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Original Article

The synergistic effects of hydroxychavicol and amphotericin B towards yeast-hyphae transition and the germination of *Candida albicans*

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الملخص

أهداف البحث: يعتبر التحول ثنائي الشكل من خلايا الخميرة إلى الواصلة أحد عوامل الفوعة الرئيسية للأنواع الخنية. في كثير من الأحيان تم الإبلاغ عن تطور المقاومة المضادة للفطريات ضد العديد من أمراض المبيضة. وبالتالي، فإن مزيجا من مضادات الفطريات المسجلة ذات المركبات النشطة بيولوجيا المشتقة من النبات قد تعزز الفعالية ضد العلاج الفطري. في هذه الدراسة، كنا نهدف إلى تحديد تأثير الهيدروكسي شافيكول، الأمفوتريسين ب ، والاثنين معا على انتقال وإنبات أنواع المبيضات الفموية.

طريقة البحث: قابلية مضادات الفطريات لهيدروكسي شافيكول والأمفوتريسين ب بشكل منفصل والاثنين معا ضد المبيضة البيضاء،والمبيضة المرطية، والمبيضة المدارية. تقنية "مايكروداييليوشن". تم حساب الحد الأدنى من التركيز المثبطة بناء على بروتوكولات معهد المعايير السريرية والمخبرية. تم تحديد التركيز الأدنى المثبط اللازم لكبح 50%، مؤشر تركيز الكسور، و التركيز التثبيطي اللازم لكبح 50% أيضا. تم استخدام قيم التركيز التثبيطي 50% كتركيز علاجي لهيدروكسي شافيكول والأمفوتريسين ب بشكل منفصل والاثنين معا لدراسة تأثير تثبيط مضادات الفطريات على انتقال الخميرة هيفا. تم حساب نسبة تكوين أنبوب الجرثومة لأنواع المبيضات على عدة فترات باستخدام اختبار اللون.

النتائج: كان الحد الأدنى من التركيزات المثبطة 50 مجموعة من الهيدروكسي شافيكول وحدها ضد أنواع المبيضات بين 120-240 ميكروغرام لكل مل بينما كان الأمفوتريسين بما بين 2-8 ميكروغرام لكل مل، على التوالي. أظهر مزيج من هيدروكسي شافيكول + الأمفوتريسين ب في 1: 1 و 2: 1 أقوى نشاط تآزري ضد المبيضة البيضاء. علاوة على ذلك، في غضون الساعة الأولى من العلاج، انخفضت النسبة المئوية الإجمالية للخلايا الإنبات بشكل كبير بنسبة 79 ٪.

الاستنتاجات: أظهر مزيج من هيدروكسي شافيكول + الأمفوتريسين ب التآزر ومنع نمو المبيضة البيضاء. أبطأت مجموعةهيدروكسي شافيكول + الأمفوتريسين ب عملية الإنبات وأظهرت تأثيرا طويلا متسقا حتى 3 ساعات بعد العلاج. سوف تمهد نتائج هذه الدراسة الطريق لامكانية فرض هذه الدراسات على الجسم الحي.

الكلمات المفتاحية: المبيضة البيضاء؛ هيدروكسي شافيكول؛ الأمفوتريسين ب؛ التآزر؛ إنبات المبيضات

Abstract

Objectives: Dimorphic transformation from yeast cells to hyphae is considered one of the major virulence factors of candidal species. The development of antifungal resistance against several candida diseases has led researchers to find plant derived alternatives. We aimed to determine the effect of hydroxychavicol (HC), Amphotericin B (AMB), and their combination (HC + AMB) on the transition and germination of oral *Candida* species.

Methods: The antifungal susceptibility of hydroxychavicol (HC) and Amphotericin B (AMB) separately and in a mixture (HC + AMB) against *Candida albicans* ATCC 14053, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 13803, and *Candida dubliniensis* ATCC MYA-2975 was determined by broth microdilution technique. Minimal Inhibitory Concentration was calculated based on the CLSI protocols. The MIC_{50}, fractional inhibitory concentration (FIC) index, and IC_{50} were also determined. The

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IC_{50} values were used as the treatment concentration of HC, AMB, and HC + AMB to study the effect of antifungal inhibition on yeast hypha transition (gemination). The germ tube formation percentage of candida species was calculated at several intervals using a colorimetric assay.

