respectively. The MIC and MFC were determined by Oxford cup dilution plate method [18]. Briefly, 50 µL of C. albicans suspensions were spread onto the solid PDA plate. 10 min later, the plate was made three holes of about 10 mm with a puncher, then Oxford cup with an outer diameter of 7.8 mm and height of 10.0 mm was placed on the hole, 200 µL of serial concentrations of CNMPFE or alcohol controls were added into each cup. All the plates were at first placed at 4°C for 20 h and then incubated at 30°C for 24 h. The diameter of growth inhibition zone was measured with a vernier caliper. All the samples were assayed for three times and calculated by statistical analysis [19]. The diameter of inhibitory circle \( \in \) [20, +∞), \( \in \) [15, 20), \( \in \) [10, 15), \( \in \) (7.8, 10) and \( \in \) (−∞, 7.8] means high sensitive (+ + + +), strong sensitive (+ + +), moderate sensitive (+ +), weak sensitive (+ +) and negative (−) [20]. The minimum inhibitory concentration of CNMPFE was defined as the maximum concentration without inhibition circle. The minimum fungicidal concentration of CNMPFE was defined as the maximum concentration with least diameter zone of inhibition.

2.5. Antifungal Activity Assay
1× MFC volume of CNMPFE was added to C. albicans suspensions and incubated at 30°C. The absorbance (A) of fungal culture solution, at different incubation time point was detected by microplate reader (Infinite F50, Tecan Group Ltd. Switzerland) at 630 nm. The antimicrobial activity of CNMPFE was calculated using the following equation [21]. \( A_0 \) stands for the absorbance of the control group.

\[ U^2 = \frac{(A_0 - A)}{A_0} \]  

(2)

2.6. Cell Growth Assay
1× MFC and 2× MFC volume of CNMPFE was added separately to C. albicans suspensions, 1× and 2× volume of 40% (v/v) alcohol was used as solvent controls. The fungi was grown at 30°C, 180 rpm. OD_{620} nm was measured by microplate reader every two hours.

2.7. Alkaline Phosphatase Assay
1× MFC and 2× MFC volume of CNMPFE was added separately to C. albicans suspensions while the 1× and 2× volume of 40% (v/v) alcohol was set as the solvent controls respectively. The C. albicans suspensions were incubated at 30°C, 180 rpm. For AKP assay, the samples of fungal culture solution were taken every one hour, centrifuged and the precipitates were retained. AKP was measured according to the kit instructions [22].

2.8. Cell Membrane Integrity Assay
Assay of cell membrane integrity was mainly through measuring the leakage of cellular contents at 260nm [23, 24]. The procedure was similar to AKP assay except that the absorbance (\( A_{260} \)) of the
supernatant was measured by ultraviolet spectrophotometer (UV-5100, Shanghai Metash instruments Co., Ltd., China).

2.9. SEM Evaluation

1× MFC volume of CNMPFE and 1×volume of 40% (v/v) alcohol solvent controls were added separately to C. albicans cell suspensions. The sterile distilled water was set as the blank control. After 12 h of incubation, the samples were collected for performing SEM assay by following procedures: 1) the sample solutions were at first centrifuged at 8000 rpm for 5 min, then the supernatants were discarded and the precipitates were retained. 2) The precipitates were transferred into 2.5% (w/v) glutaraldehyde fixation solution for 4 h at 4°C [27]. 3) The samples were washed with phosphate buffered saline (0.1M, PH 7.2) for 3 times. 4) The samples were gradually dehydrated with different concentrations of tertiary butyl alcohol (TBA, 30%, 50%, 70%, 85%, 95%,by vol.,10 min/time), 100%(v/v) TBA twice (20 min/time). 5) After dehydrated in pure TBA for 10 min at 4°C, the cells were dried in vacuum drying oven (DZF-6050, Jiaxing Cema Instruments Co., Ltd., China) [28]. 6) After ion sputtering and metal spraying (JFC-1600, JEOL, Japan), the fine structure of the samples was obtained by SEM (JSM-6510, JEOL, Japan) [29].

2.10. Statistical Analysis

Statistical significance was evaluated using one-way analysis of variance (ANOVA, SPSS software version 17.0 Inc., USA) test followed by the least significant difference (LSD) test to determine if there was a significant difference against C. albicans isolates at 0.05 level. The level of significance was set at 0.05 and 0.01. Final results were presented as mean±standard deviation of three independent experiments. For multiple comparisons, tukey correction was performed.

3. Results

3.1. Time Dependant Inhibition of CNMPFE for C. Albicans

In the time dependant inhibition assay, the CFUs of C. albicans cells were sharply reduced to zero after treatment with CNMPFE. CNMPFE was able to completely inhibit the growth of C. albicans after 2 min treatment while the CFU of sterile distilled water treatment reached 1.05×10^4 mL^-1. We can conclude that 2 min treatment of CNMPFE can completely inhibit the growth of C. albicans with the inhibition percentage of CNMPFE reached to 100% (Fig. 1).

