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To cite this article: Mohd Hafiz Mail, Wan Harun Himratul-Aznita & Md Yusoff Musa (2017) Anti-hyphal properties of potential bioactive compounds for oral rinse in suppression of Candida growth, Biotechnology & Biotechnological Equipment, 31:5, 989-999, DOI: 10.1080/13102818.2017.1348255

To link to this article: https://doi.org/10.1080/13102818.2017.1348255

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Published online: 04 Jul 2017.
Anti-hyphal properties of potential bioactive compounds for oral rinse in suppression of Candida growth

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ABSTRACT
Most oral rinses contain active ingredients with limited antifungal activity, particularly against hyphae development. This has encouraged us to search for bioactive compounds effective against preventing morphology transition of the dimorphic Candida. The antifungal activity of the selected bioactive compounds against seven species of oral-associated Candida was screened before the evaluation of their potency to inhibit hyphal formation. The identified potent inhibitor was then further investigated on its effectiveness through a brief treatment test on germination, adhesion, cellular morphology and gene expression. Bakuchiol, hydroxychavicol and pseudolaric acid B showed antifungal activity while luteolin and sakuranetin were determined to be inactive against the oral-associated Candida species at a concentration below 1 mg/mL. Hydroxychavicol was determined as a potent inhibitor against hyphal growth of Candida albicans. Hydroxychavicol delayed the germination process of C. albicans by affecting the expression of RAS1, NRG1 and HWP1 genes up to 1 h after the treatment. In addition, the treatment caused minimal changes to the cellular morphological structure. But, hydroxychavicol showed poor anti-adherence activity against the germinated cells. In summary, hydroxychavicol is one of the identified bioactive compounds that possessed anti-candida properties against oral-associated Candida species and appeared potent in inhibition of hyphae of C. albicans by affecting its ultrastructural morphology and gene regulation. This finding could suggest development of hydroxychavicol as a bioactive compound that can potentiate the activity of oral rinse in preventing the colonization of dimorphic Candida in the oral cavity.

Introduction
Candidiasis represents one of the leading cause of nosocomial infections in public health [1]. About 96% of invasive fungal infections including candidiasis are caused by genus Candida [2]. Candida albicans is the most common species isolated from oral cavity while other species are infrequently but consistently isolated [3]. Candida albicans has extensively developed its virulence traits for survival. It is a dimorphic fungus which is able to alternate between yeast and hyphae in certain environmental conditions [4]. Several lines of evidence suggest that hyphae morphology plays a key role in adhesion to epithelial cells to cause oral candidiasis [5] and it also contributes to formation of biofilms [6]. Other than C. albicans, Candida dubliniensis, Candida tropicalis and Candida lusitanae are also able to produce hyphae [7].

The morphology transition in C. albicans is controlled by regulatory genes such as Ras-like Protein 1 (RAS1) and Negative Regulator of Glucose-repressed 1 (NRG1) [8,9]. Additionally, the presence of hyphae also facilitates the adhesion activity which is mediated by Adhesin-like Hyphal Cell Wall Protein 1 (HWP1) [10]. This protein functions in generating the adhesion of C. albicans to host tissue surfaces. The existence of adhesin is essential for the Candida pathogenesis particularly in candidiasis infection [11,12]. HWP1 is also crucial to strengthen the biofilm structure [13]. Since HWP1 is hyphae-specific, its expression is highly regulated in hyphae rather than yeast form [14,15].

In the past few years, the use of oral rinse as a hygiene practice has increased dramatically. However, certain active ingredients in oral rinse such as sodium monofluorophosphate and thymol have been discovered to have weak activity against hyphal formation of C. albicans [16]. This may contribute to resistance of Candida biofilm formation in the oral cavity and limits the usage of oral rinses in the treatment of oral candidiasis as well. Many studies have now focused on searching novel bioactive compounds from natural products as an alternative to the current antimicrobial agents [17-19].
Bioactive compounds such as hydroxychavicol, bakuchoil, luteolin, pseudolaric acid B and sakuranetin have been identified to have antimicrobial properties [17,19–22]. However, actual potency of the bioactive compounds as antifungal agents particularly against dimorphic Candida remains scarce and insufficient.