Results: The MIC_{50} range of HC alone against *Candida* species was between 120-240 μg per mL while that of AMB was between 2-8 μg per mL, respectively. The combination of HC + AMB at 1:1 and 2:1 demonstrated the strongest synergistic activity against *C. albicans* with an FIC index of 0.07. Moreover, within the first hour of treatment, the total percentage of germinating cells was significantly reduced by 79% ($p < 0.05$).

Conclusion: The combination of HC + AMB displayed synergism and inhibited *C. albicans* hyphal growth. HC + AMB combination slowed the germination process and exhibited consistent prolonged effect up to 3 h post-treatment. The results of this study will pave the way for potential in vivo studies.

Keywords: Amphotericin B; *Candida albicans*; *Candida* germination; Hydroxychavicol; Synergistic

Introduction

Oral candidiasis is an infection of the lining of the oral cavity that is caused by a yeast from the *Candida* species. *Candida albicans*, isolated from the oral cavity, is the most common *Candida* species. This is essentially a dimorphic fungus that can switch its morphology from yeast to hyphae and *vice versa*. The dimorphic transition of *Candida* from yeast to hyphae is considered as one of the virulence factors responsible for tissue invasion and damage.[1,2] The hyphae play a critical role in the formation of highly heterogeneous biofilm composition, providing protection against the host immune system and antifungal drugs.[3] Since the penetration of hyphae into the epithelium causes oral candidiasis, the prevention of hyphae formation could effectively reduce the virulence capacity of *Candida* species. The overuse of broad-spectrum antibiotics, as well as lengthy and redundant treatment protocols, have led to fungal resistance towards drugs, especially antifungals. Therefore alternative, harmless, and more effective treatment options are needed to mitigate this situation.[4] The formulation of new antifungal agents may be achieved by combining an existing commercially available antifungal with another agent to produce higher antifungal activity.

Natural products, as an alternative to commercialised drugs, have attracted significant attention worldwide. The World Health Organization (WHO) have endorsed the use of natural products as alternative therapeutic options which could benefit against emerging resistance to commercially available antifungals.[5] Therefore, the development of new antifungal agents with synergistic and additive interactions in a combination therapy, has become an area of compelling research interest.

Commercialised antifungal agents such as nystatin and amphotericin B, along with bioactive compounds such as hydroxychavicol,[6] allicin/alliin[7,8] and flucytosine,[9] have been recognized to possess antimicrobial properties and are considered as drugs of choice for oral fungal infections.[10,11] However, there is a dearth of literature relating to the effectiveness of phytochemicals against the dimorphism of *Candida* species. Thus, in this study, we aimed to investigate the antifungal activities of hydroxychavicol, a natural bioactive compound, in combination with amphotericin B, against the prevalent oral *Candida* species and to determine its ability to reduce hyphal growth. The findings of this study highlight the potential of antifungal combination interactions and provide significant new information relating to the possible therapeutic uses of novel combination therapy against *Candida* species.

Materials and Methods

Stock culture preparation

Strains of *C. albicans, Candida parapsilosis, Candida tropicalis,* and *Candida dubliniensis* (ATCC 14053, ATCC 22019, ATCC 13803, and ATCC MYA-2975, respectively) were procured from the American Type Culture Collection (ATCC), (Manassas, Virginia, USA). The respective lyophilised strain was rehydrated using sterile distilled water. A 100 μL aliquot of each suspension was inoculated onto yeast extract peptone dextrose agar (YPD) and incubated for 24 h at 37 °C. The resultant colonies were cultured on YPD slants and kept refrigerated at 4 °C as a working stock. Organisms were sub-cultured regularly every 2 weeks to sustain cell viability.

Standard cell suspension

A loopful of each respective *Candida* colony was dispensed in 5 mL YPD broth and vortexed thoroughly to mix the suspension. Cell suspension turbidity was adjusted to 0.5 McFarland standard which contains approximately 1×10^6 cells per mL.

Commercialized antifungal agents and bioactive compounds

In this study, we used nystatin as a positive control and 5% dimethyl sulfoxide (DMSO) as a negative control. Amphotericin B (0.25 mg/ml) (PAA Laboratory, Austria), nystatin (Sigma Chemical Co., St Louis, MO, USA), and hydroxychavicol (1 mg/ml), were dissolved in 5% DMSO (DMSO at a final concentration of < 5% v/v did not affect the MIC).

