Anti-\textit{Candida albicans} activity and pharmacokinetics of pogostone isolated from \textit{Pogostemon Herba}

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**A R T I C L E   I N F O**

Keywords:
- Pogostone
- Anti-\textit{Candida albicans} activity
- Pharmacokinetics
- Acute toxicity

**A B S T R A C T**

The present work was designed to evaluate the \textit{in vitro} and \textit{in vivo} anti-\textit{Candida} activity of pogostone (PO), a natural product isolated from \textit{Pogostemon cablin} (Blanco) Benth. PO showed potent \textit{in vitro} activity against clinical \textit{Candida} spp. isolates tested in this study. PO and the reference drug voriconazole (VRC) were equally effective against all the fluconazole-resistant \textit{Candida albicans} strains, with MIC ranging from 3.1 \textmu g/ml to 50 \textmu g/ml. Besides, PO was fungicidal against all \textit{Candida} isolates with MFC ranging against the \textit{PO} isolates at the highest tested concentration (400 \textmu g/ml). Furthermore, oral and topical PO administration effectively reduced the fungal load in vagina of vulvovaginal candidiasis mouse models. Topical PO administration (1.0–4.0 mg/kg) demonstrated higher activity against the vulvovaginal candidiasis than VRC (4.0 mg/kg). The pharmacokinetics and safety profile of PO were also investigated. The pharmacokinetics assay revealed that PO was easily absorbed after oral administration in mice, which might account for its \textit{in vivo} anti-\textit{Candida} effect. The acute toxicity test showed that the median lethal dose of PO in mice was 355 mg/kg, which was much higher than the daily dose used for the therapeutic experiments. This study demonstrated the potential of PO as a promising candidate for the treatment of \textit{Candida} infections, particularly for vulvovaginal candidiasis.

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**I n t r o d u c t i o n**

\textit{Candida} infections, both mucocutaneous and systemic, have increased significantly in recent years (Hajieh et al. 2004; Pfaffer et al. 2006). Vulvovaginal candidosis (VVC) is one of the most common clinical manifestations of \textit{Candida} spp. that affecting 70–75% of women at least once in their lifetime, whereas 40–50% experience a recurrence (Costa-de-Oliveira et al. 2008; Mârød et al. 2002; Palmeira-de-Oliveira et al. 2009; Pauli 2006). Azole antifungal agents are the most widely used therapeutic regimen for candidiasis (Goa and Barradell 1995; Sheehan et al. 1999), particularly for VVC. However, conventional therapies are not always successful because of the significant incidence of resistance to classical antifungal agents (Coleman et al. 2010; Kremery and Barnes 2002; Pasqualotto et al. 2006). The emerging incidence of fungal infections stresses the need for the development and study of new molecules with potent action and/or prevents the development of resistance.

Essential oils from various plants have been demonstrated to possess potent antimicrobial activity. In essential oils, components that have excellent anti-fungal effects may represent a valuable therapeutic alternative for \textit{Candida} infections (Hammer et al. 1998; Van Kessel et al. 2003). Patchouli oil, the essential oil from \textit{Pogostemon cablin} (Blanco) Benth, commonly known as Pogostemon Herba, reportedly has remarkable fungistatic (or fungicidal) effects on dermatophytes (Yang et al. 2000) and \textit{Candida albicans} (Abe et al. 2003). However, little has been done to elucidate the active principle underlying its anti-\textit{Candida} activity. Pogostone (PO, C\textsubscript{12}H\textsubscript{16}O\textsubscript{4}, the chemical structure is shown in Fig. 1), a chemical marker required by law for the quality control of patchouli oil in China (Chinese Pharmacopoeia 2010), has good \textit{in vitro} activity against \textit{Penicillium}, \textit{Cryptococcus} (Kong 1986), and plant pathogenic fungi (Mo et al. 2004). However, no data has been reported on its effect on \textit{Candida} spp.
The present study aimed to investigate the in vitro anti-
*Candida* activity of PO and the in vivo therapeutic efficacy of oral versus intravaginal PO for the treatment of experimental *C. albicans* vaginitis caused by a fluconazole (FLC)-resistant isolate. Considering that pharmacokinetic properties and safety evaluations are crucial for drug development, the pharmacokinetics and safety profile of PO were also studied in mice.

