An in vitro study on the anti-adherence effect of Brucea javanica and Piper betle extracts towards oral Candida

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A B S T R A C T

Objective: The adherence of Candida to mucosal surfaces is the initial step for successful invasive process of the oral cavity. The study aimed to investigate the effect of two plant extracts on the non-specific and specific bindings of oral candida.

Methods: In the former, adsorption to hexadecane was used to measure the hydrophobic interaction of the candida cells. In the later, glass beads coated with saliva represented the experimental pellicles in specific adhesion of oral candida to hard tissue surface.

Results: Candida krusei, Candida dubliniensis and Candida tropicalis showed the highest adsorption to hexadecane at 30.23%, 26.19% and 19.70%, respectively, while the others within the range of 7–10%. All candidal species were significantly affected by the extracts (P < 0.05) with Brucea javanica exhibited more than 60% reduction of CSH than Piper betle. Candida parapsilosis showed the highest affinity in specific-bindings to pellicle with 18.72 ± 0.71 × 10³ CFU/ml. Exposing to P. betle-treated pellicle has drastically reduced the adherence of C. tropicalis, Candida albicans and C. krusei by 86.01%, 61.41% and 56.34%, respectively. B. javanica exhibited similar effect on C. tropicalis (89.86%), Candida lusitaniae (88.95%), C. albicans (79.74%), Candida glabrata (76.85%) and C. krusei (67.61%).

Conclusion: The extracts demonstrated anti-adherence activities by modifying the CSH and the characteristics of the experimental pellicle.

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1. Introduction

Human oral cavity constitutes a multitude of substrata to which microorganisms such as candida may adhere. The ability of candida to adhere to dentures is crucial in the development of an infection because the presence of this resistant microorganism may perturb the stability of the oral microflora.1–3 To establish successful colonisation, both the non-specific and specific binding mechanisms are involved. In the former, adhesion depends very much on the non-polar component of the cell wall structure while in the later, more specific receptors are involved.

The surface hydrophobicity of a cell wall (CSH) has great influence on its ability to adhere and forms biofilm on inert surfaces.4 CSH is characterised by the presence of hydrophobic proteins that are embedded in the cell wall matrix of the candida. This cell wall which is found beneath an outer fibrillar layer provides the hydrophobic interactions between the candida and host surfaces.5 Several studies have reported that
the hydrophobicity of yeast cells correlate positively with Candida-endothelial cell, Candida–Candida and Candida-immobilised binding proteins.6,7

In the oral cavity, the adherence of candida to tissue surfaces is also mediated by specific adhesion mechanism that occurs between the acquired pellicle and the candidal cells. Salivary components of the acquired pellicle promote the adhesion of pioneer candida by providing many specific receptors. Like most microorganisms, Candida possess a dynamic cell wall structure with components that are specifically designed to bind to a variety of ligands on the host cell surface via protein–protein interactions, protein–carbohydrate interactions and candida mannoprotein ligands.8 These interactions endowed with hyphae, enables candida to attach and invade tissues of the host.

Malaysia is blessed with natural products with potent and unique medicinal properties that have been reported to exhibit various biological activities. Their usage as traditional remedies is popular as there have been minimal reports on side-effect towards the host.9 Piper betle and Brucea javanica are two local plants that have been reported to possess several biological activities and used as a common ingredient in local medicines. P. betle exhibits antibacterial, anti-adhesion10,11 and antifungal12 activities, while B. javanica possess cytotoxic effect on cancer cell lines.13 Data on the antifungal effect of these plants on oral Candida is still scarce.

The study was designed with the objectives of evaluating the effect of B. javanica and P. betle aqueous extracts on the non-specific and specific adherence mechanisms of seven species of oral candida. The study focused mainly at the responses of candidal cells at the active growth stage as the physiological growth and metabolic activity of the cells are at their optimum during the log phase. Data obtained may contribute to a better understanding of the antifungal properties of these plants which can be promoted as an alternative antifungal agent.