![Figure 1](image_url)

**Figure 1.** Time dependant assay of CNMPFE for C. albicans. A: The inhibition percentage of CNMPFE at 2, 5, 10 and 20 min intervals. B: CNMPFE treatment for C. albicans after 2 min treatment. C, D, E and F represent the sterile distilled water treatment control after 2,5,10 and 20 min. Samples with drawn at the indicated times were evaluated for inhibition percentage (%). Assay was performed in PDA and incubated at 30°C for 48 h.
3.2. Determination of MIC and MFC of CNMPFE against C. albicans

The inhibition of CNMPFE on the growth of C. albicans was obvious in the concentrations of 80% (v/v) to 100% (v/v) and it was dose dependent. The MIC and MFC of CNMPFE for C. albicans was 75% (v/v) and 80% (v/v) respectively (Table 1). We also found that alcohol had inhibitory effects on the growth of C. albicans at the concentration range of 40% (v/v) to 50% (v/v) (Table 1, Fig. 2A, B). However, the antifungal effect of CNMPFE was significantly stronger than those of alcohol ($P<0.05$, $P<0.01$). In addition, from the results we could also conclude that the combined use of the extracts of medicinal plants with ethanol would synergistically enhance the antifungal effects as compared with the single ethanol treatment.

Table 1. Inhibitory Circle of CNMPFE against Candida albicans in vitro

| CNMPFE concentration (%) | 75% (MIC) | 80% (MFC) | 85% | 90% | 95% | 100% |
|--------------------------|-----------|-----------|-----|-----|-----|------|
| Relative sensitivity     | $+$       | $+$       | $+++$| $+++$| $+++$| $+++$|
| Inhibitory circle diameter (mm) | 7.82±0.2 | 12.18±0.1 | 17.11±0.2 | 21.14±0.1 | 23.02±0.1 | 26.05±0.2 |
| Alcohol concentration (%) | 37.5%     | 40%       | 42.5% | 45%  | 47.5%| 50%  |
| Relative sensitivity     | $+$       | $+$       | $+$  | $+$  | $+$  | $+$  |
| Inhibitory circle diameter (mm) | 7.81±0.1 | 11.11±0.2 | 12.09±0.1 | 13.24±0.2 | 15.31±0.1 | 18.08±0.2 |
| Blank control group      | $-$       | $-$       | $-$  | $-$  | $-$  | $-$  |
| Relative sensitivity     | $-$       | $-$       | $-$  | $-$  | $-$  | $-$  |
| Inhibitory circle diameter (mm) | 7.80±0.0 | 7.80±0.15 | 7.80±0.13 | 7.80±0.10 | 7.80±0.11 | 7.80±0.09 |

Figure 2. Determination of MIC and MFC of CNMPFE against C. albicans by Oxford cup dilution plate method. (A): CNMPFE group of Ra1, Rb1, Rc1, Rd1, Re1, Rf1 at the concentration of 100%, 95%, 90%, 85%, 80% and 75% (by vol.) respectively; Alcohol solvent control group of Ra2, Rb2, Rc2 Rd2, Re2, Rf2 at the concentration of 50%, 47.5%, 45%, 42.5%, 40% and 37.5% (by vol.) respectively; Blank control group of Ra3, Rb3, Rc3, Rd3, Re3, Rf3 were all in sterile distilled water. (B): Percent concentration-dependent inhibitory effects of CNMPFE against C. albicans. All data are presented as mean ± SD. (n=3).*$P<0.05$ was considered significant difference. **$P<0.01$ was considered extremely significant difference.

3.3. Antifungal Activity and Growth Characteristics of CNMPFE Against C. albicans

In order to determine the time dependent effects of CNMPFE on the antifungal activity of C. albicans, we used 1× MFC of CNMPFE to measure the growth characteristics of C. albicans. We found the
antimicrobial activity of CNMPFE is time dependent. With the extension of time the antimicrobial activity increased. The maximum ($U=1.301\pm0.0287$) of CNMPFE for *C. albicans* was at around 5 h, then they were declined however still stable for another 4 h (Fig. 3A). In addition, when we used two different percent concentrations of CNMPFE and 40% (v/v) alcohol (1× and 2×), we found the growth inhibition of *C. albicans* was also dose dependent, 2× MFC of CNMPFE and 2× 40% (v/v) alcohol showed better inhibition than those of 1× MFC of CNMPFE and 1× 40% (v/v) alcohol respectively (Fig. 3B).

**Figure 3.** Antimicrobial activity and growth characteristics of CNMPFE against *C. albicans*. (A): Antimicrobial activity of CNMPFE against *C. albicans*. The antifungal activity was determined by measuring $\text{OD}_{630\text{nm}}$ value of mixed fungal culture solution of CNMPFE and calculated by the equation of $U^2= (A_0-A)/A_0$. $A_0$ stands for the absorbance of the control group. (B): Growth characteristics of *C. albicans* treated with 1× and 2× MFC of CNMPFE and 1× and 2× alcohol/medium (40%, v/v) solvent control respectively.