**Materials and methods**

**Plant materials and chemicals**

The aerial parts of *P. cablin* were collected in October 2010 in Maoming, Guangdong province, China. It was authenticated by one of the authors (XPL, an experienced Pharmacognost) at the College of Chinese Medicines, Guangzhou University of Chinese Medicine, where a voucher specimen (No. 101009) was deposited. Voriconazole (VRC), FLC, and estradiol valerate were purchased from Sigma (St. Louis, MO). For the *in vitro* experiments, the compounds were dissolved in dimethylsulfoxide (DMSO) and were used for the biological testing at final DMSO concentrations less than 0.5%. For animal experiments, the compounds were formulated in polyethylene glycol 400 (PEG400, Hengshuo Chemical Company, Guangzhou, China) or thermosensitive poloxamer gel, which was prepared using the cold method (Chang et al. 2002). Briefly, polycarbophil (BF Goodrich, Cleveland, OH) was slowly added to citrate-phosphate buffer (0.1 M, pH 4.0) at 4 °C with gentle mixing. Poloxamer 407 and poloxamer 188 (2:1, wt/wt, BASF, Ludwigshafen, Germany) were then added to the polycarbophil solution and allowed to dissolve overnight at 4 °C. The compound was initially dissolved in a mixture of ethanol and PEG400 (3:5), and then added to cold poloxamer 407/poloxamer 188 solution containing 0.5% polycarbophil with gentle mixing.

**Extraction and isolation of PO**

The dried aerial parts of *P. cablin* (3 kg) were exhaustively extracted through water-steam distillation. Essential oil (10 g) was obtained and dissolved in ethyl acetate (50 ml), and then extracted five times with 4% NaOH (50 ml). The five alkaline extracts were pooled and 10% HCl was added to produce a pH 2 solution. The solution was extracted three times with ethyl acetate (200 ml); then, the ethyl acetate extracts were combined, washed five times with distilled water (400 ml), and further evaporated to dry, until a yellow oily liquid was obtained. After crystallization from normal hexane, white PO crystals (123.9 mg, yield 0.0042%) were finally obtained. The chemical structure of PO was identified by comparing its spectral data (MS, 1H and 13C NMR) with those published previously (Yang and Xie 1977). The purity of PO was >98% based on high-performance liquid chromatography (HPLC) analysis (Chen et al. 2011).

**Animals**

Six-week-old female Kunming mice (weight, 18–22 g) were obtained from the Medical Experiment Center of Guangdong Province (Guangzhou, China). A total of 200 mice were randomly assigned to each treatment or control group. Mice were maintained in a 12-h light/dark cycle under regulated temperature (22 ± 2 °C) and humidity (50 ± 10%) and fed with standard diet and water ad *libitum*. The animal experiments were conducted according to the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Animal Care and Welfare Committee of Guangzhou University of Chinese Medicine.

**Strains and media**

Clinical isolates of *Candida* species were isolated from infected patients in the department of Clinical Laboratory Center, Zhuijiang Hospital of Southern Medical University (Guangzhou, China). All isolates were identified by Guangdong Huankai Microbial Sci & Tech. Company, Guangzhou, China. *C. albicans* ATCC10231 obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) was used as a quality control strain. All the strains were maintained on Sabouraud dextrose agar (SDA) slants at 4 °C and subcultured for 48 h before use.

**Broth microdilution assay**

The minimum inhibitory concentration (MIC) of FLC was determined using a broth microdilution assay (National Committee for Clinical Laboratory Standards 2002). Briefly, 150 μl of RPMI-1640 broth containing 5 × 10^6 CFU/ml of 24 h-grown *Candida* yeast cells were dispensed aseptically into a 96-well microtiter plate. Doubling dilutions of each compound were aseptically incorporated into these wells. All the plates were incubated at 35 °C for 48 h and observed visually for growth inhibition. The MIC was determined as the lowest concentration that cause complete growth inhibition (no visible growth). Minimum fungicidal concentrations (MFCs) were determined according to the method used by Zore et al. (2011). Briefly, 5 μl of the cultures from the wells were placed on Sabouraud dextrose agar (SDA) plates. The plates were incubated at 35 °C for 48 h and number of colonies present on the plates was counted and compared with the growth in the control (without any compound). The MFC was the lowest concentration that killed 99.9% of the cells.