2. Materials and methods

2.1. Plant collection and extract preparation

Fresh P. betle leaves (100 g) and B. javanica seeds (100 g) were collected from a rural area in Sekinchan Selangor, Malaysia. The specimens were identified by a botanist from the Institute of Biological Science, Faculty of Science University of Malaya. The voucher specimens were deposited at the Herbarium of Rimba Ilmu, University of Malaya. Crude aqueous extract of the specimens was prepared according to Himratul-Aznita et al.12 The specimens were washed and oven-dried at 60–65 °C for two days. The dried specimens were homogenised in distilled water at a ratio of specimens to water of 1:10. The homogenate was heated at high temperature and concentrated to one-third of the original volume. The concentrate was filtered through a filter paper (Whatman No. 1) before it was further concentrated to a final volume of 100 ml. The decocion was then freeze-dried overnight (EYELA FDU-1200, Tokyo) and the powder was kept in sterile Falcon tubes and stored at 4 °C. A stock solution of the extract was prepared in sterile distilled water at a concentration of 200 mg/ml. Following centrifugation (Jouan A14, France) for 10 min at 8000 × g, the stock was then diluted to concentrations required for the experiment. The extract was sterilised by filtration using 0.2 μm nylon syringe filter (Millipore, USA).

2.2. Candidal strains

Seven strains of oral Candida that includes Candida albicans ATCC 14053, Candida dubliniensis ATCC MYA-2975, Candida glabrata ATCC 90030, Candida krusei ATCC 14243, Candida lusitaniae ATCC 64125, Candida parapsilosis ATCC 22019 and Candida tropicalis ATCC 13803 were purchased from the American Type Culture Collection (ATCC), USA for use in the study. Yeast Peptone Dextrose (YPD) broth (BD Difco™) was used to revive the cultures.

2.3. Determination of cell surface hydrophobicity (CSH)

2.3.1. Preparation of candidal suspension

A loopful of candidal colonies was inoculated in fresh YPD broth and incubated at 37 °C for 8 h where the log phase is attained. Following this, the cells were harvested by centrifugation at 8000 × g. Cell pellet was washed twice with PBS and resuspended in the same buffer. The cell density was adjusted at an absorbance of 0.450 at 550 nm which is equivalent to 1 × 10^7 cells/ml. Following examination using light microscope, at this log phase all cells were observed to be oval and most were in the budding stage presenting characteristic of yeast.

2.3.2. CSH of Candida strains

Determination of CSH was carried out following the protocol of Klotz et al.14 To measure the non-specific adhesion, adsorption to hexadecane was used to measure the hydrophobic interaction of the candida cells. A volume of 2 ml of cell suspension (10^7 cells/ml) of each strain was respectively dispensed into sterile glass tubes. To each tube, 2 ml of sterile saline was added to give a final volume of 4 ml. Sterile saline was used as a blank control. 200 μl of hexadecane which represented the hydrophobic surface was then added and the tubes were vigorously agitated for 1 min. The tubes were then left to stand at room temperature for 15–20 min to allow for separation of hexadecane from the aqueous phase. The lower aqueous phase of the mixture was gently aliquoted out into cuvette and the absorbance (A₀) was read at OD 550 nm. Hydrophobicity was expressed as a percentage of adsorption of the candidal cells to hexadecane. Each experiment was carried out in three independent experiments performed in triplicate to ensure reproducibility. The relative CSH was determined by the following equation:

\[
\% \text{change in } A_{550} = \frac{[A_1 - A_{0}]}{A_0} \times 100
\]

where A₀ is absorbance of the total cell suspension in the absence of hexadecane and A₁ is absorbance of the total cell in the presence of hexadecane.

2.3.3. CSH of Candida strains treated with B. javanica and P. betle extracts

Two millilitres of candidal suspension was aseptically dispensed into sterile tubes and appropriate volume of the
stock extracts was added to give final concentrations of 1, 3 and 6 mg/ml, respectively. The optical density of each tube with the extract was read to represent the initial absorbance in the absence of hexadecane (A0). Following the addition of 200 μl of hexadecane, similar procedure as above was repeated. The percentage of adsorption to hexadecane for each of the extract-treated candida was determined. Reduction in these percentages when compared to the CSH determined in the absence of the extracts was taken as the effect of the extracts on the CSH.