Therefore, the present study was designed to investigate their antifungal activities against the common oral Candida species and its virulence capability involving hyphae growth. Oral rinse that is formulated with a potent antifungal agent developed from bioactive compounds is hoped to be reliable in the prevention of oral candidiasis.

Material and methods

Candida strain

All seven Candida type strains: C. albicans ATCC 14 053, C. dubliniensis ATCCMYA-2975, Candida glabrata ATCC 90 030, Candida krusei ATCC 14 243, C. lusitaniae ATCC 64 125, Candida parapsilosis ATCC 22 019 and C. tropicalis ATCC 13 803 were purchased from American Type Culture Collection (ATCC) and the samples were delivered in lyophilized form in vials. The lyophilized cells were rehydrated in sterile distilled water, followed by inoculation onto Yeast Peptone Dextrose (YPD) agar media and incubated overnight at 37 °C. Following incubation, a single colony from the culture was used in cell suspension as recommended on the product sheet of ATCC. Following incubation, a single colony from the agar was sub-cultured onto fresh YPD agar slants before being kept at 4 °C for short-term storage stock culture. For long-term storage, stock culture was prepared in 20% (v/v) glycerol and kept at −70 °C. During the experiment, working cultures were prepared on YPD agar and maintained for two weeks at 4 °C. Routine sub-culturing was carried out before being used in any experiment to ensure the viability of cells. The growth colonies from the culture were used in cell suspension preparation.

Bioactive compounds

Bakuchiol, hydroxychavicol, luteolin, PAB and sakuranetin were purchased from ChromaDexInc., USA. All bioactive compounds were delivered in lyophilized form in vials. The bioactive compounds were dissolved in 50% dimethyl sulphoxide (DMSO) as stock solution, and kept at −20 °C for long-term storage. During the experiment, working solution was freshly prepared through dilution of stock with sterile distilled water to obtain 4 mg/mL of the respective bioactive compounds in 1% DMSO. A 0.12% chlorhexidine gluconate (CHX) was used as positive control in the study.

Media

YPD agar and broth were used as the growth media for all Candida strains in the study. The media was initially dissolved in distilled water before further sterilized at 121 °C for 15 min in an autoclave. The sterilized YPD agar media was poured into 15 mm agar plates while the sterilized broth media was kept in Schott bottle and stored at 4 °C for later use.

An induction medium was prepared to stimulate the germination of Candida cells. The induction media is a mixture of YPD broth with 10% fetal bovine serum (FBS) and named as YPDS. During preparation, YPD growth media and FBS were sterilized separately. YPD was sterilized through heat sterilization using autoclave while FBS was sterilized through microfiltration using 0.22 μm membrane filter. Following sterilization, YPD was left to cool to about 50–60 °C before being added with 10% FBS to produce YPDS. YPDS was freshly prepared each time prior to the experiment.

Standard cell suspension

Candida cell suspension was adjusted to a 0.5 McFarland standard which contain approximately 1 × 10^6 yeast cells/mL. Briefly, a loopful of Candida colony from fresh culture agar was inoculated into 5 mL of YPD broth and mixed by vortex. The turbidity of cell suspensions was then adjusted spectrophotometrically to achieve an optical density (OD_{550 nm}) of 0.144 which is equivalent to the 0.5 McFarland standards.

Minimum inhibitory concentrations (MIC) determination

In our study, the MIC was determined using Clinical and Laboratory Standards Institute M38-A2, a reference method for broth dilution antifungal susceptibility testing with some modifications [23]. A 50 μL of YPD broth was dispensed into 96 wells microdilution plate marked as Well 1 (W1) to Well 12 (W12). Following this, 50 μL of the working solution (4 mg/mL) of the bioactive compounds was added into W1 and twofold serial dilution was carried out from W1 to W10. CHX was used as positive control in W11 and W12, which contained only YPD broth, represented the negative control. A 50 μL of standard yeast cell suspension was added into each well (W1–W12). Hence, the final concentrations of the compounds in W1–W10 were 1000, 500, 250, 125, 62.5,
overnight at 37 °C and then incubated at 37 °C for 3 h to induce germination. After incubation, the method of crystal violet staining (colorimetric assay) [24] was then used to determine IC50 value. In this context, IC50 was the concentration of compounds required for 50% inhibition of germinated Candida strains. IC50 and 95% confidence interval (CI) of the IC50 were determined using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Triplicate samples were performed for each test concentration and the IC50 value reported in the study was the median of three independent tests to ensure reproducibility of research data.