Culture media

YPD broth and agar were used to grow *Candida* strains for experimentation. Thereafter, an induction medium was prepared to galvanize the germination process by adding 10% foetal bovine serum (FBS) in YPD broth; this was referred to as yeast peptone dextrose serum (YPDS). To prepare sterile

YPDS, FBS solution was microfiltered using a 0.22 μm membrane filter. In contrast, YPD was sterilized using a heat sterilization technique. After the sterilisation process, 10% FBS was added to cooled YPD (at 50–60 °C) to produce YPDS. This was prepared fresh before every experiment.

Antifungal susceptibility testing and drug synergy

A two-fold microdilution broth method was used to determine the MIC. For this, we used a 96-well microdilution plate containing 100 μL of YPD broth in each well. The dispensed wells were labelled 1 to 12 (W1–W12). Two-fold serial dilution was performed from W1 to W10 and 100 μl of antifungal agent/bioactive compound was added. Nystatin was added in W11 as a positive control whereas YPD was added to W12 as a negative control. Next, 20 μl (1×10^6 cells per mL) of *Candida* cell suspension was dispensed to the wells designated as W1–W12. The final concentrations of AMB that were used in W1–W10 were 125, 63, 31, 16, 8, 4, 2, 1, 0.5, and 0.2 μg/ml respectively. The final concentrations of HC were 500, 250, 125, 63, 31, 16, 8, 4, 2 and 1 μg/ml in the respective wells. In this context, the lowest concentration of amphotericin B (an antifungal agent) or bioactive compound of hydroxychavicol that inhibited 50% of microbacterial growth was designated as the MIC_{50}. The reported data represented the mean value of at least three independent tests.

The bioactive compounds that exhibited antifungal properties were further analysed for their synergistic effects in combination with amphotericin B based on the MIC_{50} results obtained. The following formula was used to measure the fractional inhibitory concentration (FIC) index:

$$\text{FIC index} = [MIC_{50}(A_{comb})/MIC_{50}(A_{alone})] + [MIC_{50}(B_{comb})/MIC_{50}(B_{alone})]$$

The combined bioactive compound and antifungal agent are known to exhibit a synergistic effect. This means that the FIC indexes were ≤ 0.5; however, when the FIC was >0.5 but <1.0, this showed partial synergy, additive when the FIC was equal to 1.0, and indifferent when the FIC was >1.0 but <4.0. When the value of FIC was $\geq \times 4.0$, then this was referred to as antagonistic.[12]

Based on the obtained results, the antifungal combinations that showed synergistic effects were further investigated to identify their effects on *Candida* germination.

The inhibitory activity of AMB + HC against germination

Screening of germinated Candida

Yeast cells were incubated in YPDS induction media at 37 °C for 3 h. Next, the required amount of each respective sample was carefully transferred to the counting chamber of a haemocytometer slide. These slides were viewed under a light microscope with a 10× objective lens. For the enumeration of germinated and non-germinated *Candida* cells, an area containing a total cell count of 100 cells was used and percentages were calculated. An average of four observations were used to determine the total cell count, including fully germinated cells (cells in which germ-tube length was greater than or equal to the blastospore diameter) and/or partially germinated cells with pseudohyphae (delayed cell cycle progression with a maximum constriction between the mother and daughter cells).

(IC_{50}) median inhibition concentration on germination

The median inhibitory concentration (IC_{50}) is the concentration required to inhibit at least 50% germination in *Candida* cells. This value was used as the treatment concentration to determine the effect of antifungal inhibition on yeast–hypha transition (germinated *Candida*). In the present study, YPDS was used as the induction media and all *Candida* species were first screened for germination and incubated for 3 h at 37 °C. For the next experiment, only strains with positive germination were tested. A 96 well microdilution plate was used containing 50 μl of YPDS (diluted by two-fold). Thereafter, to induce the germination process, the plate was incubated at 37 °C for 3 h with a standard yeast cell consisting of 50 μl suspension. After incubation, a colorimetric crystal violet assay was used to measure the IC_{50} value using GraphPad Prism software (GraphPad Software version 3.00 for Windows, San Diego, CA, USA). To limit the possibility of bias and for the sake of reproducibility, three independent experiments with each of three biological triplicate samples were performed.