The MIC breakpoints for FLC were used as described in previous studies (Barry and Brown 1996; Sandven 1999). Isolates that exhibiting MICs <8 μg/ml, 16–32 μg/ml, and ≥64 μg/ml were classified as susceptible, susceptible-dose dependent (S-DD) and resistant, respectively.

**Mouse vaginal model**

A mouse model was used for the experimental vaginal infection as previously described (González et al. 2009). To induce pseudodestrus during infection, the mice were injected subcutaneously with 1 mg/ml of estradiol valerate in 0.05 ml of sesame oil 6 d before inoculation with *C. albicans* ATCC10231. Thereafter, the estradiol treatments were administered every other day. Infection of the vaginal canal was initiated by inoculating the mice intravaginally with 3.5 × 10^6 CFU/ml stationary-phase *C. albicans* ATCC10231 in 20 μl of phosphate-buffered saline (PBS). On days 3, 5, 7, 11 and 15 after the infection, vaginal swabs were obtained to estimate the *Candida* load. In addition, the mice were sacrificed at the aforementioned time points (3 mice per group at each time point) and the vagina of each mouse was removed to examine for the vaginal pathology.

Each alginate swab was placed in 0.9 ml of sterile saline. Serial 10-fold dilutions were made and 100 μl was placed on SDA supplemented with 0.5% chloramphenicol (wt/vol) to quantify the CFU/ml. The vaginal tissues were fixed in Carnoy’s fixative,
embedded in low–melting point paraffin, sectioned at 5 mm thickness, and stained with periodic acid Schiff (PAS) for fungal visualization.

PO was applied intravaginally once daily at 1, 2, and 4 mg/kg of body weight when used at concentrations 80, 160, and 320 times the MIC, respectively. VRC was applied intravaginally once daily at 4 mg/kg when used at 640 times the MIC. In addition, PO was administered once daily at 20, 40, and 80 mg/kg of body weight by oral gavage post-infection. VRC was administered once at 40 mg/kg of body weight by oral gavage. The control mice were infected but received a drug vehicle (7% PEG400) orally or (drug-free thermosensitive gel) intravaginally.

**Pharmacokinetic analysis**

For the intravenous pharmacokinetic study, the dosage form of PO was prepared as follows: 100 mg of PO (accurately weighed) was dispersed in physiologic saline. The pH was adjusted to 8.5 with sodium hydroxide and hydrochloric acid, and then the solution was diluted with physiologic saline to obtain a 10 mg/ml solution. Specified doses were delivered at a dose of 20 and 40 mg/kg. Two groups of mice were injected intravenously via the tail vein, and blood samples were collected before injection and at 2 min to 150 min after injection. The blood samples were immediately centrifuged at 1500 rpm for 10 min and stored at −20 °C until HPLC analysis. For the oral pharmacokinetic study, the dosage form of PO was prepared similar to that of the intravenous injection. PO was administered to the mice at 40 and 80 mg/kg doses by oral gavage. Blood samples were collected from the vena cava using heparinized syringes at 2, 5, 10, 15, 20, 25, 30, 35, 40, 60, 90, 120, 150, 180 and 240 min after drug administration. The blood samples were treated the same as that for the intravenous injection.

The following pharmacokinetic parameters were calculated using a two-compartment model for data analysis (Drug and Statistics ver 2.0 Program, Shanghai, China): the area under the plasma concentration–time curve from time zero to time infinity (AUC), the total body clearance (Cl), the mean residence time (MRT), the terminal elimination half-life (T1/2), and the apparent volume distribution at equilibrium (V1). The observed peak plasma concentration (Cmax) and the corresponding time (Tmax) were directly obtained from the raw data. The absolute bioavailability (%) was estimated according to the following equation: % = (AUCoral × Doseoral)/(AUCi.v. × Dosei.v.) × 100%, where AUCoral and AUCi.v. are the areas under the plasma concentration-time curve, and Doseoral and Dosei.v. are the actual dosages received following oral and intravenous administration, respectively.