**Assessment of potent inhibitor against germinated Candida**

Since the usage and recommendations for using oral rinse for each gargle are practically between 1 and 2 min, we take into consideration using similar exposure period of the bioactive compounds in this study. This experiment is basically assessing the post effect on germinated Candida cells after being exposed to the potent inhibitor for 2 min with focus on germination inhibition, cellular morphological changes, adherence inhibition and gene expression changes.

In this experiment, sample preparation for cell treatment was prepared by incubating 1 mL of standardized yeast cells suspension in the presence of the compound at IC50 (treated) for 2 min at 37 °C in the incubator. After incubation, the cells were subsequently washed twice with PBS and collected by centrifugation at 3000 g at 37 °C for 10 min. Untreated yeast cells of Candida, which act as control of the experiment, were prepared similarly without the addition of the compound.

**Germination inhibition**

The washed cells were suspended in 1 mL of induction media and 100 μL of the samples were then added into 96 well plates and incubated at 37 °C. The percentage of germ tube formation was checked every hour for the first 3 h of incubation using the crystal violet staining (colorimetric assay). After the absorbance reading at 590 nm wavelength, fold change of the germination for the treated sample was calculated as follows: (Abs<sub>treated</sub> — Abs<sub>control</sub>) / Abs<sub>control</sub>.

**Cellular morphology**

The washed cells were suspended in 1 mL of induction media and incubated at 37 °C. After 3 h of incubation, the cells were washed again with PBS twice prior to immersion in 1 mL mixture of gluteraldehyde 8% and Sorenson phosphate buffer solution (1:1 ratio) for 1 h for fixation. After that, the solution was discarded and the samples were washed with a mixture of Sorenson phosphate buffer solution and distilled water (1:1 ratio). Samples were further immersed in a mixture of osmium tetroxide 4% and distilled water (1:3 ratio) at 4 °C for 14 h in fridge to coat the cells. After pipetting out the solution, the samples were then immersed for 15 min in the following series of solutions: distilled water, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% ethanol in the fume hood for dehydration steps. Following that, samples were immersed in several respective ratios of a mixture of ethanol (100%, v/v) and acetone (100%, v/v) with a ratio of 3:1, 1:1, 1:3 for 20 min. Samples were immersed in acetone 100%, v/v for 20 min for four times and dried using critical drying point technique. Once the samples were completely dried, they were mounted on aluminium stubs, with copper tape and coated with gold under low-pressure with an ion sputter coater. Three samples from each treatment were visualized with a scanning electron microscope in high-vacuum mode at 15 kV and the images processed. Any considerable changes on the morphology between the untreated and treated cells were taken into account.

**Adherence inhibition**

In the experiment, unstimulated saliva was collected by expectoration from a healthy donor between 8 am and 11 am and kept in ice-chilled test tube. A single donor was used throughout the study to minimize variations in saliva composition that may arise between different individuals. The donor was asked to gargle with distilled water for a few seconds to clean and reduce bacterial contamination before the collection begins. The saliva
was then clarified by centrifugation (17,000 g) for 30 min and the pelleted debris was discarded. The clarified saliva was then sterilized through microfiltration using 0.22 μm membrane filter. Collection from different batches was then pooled and stored at −20 °C until further use.

A 100 μL of the saliva was added in 96 well plates for 1 h to allow the formation of salivary pellicle in the wells. The saliva was then removed by gentle pipetting and the formed salivary pellicle was then incubated with 100 μL of the compound (treated) at IC50 for 2 min. Untreated salivary pellicle, which acts as the control of the experiment, was prepared similarly without the addition of the compound. The salivary pellicles were subsequently washed twice with PBS by pipetting. A 100 μL of 1 × 106 cells per millilitre of germinated cells, which was prepared prior to the experiment was added into the wells and then incubated at 37 °C. The adherence of germinated cells to the salivary pellicle was checked every hour for the first 3 h of exposure using the crystal violet staining (colorimetric assay). After the absorbance reading at 590 nm wavelength, fold change of the adherence for the treated sample was calculated as follows: (Abscontrol − Abs treated)/Abscontrol.