### 3.4. Antifungal Activity and Growth Characteristics of CNMPFE Against *C. albicans*

In order to determine the inhibitory mechanisms of CNMPFE against *C. albicans*, we tested AKP activity, ultraviolet absorbance and electric conductivity of the fungal solutions to find if they had impacts on the cell membrane integrity and permeability. We found that all the three parameters increased when treated with CNMPFE and alcohol as compared with their counterparts. AKP activity reached the peak value of $5.11\pm0.14$ King unit/100mL after being treated by 2× MFC of CNMPFE for 5 h (Fig. 4A). The ultraviolet absorbance of the treatment groups of 1× MFC and 2× MFC of CNMPFE was higher than those of the control alcohol groups of same concentration (Fig. 4B). The electric conductivity of fungal culture solution treated with 1× MFC and 2× MFC of CNMPFE reached $14.61\pm0.097$ ms/cm and $15.51\pm0.094$ ms/cm while the control groups of 1× and 2× 40% (v/v) alcohol reached $13.86\pm0.090$ ms/cm and $14.81\pm0.096$ ms/cm respectively (Fig. 4C). All three results revealed the disruptions of the cell membranes and the integrities of the cells were damaged.

### 3.5. SEM Evaluation of Cellular Ultra Microstructure

By SEM, we found that the cellular surface was smooth and the morphological structure was intact for the untreated control *C. albicans* (Fig. 5A1, A2, A3). After 12 h treatment with 1× MFC of CNMPFE, the fungi were seriously shrunk and deformed, we could observe extremely rough surface, much more than those of 1× 40% (v/v) alcohol (Fig.5B1, B2, B3). Moreover, the cell wall and membrane was seriously ruptured, the cytoplasm was leaked out of the cell and the conidia were scattered or/and even broken (Fig. 5C1, C2, C3). In addition, 1× MFC of CNMPFE had more obvious damage for *C. albicans*. The results further confirmed the antifungal effects of CNMPFE and were consistent with our previous conclusions that the damage of the cell wall and membrane and the leakage of cytoplasm were the preliminary mechanisms of the inhibition of CNMPFE against *C. albicans*.
Figure 4. Effects of CNMPFE on the membrane integrity and permeability of *C. albicans*. A, B and C were the determination of AKP activity, ultraviolet absorption and electric conductivity of the fungal solutions respectively. All the data were presented as mean ± SD. (n=3).

4. Discussion and Conclusions
Alcohol was well known as a generally used organic solvent and disinfectants, the extract of medicinal plants with alcohol not only enhance the leaching rate of efficient components but synergistically enhance the antifungal effects of CNMPFE. AKP not only participates in the metabolism of calcium and phosphorus to maintain the rational proportions in the body, but also relates to the secretion and synthesis of the proteins [22]. Under normal circumstances, the AKP activity of the cells almost can't be detected by the assay of the outside fungal culture solution, however, if the cell wall and membrane were damaged, the cell inside contents and AKP will be leaked out of the cells and can be measured [23, 30]. By detecting the changes of AKP activity outside the cells, we can detect the integrity or damage of the fungi cell wall and membrane. Also, when the fungi suffer from foreign stress, the protection of the fungi barrier would be broken, and the internal electrolytes would be leaked to medium and the electrical conductivity would be raised [31]. Here we found the increasing changes of AKP activities when treated with CNMPFE indicating the possible increase of membrane permeability and the leakage of the cytoplasm. The results were also confirmed by our cellular in vitro ultraviolet absorption and electric conductivity assay. After treatment with CNMPFE, the ultraviolet absorption at $A_{260}$ nm and the electric conductivity of the fungal culture solution increased significantly. This was consistent with Qian et al.'s work, who found that the increase of electric conductivity of the cultured bacterial medium was possibly related to the damage of the structure of the cell membrane [32]. The positive relation between the concentrations of CNMPFE and the inhibitory effects on the cells indicates the dose-time dependence of CNMPFE against *C. albicans* [33, 34].

SEM is a suitable method for the observation of the cellular ultra-structure [8, 35]. As expected, the damaged structures found in the cells supported our previous results of increased AKP activities, ultraviolet absorption and electric conductivities when treated with CNMPFE. Furthermore, cytoplasm...
had been leaked out of the cells and conidia had been scattered or broken and led to the death of some fungi [35].

![Figure 5. SEM observation of C. albicans treated with CNMPFE for 12 h. A1, A2, A3 were the C. albicans treated with sterile distilled water as the blank control group; B1, B2, B3 were the C. albicans treated 1× alcohol/medium (40%, v/v) as the alcohol control group; C1, C2, C3 were the C. albicans treated in 1× MFC of CNMPFE. The broken parts of the C. albicans were pointed by the black arrow.](image)

Even though CNMPFE was not the most efficient antifungal products as compared with antibiotics, however, in our previous work, we also found the products had strong inhibitions for other microbes including *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Aspergillus niger*. In addition, CNMPFE had significant effects on pain releasing, wound healing and anti-inflammation, the combined multiple functions of CNMPFE would make it the very good antifungal products for disease prevention and therapy.

5. **Conflict of Interest**
Mingzhu Song, Xirui Wang and Canquan Mao contributed equally to this work, as co-author of the first author. All authors have no conflicts of interest.

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