Antifungal inhibition testing on germinated Candida

The sample for cell treatment was prepared by incubating 1 mL of 1×10^6 cells/ml *Candida* cells suspension in the presence of the compound at IC_{50} (treated) for 15, 30 and 60 min at 37 °C. Following incubation, the cells were centrifuged (at 3000 g at 37 °C for 10 min) and washed twice with PBS. Raw (untreated) *Candida* cells will be used as a control and were prepared in the same way without any adjunctive compound.

Next, 100 mL of each sample was placed in 96-well plates after washed cells were suspended in 1 mL of YPDS induction medium; then, the plates were incubated at 37 °C. For the first 3 h of incubation, the proportion of germ tubes that were budding was monitored every hour. Thereafter, colorimetric crystal violet assays were used to record germination at 1 h, 2 h and 3 h. *Candida* cells were first exposed to the IC_{50} of HC + AMB at 15, 30 and 60 min. Absorbance readings at 590 nm wavelength was determined, and the fold changes of the germination for the treated sample was evaluated using the following formula:

$$(Abs_{control} - Abs_{treated})/Abs_{control}$$

The proportion (%) of germ tube formation was then calculated as follows:

$$\%\ \text{Germ tube formation}_{treated} = (Abs_{treated}/Abs_{control}) \times 100\%$$

The proportion (%) of controls was considered as 100%. Thus, when calculating the percentage of inhibition, the difference of germ tube formation control (in %) to the % germ tube formation in the treated group was calculated.

Table 1: Antifungal susceptibility of hydroxychavicol (HC) and amphotericin B (AMB) against *Candida* species.

	C. parapsilosis ATCC 22019	*C. albicans* ATCC 14053	*C. dubliniensis* ATCC MYA-2975	*C. tropicalis* ATCC 13803
HC alone	120	240	120	240
AMB alone	8	8	4	4
HC + AMB (1:1 ratio)	$4_{HC} + 1_{AMB}$	$2_{HC} + 0.5_{AMB}$	$2_{HC} + 0.5_{AMB}$	$4_{HC} + 1_{AMB}$
FIC index	0.16 = SYN	0.07 = SYN	0.26 = SYN	0.27 = SYN
HC + AMB (2:1 ratio)	$2_{HC} + 0.5_{AMB}$	$2_{HC} + 0.5_{AMB}$	$4_{HC} + 1_{AMB}$	$8_{HC} + 2_{AMB}$
FIC index	0.08 = SYN	0.07 = SYN	0.28 = SYN	0.53 = IND

IND, antagonistic; SYN, synergistic.

Table 2: The median inhibitory concentration IC_{50} of the antifungal combination against germination in *C. albicans*.

Species	MIC combination of HC + AMB	IC_{50} combination of HC + AMB
C. albicans	$2_{(HC)} + 0.5_{(AMB)}$	$4_{(HC)} + 1_{(AMB)}$

Results

Determination of antifungal activity

The minimum inhibitory concentration (MIC_{50}) of HC and AMB when tested separately and as a mixture against *Candida* strains is shown in Table 1. The MIC_{50} of HC alone against *Candida* species ranged from 120 to 240 µg per mL while that of AMB ranged from 2 to 8 µg per mL, respectively. The combination of HC and AMB at equal ratio resulted in a synergistic interaction against *C. parapsilosis, C. albicans, C. dubliniensis,* and *C. tropicalis*. The combination of HC + AMB at ratios of 1:1 and 2:1 demonstrated the strongest synergistic activity against *C. albicans* with a FIC index of 0.07. At a HC + AMB ratio of 2:1, the FIC index for *C. parapsilosis* was 0.08; this compared to 0.16 when the HC + AM ratio was 1:1. Against *C. tropicalis*, a synergistic affect was observed at a ratio 1:1 of HC + AMB (FIC index = 0.27); however, the interaction was found to be antagonistic (FIC index = 0.53) at a ratio of 2:1 HC + AMB. In contrast, HC + AMB was found to be synergistic at a ratio of 1:1 and 2:1 towards *C. dubliniensis*.

Germination of Candida

C. albicans was the only *Candida* species that exhibited germination; this yielded 89% of germinated cells within 3 h of incubation in induction medium. The other three *Candida* species failed to exhibit any dimorphic changes; instead, these demonstrated an increase in yeast size and cell number. *C. tropicalis* and *C. dubliniensis* failed to germinate in the tested conditions despite being reported as potentially germinating species. The IC_{50} of germination was determined for the combination of HC + AMB (Table 2).