Gene expression

Total RNA extraction and cDNA synthesis. The washed cells were suspended in 1 mL of induction media and incubated for 3 h at 37 °C. The cells were washed again with PBS twice prior to RNA extraction process every hour of incubation period, from first to third hour. Total RNA was extracted using RiboPure™ Yeast RNA Purification Kit according to the manufacturer’s instructions. The concentration of total RNA was standardized to 5 μg by sample dilution before use in cDNA synthesis step using SuperScript® VILO™ cDNA Synthesis Kit.

Development of primers. Candidate genes that are related to hyphae – RAS1, NRG1 and HWP1 were selected for gene expression analysis. DNA sequences of the related genes were obtained from the Candida Genome Database (www.candidagenome.org). Primers (Table 1) were designed using National Center for Biotechnology Information Primer-Blast online software. The housekeeping gene act1 (ACT1) was used as a control for normalization in real-time PCR experiments. Universal primer of ACT1 for yeast was obtained from previous study [25]. For optimization purposes, the primers were subjected to standard PCR reaction.

Real-time PCR. Real-time PCR was performed in MicroAmp® Fast 8-Strip, 0.1 mL and run using the 7500 Real-time PCR System. The SYBR® green assay was used as a dye for the quantification of PCR product using SYBR® Select Master Mix kit. Real-time PCR reaction was performed at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Following PCR, samples were subjected to incubation of an ascending temperature starting from 60 to 95 °C to obtain melt curves. Control reaction was performed with RNA that had not been reverse transcribed in order to ensure that no genomic DNA amplified during the PCR reactions. Triplicate reactions were performed for each sample. RT-qPCR data analyses were performed using Applied Biosystems 7500 Fast Real-Time PCR System Software v2.3 after data collection from this experiment. Fold changes of gene expression were determined through double delta CT (2−ΔΔCT) value and relative quantification value was plotted in graph with comparison between treated and untreated sample for each incubation time.

Data analysis

Every test was run in triplicate and the data was recorded and analysed using IBM SPSS statistics v. 20 software with significance value acceptance p < 0.05.

Results and discussion

MIC of the selected bioactive compounds

MIC of the selected bioactive compounds against the Candida strains was determined as shown in Table 2. Three out of five selected bioactive compounds: bakuchiol, hydroxychavicol and PAB had antifungal activity against all seven Candida strains. Bakuchiol exhibited the most effective antifungal activity with the lowest range of MIC (31.3–125 μg/mL) followed by PAB (3.9–250 μg/mL) and hydroxychavicol (250–500 μg/mL). MIC of both luteolin and sakuranetin was unsuccessfully determined at concentration ≤1 mg/mL. Luteolin and sakuranetin were previously described to be active against dermatophytes and rice blast fungus [19,20]; however, both were found to be inactive against the Candida species tested in our study. This finding suggests that both compounds, luteolin and sakuranetin,

Table 1. Details of forward and reverse primer of ACT1, RAS1, NRG1 and HWP1 used for gene expression analysis.

<table>
<thead>
<tr>
<th>Sets</th>
<th>Gene</th>
<th>Direction</th>
<th>Primers (5’ – 3’)</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACT1</td>
<td>F</td>
<td>CGATACGGTTCCTGATG</td>
<td>52.5</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>RAS1</td>
<td>R</td>
<td>CTCCTAGCTTCTGATAC</td>
<td>50.6</td>
<td>212</td>
</tr>
<tr>
<td>3</td>
<td>NRG1</td>
<td>R</td>
<td>AAAACCTACCCGGTCCTCA</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HWP1</td>
<td>R</td>
<td>TGGGTCTTCACTGGTGGT</td>
<td>59.8</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>R TGGGTCTTCACTGGTGGT</td>
<td>59.7</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>R GCAGATGGTTCAGTGGTG</td>
<td>58.5</td>
<td></td>
</tr>
</tbody>
</table>
are not anti-candida agents. Based on the chemical structure, the selected bioactive compounds are categorized into three different groups which is terpene (bakuchiol and PAB), flavonoid (luteolin and sakuranetin) and phenylpropanoid (hydroxychavicol). In previous reports, most of the terpenes and phenylpropanes are known to be active against isolates of Candida species [26,27] but certain flavonoids need to be coupled with other commercial drugs to be fully active against the species [28].