**Acute toxicity test**

The median lethal dose (LD50) of PO was determined in mice according to method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949). Female mice fasted over night were randomly divided into six groups of ten female mice. A graded dose of each of PO (0, 250, 297, 354, 420, 500 mg/kg) was separately administered orally to the mice in each of the test groups. Each of the mice in the control group was intragastrically given the same volume of vehicle (7% PEG400) only. After administration, the mice were then allowed free access to food and water, and observed under open-field conditions for 2 weeks, and the number of deaths and signs of clinical toxicity within this period of time were recorded. The final LD50 value was calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose, i.e. the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded.

**Statistical analysis**

One-way ANOVA and a Dunnett’s test were applied to compare the differences between groups. p-Values <0.05 were considered significant.

**Results**

**FLC susceptibilities of clinical Candida isolates**

The clinical Candida spp. isolates (n = 19) tested in this study exhibited differential sensitivity toward FLC (Table 1). The C. albicans (n = 16) and Candida guillermondii (n = 1) isolates were resistant (>64 µg/ml), whereas the Candida famata (n = 1) isolates were susceptible. One Candida parapsilosis isolate was S-DD to FLC.
The standard strain (C. albicans ATCC10231) included in this study was resistant (>64 µg/ml).

**In vitro anti-Candida activity of PO**

The efficacies of PO and VRC were tested against 19 clinical *Candida spp.* isolates (differentially sensitive to FLC) and one standard FLC-resistant *C. albicans* strain (Table 1). The MIC of PO and VRC against the standard *C. albicans* strain were 12.5 µg/ml and 6.25 µg/ml, respectively. Among the 16 clinical *C. albicans* isolates tested, two showed an MIC for PO of 50 µg/ml, eight required 25 µg/ml PO, two required 12.5 µg/ml PO, one required 6.25 µg/ml, and three required 3.13 µg/ml PO. The MICs for VRC against these clinical *C. albicans* isolates were also between 3.13 µg/ml and 50 µg/ml. Moreover, the other *Candida spp.* exhibited from 12.5 µg/ml to 100 µg/ml of PO as MICs. By contrast, VRC showed higher activity than PO against these *Candida* isolates. Furthermore, PO was fungicidal against all isolates tested in this study between 50 µg/ml and 400 µg/ml. However, VRC was fungistatic and the highest concentration (400 µg/ml) failed to elicit a fungicidal effect against these *Candida* spp. isolates.

**Fungal load in mouse vaginal model**

Fig. 2A shows the results of the intravaginal PO administration for the treatment of vaginal infection. Compared with the...
control group, topical treatment with PO (1–4 mg/kg) and VRC (4 mg/kg) significantly decreased the fungal load starting from 7 days post-infection (dpi) \( p < 0.05–0.0001 \). Moreover, the fungal load in vagina in the group treated with 4 mg/kg PO was significantly lower than in the group treated with 4 mg/kg VRC within 11 days dpi \( p < 0.05 \). Fig. 2B shows the results of the orally administered PO for the treatment of vaginal infections. Compared with the vehicle control group, the oral administration of PO (20–80 mg/kg) and of VRC (40 mg/kg) significantly reduced the fungal load in mice starting within 11 dpi \( p < 0.05–0.0001 \). The topical and oral PO treatments reduced the fungal load in a dose-dependent manner.

**Histopathologic examination**

Histopathologic examination was performed to maximize the recovery and visualization of the microorganisms during the treatment time. Fig. 3 shows that except for samples from 3 dpi, the samples from all groups at other time points had variable signs of infections. After the vehicle treatment, the control groups showed visible *Candida* blastospores and pseudohyphae infiltrating epithelial cells and the number of visible microorganisms increased with time. After 7 days of topical treatment with PO and with VRC, the number of visible microorganisms gradually decreased. Similarly, after 11 days of oral treatment with PO and with VRC, the number of visible microorganisms gradually decreased.