Brief exposure to HC + AMB affected germination activity

The effect of an IC_{50} concentration of HC + AMB on the 1st, 2nd and 3rd hour of germinating cells was measured at 15 min, 30 min, and 60 min post-exposure respectively (Figure 1).

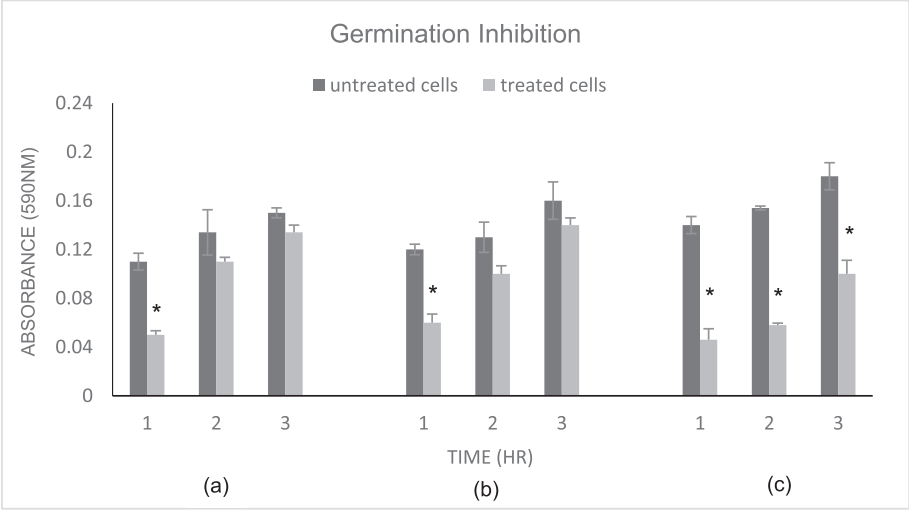

Figure 1: The reduction of germination in *C. albicans* within 3 h of incubation time after exposure to HC + AMB. (a) 15 min treatment of HC + AMB. (b) 30 min treatment of HC + AMB. (c) 60 min treatment of HC + AMB.

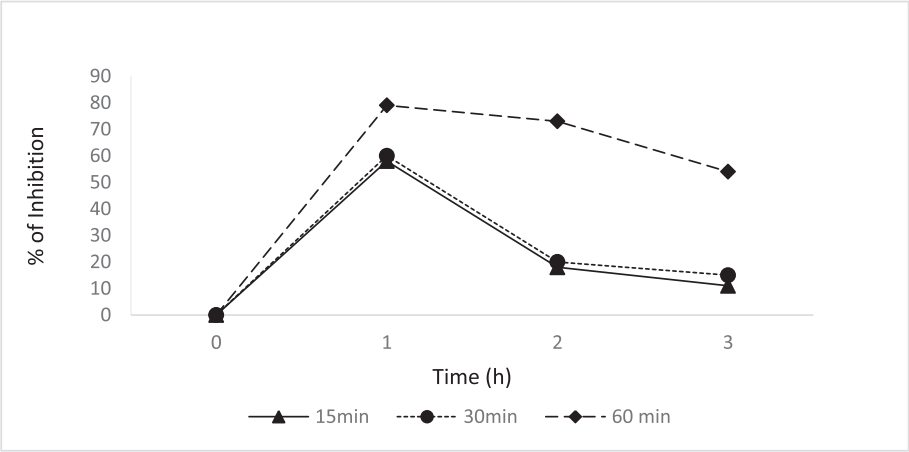

Figure 2: The post-antifungal effect of HC + AMB on *C. albicans* germination within 3 h of incubation. Drug exposure at 15, 10 and 60 min.

After 15 min of brief exposure to HC + AMB, the fold changes of inhibition for the 1st, 2nd and 3rd hour post-treatment were significantly reduced to 0.6, 0.2, and 0.1 when compared to the untreated sample, respectively ($p < 0.05$) (Figure 2). The total percentage of germinating cells was reduced by 60% after 1 h of treatment. Afterwards, a 20% and 15% reduction was observed at the 2nd and 3rd hour post-treatment, respectively (Figure 2).

For the 30 min exposure, the fold changes of inhibition after the 1st, 2nd and 3rd hour post-treatment were 0.67, 0.2, and 0.15, respectively (Figure 1); this was similar to the 15 min treatment. Germ tube reduction at 2nd and 3rd hour was not significant ($p > 0.05$).