**Identification of potent inhibitor**

Out of the seven Candida strains, only one strain – C. albicans was positive in germination. Candida albicans produced 98% of germinated cells in the induction media within 3 h incubation period. Other strains did not display any morphological changes but showed an increased cell number and grew in budding yeast form. The bioactive compounds that showed antifungal activity in earlier experiment were further subjected to germination inhibition of C. albicans.

IC$_{50}$ of the antifungal bioactive compounds and CHX on germination were determined as shown in Table 3. CHX demonstrated the most effective inhibition activity with the lowest IC$_{50}$. Among the bioactive compounds, hydroxychavicol showed the lowest IC$_{50}$.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Candida albicans ATCC 14053</th>
<th>Candida dubliniensis ATCC MYA-2975</th>
<th>Candida glabrata ATCC 90030</th>
<th>Candida krusei ATCC 14243</th>
<th>Candida lusitaniae ATCC 64125</th>
<th>Candida parapsilosis ATCC 22019</th>
<th>Candida tropicalis ATCC 13083</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakuchiol</td>
<td>125</td>
<td>250</td>
<td>125</td>
<td>62.5</td>
<td>62.5</td>
<td>62.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Hydroxychavicol</td>
<td>250</td>
<td>31.3</td>
<td>125</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Luteolin</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
</tr>
<tr>
<td>Pseudolaric acid B</td>
<td>3.9</td>
<td>3.9</td>
<td>250</td>
<td>250</td>
<td>125</td>
<td>15.6</td>
<td>125</td>
</tr>
<tr>
<td>Sakuranetin</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
<td>ND$^{b}$</td>
</tr>
</tbody>
</table>

Note: ND = Not determined.

MIC was unsuccessful determined at concentration $\leq$ 1 mg/mL.

Each compound has tended to act as an inhibitor against germination. However, certain compounds such as bakuchiol and PAB have a higher concentration range of IC$_{50}$ which is greater than the MIC determined. This variance may indicate the capacity of certain compounds in possessing limited and inconsistent inhibitory activity. In order to describe the differences better, a previous study [29] has formulated a calculation based on IC$_{50}$/MIC ratio. This ratio can be used to determine the potency of each agent in the inhibition of either budding yeast or germination of hyphal growth. It has suggested that an agent preferentially inhibits hyphal growth if the IC$_{50}$/MIC ratio is less than 1. If the ratio is more than 2, an agent is considered to have low ability to inhibit hyphal growth, but exert their activity by inhibiting the budding yeast growth. In contrast, if the ratio is between 1 and 2 an agent is considered to have approximately equal effects on both activities.

Hydroxychavicol was considered to have higher potency in the inhibition of hyphal growth with ratio below 1 while bakuchiol and PAB were considered to be more potent in inhibiting the growth of yeast bud with ratio more than 2. CHX has an equal inhibition effect on both budding and hyphal growth with ratio between 1 and 2 (Table 4). What makes the potency different

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (mg/mL)</th>
<th>MIC (mg/mL)</th>
<th>IC$_{50}$/MIC ratio</th>
<th>Preferential growth inhibition</th>
<th>Based on IC$_{50}$/MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakuchiol</td>
<td>642.6</td>
<td>125</td>
<td>5.1</td>
<td>Budding yeast</td>
<td></td>
</tr>
<tr>
<td>Hydroxychavicol</td>
<td>151.7</td>
<td>250</td>
<td>0.6</td>
<td>Hyphal growth</td>
<td></td>
</tr>
<tr>
<td>Pseudolaric acid B</td>
<td>502.9</td>
<td>3.9</td>
<td>128.6</td>
<td>Budding yeast</td>
<td></td>
</tr>
<tr>
<td>*Chlorhexidine gluconate (CHX)</td>
<td>15.8</td>
<td>9.4</td>
<td>1.7</td>
<td>Budding yeast and hyphal growth</td>
<td></td>
</tr>
</tbody>
</table>

Note: Determination is based on IC$_{50}$/MIC ratio. This ratio determines the relative potency of each agent to inhibit budding and hyphal growth: (1) ratio $<1$ (agents which preferentially inhibit hyphal growth), (2) ratio of 1–2 (those with approximately equal effects on both) and (3) ratio $>2$ (agents with a lower ability to inhibit the hyphal growth but exert their activity by inhibiting the budding yeast growth).
between the compounds could be possibly derived from their specific action mechanism on the biological target.