**Pharmacokinetic analysis**

Fig. 4A shows the mean plasma concentration–time profiles after intravenous PO administration at 40 and 20 mg/kg. Table 2 summarizes the pharmacokinetic parameters of the two doses. Following intravenous doses of 20 and 40 mg/kg, the plasma concentration increased with the dose; however, the increase in AUC was not proportional to the increase in dosage. The ratio of the mean AUCs was 1:1.6, whereas the dose increased at a ratio of 1:2. In summary, the results suggest that the pharmacokinetics of intravenously injected PO increase non-proportionally between 20 and 40 mg/kg.

**Table 2**

<table>
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<th>Parameters</th>
<th>Intravenous administration</th>
<th>Oral administration</th>
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<tbody>
<tr>
<td></td>
<td>40 mg/kg 20 mg/kg 80 mg/kg 40 mg/kg</td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>47.24 24.77 29.34 13.67</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>2.00 2.00 25.00 25.00</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>31.30 12.04 53.31 51.96</td>
<td></td>
</tr>
<tr>
<td>AUC(0–1) (µg/ml min)</td>
<td>1065.64 657.61 1670.08 759.94</td>
<td></td>
</tr>
<tr>
<td>AUC(0–∞) (µg/ml min)</td>
<td>1077.00 663.60 1743.89 825.75</td>
<td></td>
</tr>
<tr>
<td>MRT(0–1) (min)</td>
<td>21.37 19.46 64.00 55.18</td>
<td></td>
</tr>
<tr>
<td>MRT(0–∞) (min)</td>
<td>23.20 20.26 74.71 71.10</td>
<td></td>
</tr>
<tr>
<td>VI (l/kg)</td>
<td>0.76 0.74 1.42 3.72</td>
<td></td>
</tr>
<tr>
<td>CI (l/min/kg)</td>
<td>0.03 0.03 0.04 0.04</td>
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Fig. 4B shows the mean plasma concentration-time profiles after oral PO administration at 40 and 80 mg/kg. The pharmacokinetic parameters of the two doses are listed in Table 2. PO was detectable in the plasma up to 240 min and 180 min following its administration at 80 and 40 mg/kg, respectively. The elimination $T_{1/2}$ of orally administered PO was 53.3 min (80 mg/kg) and 51.9 min (40 mg/kg). The increase in AUC was proportional to the increase in dosage. The $F\%$ value is listed in Table 3. The final oral $F\%$ of PO in mice was 71.39%, the average of the $F\%$ values.

**Acute toxicity**

Oral administration of stepwise, graded doses of PO in female mice gave an LD$_{50}$ value of 355 mg/kg (95% CI: 325–387 mg/kg). During the time of observation the animals exhibited decreased mobility, respiratory distress (gasping) with eventual immobility but no convulsions or loss of righting reflex prior to death.

**Discussion**

Pogostemonis Herba is the dried aerial part of *P. cablin* (Blanco) Benth and has traditionally been applied for internal use in Chinese medicine to treat common colds, diarrhea and vomiting (Chinese Pharmacopoeia 2010). The topical application of Pogostemonis Herba in treating for VVC has long been in existence because of its potent therapeutic effect on VVC (Wang 1997). However, the individual constituents of Pogostemonis Herba have not been tested for anti-Candida activity.

In this study, we evaluated the anti-Candida potential of PO isolated from Pogostemonis Herba. PO exhibited potent in vitro activity against *C. albicans* isolates. Moreover, PO was as effective as VRC against FLC-resistant *C. albicans* strains. In addition, PO exerted a fungicidal effect against the tested clinical Candida isolates. Fungicidal activity is a desirable quality for antifungal agents because unlike fungistic agents, it kills the pathogen rather than inhibiting its growth. Most of the currently available azole drugs are fungistatic and fail to eliminate the pathogen completely, particularly in *C. albicans* infections.