At the 60 min exposure time, the fold changes for the 1st, 2nd, and 3rd hour post-treatment were 0.7, 0.22, and 0.2, respectively (Figure 1). The proportions of germinated cells were reduced by 79%, 73% and 54% within the 1st, 2nd, and 3rd hour post-treatment (Figure 2).

Discussion

Candida species are among the most prevalent and opportunistic microorganisms that are responsible for oral fungal infections.[10] This genus contains approximately 200 species; eight of these are found in the human body. With a detection rate of between 20% and 75% in the general population, *C. albicans* is the most prevalent form that is detected in patients with oral candidiasis.[13] This condition generally appears as a mild infection of the mucosal membranes, but when severe, can become invasive and difficult to treat.[14] The incidence of *Candida* infections has increased significantly over recent years, particularly in immunocompromised patients.[15] Traditionally, the medications prescribed by medical and dental practitioners belong to the four categories of antifungal drug: azoles, polyenes, echinocandins and allylamines. These antifungals are only partially effective and are considered as a form of adjunct therapy; furthermore, these drugs have been known to cause serious clinical challenges.[16] This situation has led scientists to study and develop a unique class of antifungal agents or potential compounds that are biocompatible, more efficient, and improve the management of *Candida* infections. There are many plants that are safe for consumption and possess potential bioactive compounds that exhibit activity towards recovery from various inflammatory conditions. Thus, various plant-derived components have been proactively investigated with regards to their use as antifungals. In this study, bioactive compounds of hydroxychavicol and a commercial antifungal agent (amphotericin B) were chosen for antifungal screening against *C. albicans, C. parapsilosis, C. tropicalis* and *C. dubliniensis*.

From the MIC_{50} data acquired, it was evident that HC exhibited antifungal properties against different species of *Candida*. Hydroxychavicol is known to be associated with multiple health benefits and has been investigated for its antimicrobial, antioxidant and anti-inflammatory properties.[17,18] Over recent studies, researchers have employed HC to induce apoptosis and to inhibit the growth of malignant pancreatic cells,[19] colon cells,[20] breast cells[21] and myelogenous leukaemia cells.[22] With regards to the oral cavity, *in vitro* studies have been conducted to assess the capability of HC to control microbial growth.[23] HC demonstrated inhibitory effects towards *Streptococcus intermedius* and *Streptococcus mutans*.[24] In the recent pandemic, HC was investigated *in silico* with regards to its ability to control the growth of oral fungal infections.[25] HC has also proven to be a promising agent against *C. albicans*.[26] None of the previously conducted studies investigated the role of HC in the inhibition of germination in species of *Candida*. Similarly, studies have revealed that the MIC value of HC is considerably high against microbial species; this is why its application in a clinical context might not be feasible.[27] Our study investigated the role of HC against *Candida* species alone and in combination with another agent with a view of enhancing its properties.

In this study, we used polyene antifungal agent, amphotericin B (AMB), which has been proven clinically to treat invasive mycotic infections for the last 60 years and is still the drug of choice for most dental practitioners.[28] This drug inhibits cell division by attaching to the cell walls of both mature and daughter cells at the budding stage by binding to ergosterol (a principal sterol in the cell membrane of fungi),

thus leading to depolarization, metabolic disturbances and fungal cell death.[29,30] However, because of its poor permeability across membranes, an increased amount of AMB must be administered to patients, often resulting in severe side effects.[31] The long-term use of AMB has been linked with renal toxicity and other acute conditions such as fever and nausea. In addition, intravenous AMB may lead to liver damage.[32] To overcome the severity of side effects, some lipid-associated formulations, such as liposomal-AMB and AMB-lipid complex were developed; however, their use is still questionable.[33] AMB was then combined with other drugs, such as isavuconazole,[34] Posaconazole,[35] and Voriconazole,[36] to enhance efficacy and reduce adverse effects. The combination strategy is often effective and beneficial for both the pace and intensity of microbial damage. Typically, every medicine has a different mode of action and by combining more than one drug, we could direct medications at multiple targets in a host cell. This multi-targeting strategy could potentially reduce the probability of drug resistance.[37] The advancement of research in medicinal plants/herbs, or bioactive natural products, is essential for the treatment of oral fungal infections.[38] Bioactive elements could help increase the intracellular concentrations of antimycotics by strengthening their efficiency, blocking efflux transporters (pumps), and preventing yeast morphogenesis.[39] Our research tested the combination of AMB with an organic agent because natural compounds are free of toxicity. Therefore, the combination of AMB with HC should be investigated as a breakthrough antimycotic combination because it exhibited synergistic effects at lowest ratio against *Candida* spp.