In biological reactions, hydroxychavicol and CHX are capable to alter the cell membrane structure and disrupt the permeability barrier of the membrane [17,30]. Consequently, the injured cells are incapable to perform normal biological activity and indirectly inhibit the germination as well as budding growth. PAB is known to inhibit the synthesis of mitotic spindles, collective spindle fibres formed during mitosis [31]. Mitosis is crucial during cell growth in living yeast, but its process is variable in different growth mode. During budding growth, yeast cells will initially undergo mitosis stage before further buds off daughter from parent cells [32]. In germination, yeast cells will start to germinate before producing elongated tube through mitosis stage [33]. These factors may explain the inefficient activity of PAB against germination. From this observation, it is suggested that distinct mechanisms of the mitosis in yeast growth modes affect the activity of certain compounds.

**Hydroxychavicol suppressed germination**

Hydroxychavicol exhibited suppression effect on the germination activity of *C. albicans*. Significant inhibition can be seen within the first hour with 0.4 fold reduction but not in the second and third hour post-treatment with 0.1 fold reduction compared to the untreated sample (Figure 1). The presence of hydroxychavicol during the brief treatment delayed the process of elongated tube production but then it started to return to its normal biological activity after the first hour post-treatment. Hydroxychavicol may inhibit the growth of cells and cause injury by disrupting the cell membrane [17]. However, the cells are able to recover within a certain period after hydroxychavicol was removed and continue germinating. Since the effect of 2 min exposure was 1 h inhibition, we expected that longer time inhibition could be achieved when the exposure time increased.

**Hydroxychavicol caused minimal changes to cellular morphology**

The activity of hydroxychavicol was further studied for their effect on the morphological appearance of the germinated cells by using a scanning electron microscope and the result was shown in Figure 2. For the untreated control sample, it can be concluded that the cells had developed the optimal size, rounded shape and had a smooth surface of the germinated tube. Following 3 h treatment with hydroxychavicol, similar observation was seen in the untreated sample but some of the cells have minimal changes such as constriction at the germ tube neck and had rough surface morphology on the whole surface of its body and tail. Hydroxychavicol may cause slight environmental stress towards cell growth, which would restrict the ions and nutrients uptake mechanisms that normally take place on the cell surfaces. Some hyphal cell wall constituents which are less firmly bound to the rigid glucan-chitin network may be lost during the treatments which then caused uneven cell surface. Previous study has suggested that the loss of a few cell wall components could lead to the loss of structural integrity [34].

**Anti-adherence of hydroxyhchavicol**

Oral surface comprises of salivary pellicle, a covering protein film that has a selective binding of glycoproteins. Cell membrane of *C. albicans* has non-polar cell surface proteins and the cells are able to bind to the pellicle through hydrophobic interaction. Rationally, if the cells are in hyphae form, it has more adherence capacity to pellicle layers due to the large surface area of the elongated membrane.

Treatment of the experimental pellicle with hydroxychavicol at IC₅₀ (152 μg/mL) was found to exhibit insignificant reduction in the adhering capacity of the germinated *C. albicans* strain (Figure 3) with 0.01 fold reduction after the first, the second and the third hour post-treatment. We suggest that hydroxychavicol has poor anti-adherence effects against the germinated *C. albicans*. It has been described that certain antifungal agents were able to alter the protein receptors on salivary pellicle resulting in failure recognition of *Candida* cells [35]. Furthermore, physiochemical forces from cell surface hydrophobicity (CSH) of the cells may be implicated in assisting its adherence to treated pellicle. In a previous report, some researchers have suggested that CSH is directly involved in adherence since it eases the
binding process of microbes to epithelial cells [36]. In this case, the CSH was increased in germinated cells since the elongated tube has large surface area compared to yeast form. This situation increases the physiochemical forces and strengthens the binding affinity of the cells even to the treated salivary pellicle.