The considerable *in vitro* efficacy of PO against FLC-resistant *C. albicans* encouraged us to test its activity *in vivo*. The estrogen-dependent vaginal candidiasis mouse model is considered a reliable model for the *in vivo* evaluation of antifungal drugs (González et al. 2009; Intini et al. 2003; Mondello et al. 2006). This model can be effectively utilized to study various aspects of vaginal candidosis (Hamad et al. 2006). Therefore, we used this model to evaluate the effects of PO based on fungal load and histologic examinations. In addition, the route of drug administration determines the outcome of therapy (Robinson and Bologna 1994). Thus, both topical and oral treatments were applied in this study. The results suggest that both topical and oral PO administration effectively reduced the fungal load in the vagina of infected mice. Moreover, the topical PO treatment was more effective than the oral treatment, even though the effects of both were statistically compared with the control. The topical treatment significantly reduced the *Candida* load within 7 dpi, whereas the oral treatment reduced the *Candida* load at 11 dpi, which likely provided opportunity for the topical PO treatment to reach the *C. albicans* isolate in the vagina, unlike the oral treatment. In vaginal candidiasis (Robinson and Bologna 1994), topical treatment is thought to be the most effective route for drug administration because of the remarkable capacity for drug absorption in the vaginal wall. Furthermore, the carrier system in the vaginal cavity plays an important role in drug delivery (Ahmad et al. 2005; Intini et al. 2003). In this study, a mucoadhesive thermostensitive poloxamer gel was used as a carrier for the topical treatment because it is reportedly an efficient transdermal delivery system with low cellular toxicity (Chang et al. 2002). Furthermore, during the experiment, few preparations leaked from the vagina after application. Thus, the mucoadhesive thermostensitive poloxamer gel increases the retention time of PO in the vagina.

The therapeutic effect of PO on VVC was further confirmed by the histologic examination using PAS staining. PAS staining has a high specificity for detecting *Candida* (Kumar et al. 2009). Given that PAS staining only works on living fungi, it is widely used in the qualitatively assessment of the anti-fungal effect for antifungal drug discovery (Intini et al. 2003). The results showed that the *Candida* microorganisms visualized in the vagina swabs of the PO-treated and VRC-treated mice decreased with time. However, the *Candida* microorganisms in the vagina of the vehicle-treated mice increased with time. These results indicate that PO has in vivo anti-Candida activity. To understand further the *in vivo* anti-Candida efficacy of PO, we investigated its pharmacokinetics and safety profile. After PO was orally administered to the mice at 40 and 80 mg/kg, the $C_{\text{max}}$ values for PO were higher than the corresponding MIC in the ATCC10231 isolate. This result may account for the significant effect of oral PO administration on VVC. Moreover, the mean AUC increased proportionally with the dose, which was consistent with the dose-dependent effect of PO on the fungal load in the vagina. In addition, the absolute bioavailability of PO indicates that PO is easily absorbed after oral administration, which provides a theoretical basis for its orally administered forms. However, PO suffers from a rather short $T_{1/2}$ (approximately 51–53 min), which indicates that more frequent administration is required to obtain the optimum

**Table 3**

The oral absolute bioavailability of PO in mice at different levels.

<table>
<thead>
<tr>
<th>p.o. groups</th>
<th>%</th>
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<tbody>
<tr>
<td>80 mg/kg</td>
<td>80.96</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>76.67</td>
</tr>
</tbody>
</table>

| i.v. 40 mg/kg | 65.70 |
| i.v. 20 mg/kg | 62.22 |

**Fig. 4.** Plasma concentration profiles of PO after intravenous injection (A) and oral administration (B). Each data represents the mean ($\pm$SD) concentration of six mice.
therapeutic response. In our study, an acute toxicity test was conducted to evaluate the safety of PO. The LD₅₀ of orally administered PO in mice was 355 mg/kg. Thus, the daily dose of PO in our experiments was extremely low that no toxicity was observed on the vaginal mucosa.

In summary, the efficacy, pharmacokinetics, and safety profile of PO indicate that it is a promising potential treatment for Candida infections, particularly for VVC. In addition, PO cannot only be easily isolated from Pogostemon Herba cultivated extensively in Asia and West Africa (Kong 1986; Li et al. 2011), but also can be chemically synthesized using the materials which are readily accessible (Uijhara et al. 2002). These findings suggest that PO might be a good candidate for the development of new anti-Candida agents. Further studies are needed to increase the potential application of PO in vivo and to elucidate the mechanisms of action underlying the anti-Candida activity of PO.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grant No. 81173534 from the National Natural Science Foundation of China, grant No. 20111234 from Administration of Traditional Chinese Medicine of Guangdong Province of China, grant No. 2009A031000014 from Science and Technology Planning Project of Guangdong Province of China, and supported by Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2011).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2012.08.008.

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