3. Determination of adherence to saliva-coated surface

3.1. Collection of saliva

Unstimulated saliva obtained from a healthy donor was collected into ice-chilled sterile test tubes. A single donor was used throughout the study to minimise variations that may arise between individuals. The donor was first asked to rinse the mouth with distilled water before the beginning of collection at 9.00 am to 11.00 am, to reduce bacterial carriage from the oral cavity. The saliva was then clarified by centrifugation at 17,000 × g for 30 min. The pelleted debris was discarded and the clarified saliva was further filter-sterilised and stored at −20 °C prior to use.

3.2. Preparation of salivary pellicle

An artificial mouth (NAM) model (Fig. 1) was set up and preparation of experimental acquired pellicle was performed following a standard procedure described by Rahim et al.15 Ten sterile glass beads were coated with saliva by allowing clarified sterile saliva to flow into the NAM model for 2 min at a constant rate of 0.3 ml/min to allow for the formation of the experimental acquired pellicle on the glass beads. Sterile distilled water was then run into the system to rinse excess saliva from the glass beads. Salivary components that got deposited on the glass beads will act as specific binding receptor in the acquired pellicle.

3.3. Adherence of Candida to salivary pellicle

To measure for the specific adhesion, glass beads coated with saliva represented the experimental pellicles in specific adhesion of oral candida to hard tissue surface. Candidal suspension of 10⁶ cells/ml was pumped into the NAM model and left to circulate overnight (37 °C) to allow for the adhesion of the candida to the experimental pellicle. Determination of adhering cells was conducted by transferring the glass beads into sterile microcentrifuge tube containing one millilitre of phosphate-buffered saline (PBS). Following sonication for about 15 s, the dislodged candida was serially diluted to 10⁻⁷ in preparation for agar plating. 100 μl from each diluted tube was drawn and plated on three separate YPD agar plates. Following incubation at 37 °C for 24–48 h, the colony forming unit (CFU) was enumerated. Based on the formula given, the population of candida adhering to the salivary pellicle on the glass beads was calculated and determined:

\[ \text{Total CFU (ml)} = \frac{\text{Number of colonies formed}}{\text{Dilution factor} \times \text{volume used (ml)}} \]

3.4. Adherence of Candida to extract-treated salivary pellicle

In this procedure, 10 ml of the respective extracts at 6 mg/ml was introduced into the NAM model for 2 min to treat the experimental pellicle. Similar candidal concentration in Section 3.2 was then pumped into the model and left to circulate overnight at a constant flow rate of 0.3 ml/min. The population of adhering candida was determined based on CFU counts following procedures similar as in Section 3.3. The anti-adherence effect of the extract was indicated by the reduction in the number of candida adhering to the extract-treated pellicle when compared to the untreated pellicle determined in Section 3.3.

3.5. Statistical analysis

All data obtained were computed and expressed as mean ± standard deviations (SD) from three independent experi-

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**Fig. 1 – An illustration of the Nordini Artificial Mouth (NAM) model.**
ments performed in triplicate \((n = 9)\). Statistical analysis was performed using SPSS software (version 17.0). Independent t-test was used to compare the significant difference between the adsorption of candidal cells to hexadecane following exposure to \(C. javanica\) and \(P. betle\). One-way ANOVA and post hoc test Dunnett’s T3 were also used to compare the significant difference between the groups. A P-value of <0.05 was considered as statistically significant.

4. Results

4.1. Cell surface hydrophobicity of oral candida

All seven candida species demonstrated varying degree of CSH. \(C. krusei\), \(C. dubliniensis\) and \(C. tropicalis\) can be categorised as highly hydrophobic among the others (Fig. 2). The other four species that includes \(C. glabrata\), \(C. albicans\), \(C. lusitaniae\) and \(C. parapsilosis\) exhibited significantly lower CSH at less than 10% \((P < 0.05)\).