There are several clinical trial models that could be used to study the effects of different agents when used in combination. Drug synergy has its roots in ancient Chinese medicine, approximately 100 years ago.[40] A number of methods have been use to assess such synergy, including Time-kill, E test, and checkerboard.[41,42] The generally accepted criterion, and very simple test as to whether a combination is synergistic or not, is to calculate the fractional inhibitory concentration index (FIC) value.[43] Checkerboard evaluation has showed that the combination of HC + AMB produced MIC_{50} values that exhibited synergistic effects against all four *Candida* species with a ΣFIC index of $\geq$0.5.

Our analysis found that HC and AMB, when combined at an equal ratio, produced synergistic effects against *Candida* species, and when combined at a ratio of 2:1, the antifungal effects were maximised. It was previously reported that HC alters the membranes of the host cell, thus causing a disruption in the permeability barrier.[44] Other *in vitro* studies suggested that HC inhibits mycelial growth and spore germination.[6] With reference to commercial antifungals, polyenes bind to the fungal cell membrane *via* ergosterol, the main lipid component. Antifungal agents target specific enzymes in the ergosterol pathways to penetrate the cell membrane. AMB has the capacity to attach to ergosterol and create pores that induce the leakage of monovalent ions.[45] The goal of using synergistic agents is to enhance their therapeutic effects. Our study indicates that the combination with AMB could possibly reduce the required dosages of each agent and potentially lower drug-induced toxicities. Although our study revealed the inhibitory effects of HC + AMB against *Candida* species, more detailed investigations are now needed to investigate the exact mechanisms underlying the synergistic interaction between the two agents.

In the present study, we investigated the effect of HC + AMB on the morphogenesis of *Candida* species. Yeast-to-hypha transition can be induced by a wide range of media and environmental conditions *in vitro*. Environmental factors, such as temperature, pH, carbon starvation, growth medium, and low oxygen concentration, are known to play a vital role in the induction of hyphae formation.[46] A wide range of chemical substances, such as proline, *N*-acetyl glucosamine, and alcohols, also contribute to hyphae formation. Hyphae formation begins within minutes at 37 °C at 5–20% serum in liquid, and within hours on solid media.[47] YPDS medium containing YPD and supplemented with 10% foetal bovine serum (FBS) was chosen for the assays used in our present research; this was because of its ability to effectively induce germ tube formation. YPDS, in combination with a rise in temperature to 37 °C, and a pH of 7.4, is known to induce the growth of hyphae in the most robust manner.[47]

We tested several *Candida* species for germ tube formation under conditions which closely resembled the environment of the human body (the presence of serum and incubation at a temperature of 37 °C). Out of the four *Candida* species investigated, only *C. albicans* exhibited germination. *C. albicans* has the ability to grow in a variety of environmental conditions and unlike other *Candida* species, it can adapt and thrive in the most difficult of environments. This most likely arises from the varied sensing mechanisms adopted by the strains with regards to environmental nutrients; these mechanisms control how active their germination is. Studies have suggested that the reason for this difference might be the expression of predominantly virulent genes. *C. albicans* relies on the release of agglutinin like sequence (ALS) and aspartic proteinases (SAP), whereas hyphae-specific genes such as *Hyr1* and *Als3* are missing in *C. dubliniensis*.[48] Literature suggests that other forms of *Candida* are undergoing reductive evolution when compared to *C. albicans*. Thus, it is not surprising that only *C. albicans* was able to produce hyphae under our experimentally controlled conditions.