**Transcriptional response of RAS1, NRG1 and HWP1 gene to hydroxychavicol**

*Candida albicans* change its morphology from yeast to hyphae through germination process. In untreated condition normal yeast-hyphae transition involved *RAS1* gene that works as a signalling transducer for cAMP-PKA pathway [37] being upregulated when the cells being cultured in YPDS [38]. As a result, cAMP signalling pathway is activated. The upregulation of *RAS1* has indirectly downregulated the expressions of *NRG1*, a repressor gene which blocks the transition activity of *Candida* yeast [39]. Thus, the lowered expression of repressor gene *NRG1* has encouraged yeast transition activity and resulted in germination. Changes in the regulatory genes have finally resulted in a higher expression of hyphae specific genes including *HWP1*. *HWP1* is a gene that encodes for a cell wall adhesion protein which helps *C. albicans* to attach strongly to oral surfaces [40].

In our study, we found that the relative expression of the genes changed with time in hydroxychavicol-treated cells (Figure 4). During the treatment (0 h), expression of these genes were initially downregulated with 0.68, 0.90 and 0.71 fold changes for *RAS1, NRG1* and *HWP1*, respectively. However, the expression of *RAS1* was initially upregulated with 1.40 fold change both within the first and second hour but was then downregulated with 1.0 fold change within the third hour post-treatment; the expression of *NRG1* was upregulated within the first hour with 1.40 fold changes but was then downregulated with 1.0 and 0.87 fold changes within the second and third hour post-treatment, respectively. In contrast, expression of *HWP1* was downregulated with 0.96 fold change within the first hour and then began to show signs of upregulation with 1.53 and 1.19 fold changes within the second and third hour post-treatment, respectively.

Apart from causing damage to the cell membrane [17], we found that hydroxychavicol-inhibited cell
germination by regulating the hyphae-related genes expression (Figure 5), especially on RAS1 which caused the inhibition to the growth of cells. Since RAS1 plays an important role in cell sustainability [41], this could be the reason why the cells experienced immediate upregulation of RAS1 gene for restoration of its normal physiological condition after the exposure to hydroxychavicol. Within this period, biological processes including cell cycle progression and life span were recovered by upregulation of RAS1 gene expression [42,43].

The downregulation of RAS1 gene expression lead to the inactivation of cAMP-PKA signalling pathway. However, with time, the expression of NRG1 was found to be downregulated and cells begin to regain its ability to germinate. This finding has answered the reason why the number of germinated cells was substantially elevated in the second and third hour after exposure to hydroxychavicol.

HWP1 initially downregulated during treatment but regained to its normal level of expression after the first hour and continued to be upregulated with time. Thus, the cell begins to produce an abundance of hyphal protein to support the growth of hyphae.

Our study has demonstrated that bakuchiol and PAB are antifungal agents against Candida strains but did not act as anti-hyphal agents. However, hydroxychavicol was found to possess anti-hyphal characteristic but was insufficient to eliminate C. albicans from salivary pellicle at dosage of 152 μg/mL. The possible mechanism of hydroxychavicol to inhibit the hyphal growth is by (1) altering the cellular morphology which lead to the unproductive cells and (2) delaying germination process by changing the expression of hyphae-related genes such RAS1, NRG1 and HWP1. Our finding strongly suggests that hydroxychavicol is effective against Candida species such C. albicans and this could support its usage in oral rinse to control and prevent candidal infection especially among target users like cigarette smokers [44] and immunocompromised patients [45].

Figure 4. Relative expression of RAS1, NRG1 and HWP1 genes of treated and untreated C. albicans.
Conclusions

In this study, we found that hydroxychavicol is one of the identified bioactive compounds that possessed antifungal properties against oral-associated Candida species, potent in inhibition of hyphae development of C. albicans by affecting the morphological and gene regulation. Since hydroxychavicol has poor anti-adherence activity against germinated cells, we recommend further study of the synergistic potential with other bioactive compounds to improve its properties towards preventing colonization of C. albicans in the oral cavity.

Acknowledgments

We are highly gratitude to the Balai Ungku Aziz Research Laboratory (BUARL), Faculty of Dentistry, University of Malaya for research facilities.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the University of Malaya under the High Impact Research (HiR) [grant number UM.C/HiR/MoHE/DENT/17].

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