With regards to pathogenicity, switching between yeast and hyphal growth very important.[49] While yeast cells play a critical role in spreading and the initial colonisation of host surfaces, hyphae have been hypothesised to play a significant role in adhesion, invasion, and morphological and biochemical features.[50] These findings were reported in a study which also found that the germ tube represented the early phase of hyphal growth. Therefore, limiting the germination process may potentially assist in the prevention of hyphae-related diseases, such as oral candidiasis.[50] By analysing germination outcomes, *C. albicans* was selected to determine the IC_{50} value, which was then used to study the subsequent effect on germination, adhesion, cellular morphology, and gene expression. As the combination of HC + AMB at a ratio 1:1 exhibited strong synergistic effects similar to those seen at a ratio of 2:1, we used the 1:1 ratio further to limit germination. The ability of this combination to act as an inhibitor was assessed by determining the concentration required to halt 50% of germination. According to a previous study,[51] the germ tube is the early

stage of hyphal growth; therefore, halting the germination process could help to prevent diseases related to the presence of hyphae. The IC_{50} concentration range for the HC + AMB combination was higher and extended beyond the MIC value. This disparity could mean that specific chemicals have inconsistent and restricted inhibitory efficacy.

The antifungal activities of AMB and HC could present a step towards explaining the relationship between fungicidal or fungistatic properties towards the yeast-hyphae transition. According to a previous study, AMB is highly fungicidal against *Candida* genera, while HC exhibits fungistatic activity. Therefore, fungicidal drugs hinder the yeast-hyphae shift at sub-MIC, whereas fungistatic drugs inhibit yeast-hyphae transition only at much higher concentrations than their MIC.[52] However, our combination data showed that both the concentration of inhibition transition (IC_{50}) of fungicidal (AMB) and fungistatic (HC) activity was higher than their MIC concentration. This variation can be a sign that some drugs have variable and reduced inhibitory efficacy. The IC_{50} of the combination of HC + AMB on growth was then ascertained, as shown in Table 2.

The process of elongated tube production was hindered by the presence of HC + AMB during the short period of treatment. However, the recovery of cells started within a definite period after HC + AMB was removed and germination continued after the 1st hour post-treatment. AMB is known as a fungicidal agent and is expected to produce a prolonged effect; however, in combination with fungistatic HC at 15- and 30-min of treatment, it produces a short post-antifungal effect on germination. However, when cells were exposed to HC + AMB for 60 min, this combination exhibited a consistent prolonged effect of 79%, 73%, and 54% within a 3-h incubation period. This occurrence could be due to the combination of fungicidal and fungistatic agents acting in a slow manner and requiring long exposure (e.g., 1 h) to inflict a prolonged crippling effect on the cell.

Amphotericin B is also known to interact with the transition of *C. albicans* from yeast to hyphal morphogenesis.[53] Moreover, this drug was also shown to inhibit the transition from yeast to filamentous growth at sub-MIC concentrations. Hydroxychavicol may be involved in cell injury through interference of the cell membrane, thus causing the excessive loss of extracellular matrix and restraining the growth of cells.[44] Modifications in the cell membrane may cause a disturbance in cellular morphogenesis and cell elongation and may be responsible for the combination of HC + AMB to suppress hyphal development.

In biological processes, hydroxychavicol and AMB interact with ergosterol in the cytoplasmic membrane of fungi to form pores that allow the vital nutrients and ions to escape from the cellular environment.[54] The depletion of vital nutrients and ions leads to the apoptosis of fungal cells. As a result, the damaged cells are unable to carry out normal biological functions and inadvertently obstruct budding growth. The use of natural items in conjunction with antifungal medications is a successful strategy to combat invasive fungal infections and microbial resistance. The results of our study will pave the way for future researchers to develop strategies for improving healthcare using natural organic ingredients. Further studies, including clinical trials, are recommended to adjust doses for common fungal oral conditions.

Conclusion

In the present study, we aimed to establish the efficacy of short exposures of sub-inhibitory concentrations (MIC_{50}) of an antifungal combination on the germination of *C. albicans*. The determination of post-antifungal effect could be useful as an *in vitro* tool together with antifungal susceptibility testing to provide a better understanding of the activity of antifungal agents. Thus, drug combination research may yield new medicines for the treatment of diseases caused by *C. albicans*.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This article did not involve human participants or animals.

Consent

All authors provided consent for the publication of this article.

Authors contributions

Conceptualization, WHAWH and AF; Data collection, AF and CON-Z; Investigation, AF and CON-Z; Methodology, AF, CON-Z and NUMA; Project administration, WHAWH and AF; Resources, WHAWH; Software, AF and CON-Z; Supervision and Visualization, WHAWH; Writing − original draft, AF; Writing − review and editing, AF and NUMA. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Data availability statement

The data for this study will be made available upon reasonable request.

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