4.2. Effect of \(C. javanica\) and \(P. betle\) on CSH of oral candida

Both extracts were found to have reducing effect on the CSH of all seven candida species \((P < 0.05)\). Compared to the untreated, \(C. javanica\) has drastically reduced the CSH of all candida species by more than 50% \((C. albicans 82.08\%, C. krusei 81.52\%, C. lusitaniae 74.60\%, C. parapsilosis 72.24\%, C. tropicalis 60.57\%, C. dubliniensis 58.64\%, C. glabrata 53.31\%) (Fig. 3). Exposing the candidal to higher concentrations of \(C. javanica\) has further reduced the CSH by more than 90% \((P < 0.05)\) indicating the reduction effect was concentration-dependent. Similar pattern of reduction in the CSH of all candida was also observed following treatment with \(P. betle\).

Between the two extracts, \(C. javanica\) showed relatively stronger effect on CSH compared to \(P. betle\) (Fig. 3). At 1 mg/ml for instance, \(C. krusei\) and \(C. tropicalis\) treated with \(C. javanica\) showed more reduction of CSH compared to treatment with \(P. betle\) extract. Independent t-test showed that the means percentage adsorption of \(C. javanica\)-treated \(C. albicans\), \(C. dubliniensis\), \(C. krusei\), \(C. parapsilosis\) and \(C. tropicalis\) to hexadecane was very much reduced and differ significantly \((P < 0.05)\) from those treated with \(P. betle\).

4.3. Adherence affinities of Candida species to experimental pellicle

It was observed that different species showed variation in their adhering capacity to the experimental pellicle. \(C. parapsilosis\) adhered with the highest capacity at 18.72 ± 0.71 × 10⁴ CFU/ml, followed by \(C. lusitaniae\) \((14.39 ± 2.38 × 10⁴ CFU/ml), C. tropicalis\) \((8.58 ± 0.53 × 10⁵ CFU/ml), C. albicans\) \((6.22 ± 0.87 × 10⁵ CFU/ml)\) and \(C. glabrata\) \((7.17 ± 1.87 × 10⁵ CFU/ml). The adhesion of \(C. dubliniensis\) and \(C. krusei\) were comparatively lower at 2.76 ± 0.10 × 10⁵ CFU/ml and 2.13 ± 0.38 × 10⁵ CFU/ml, respectively. These adhering populations will be taken as the maximum \((100\%)\) adhering capacity of the experimental pellicle for each of the \(Candida\) species.

4.4. Anti-adherence effect of \(C. javanica\) and \(P. betle\) extracts

The anti-adherence effect of the extracts was measured by the reduction in the percentage of adhering candida to the extract-treated experimental pellicle. Fig. 4 shows that the strength of anti-adherence activity of both extracts was species-specific. The adherence capacity of five out of seven \(Candida\) species was reduced by more than 50% when the experimental pellicle was pre-treated with \(C. javanica\) extract prior to the adhesion of the candidal cells. Thus, the adherence of \(C. krusei\), \(C. glabrata\), \(C. albicans\), \(C. lusitaniae\) and \(C. tropicalis\) were significantly \((P < 0.05)\) reduced on the extract-treated pellicles. The reduction in adhesion of \(C. parapsilosis\) and \(C. dubliniensis\) were comparatively lower at 49.0% and 27.9%, respectively. Similar pattern of anti-adhesion effect but of lower degree was also demonstrated by the extract of \(P. betle\) \((C. tropicalis 86.0\%, C. albicans 61.4\%\) and \(C. krusei 56.3\%). The adhesions of the other candida were lower with \(C. glabrata\) the least at 12.4%.

5. Discussion

Adherence which involves specific and non-specific adhesion is a key attribute of virulence among \(Candida\) species for successful colonisation and infection of the oral surfaces. Specific adhesion involves the interaction between receptors in acquired pellicle covering the oral tissues and the surface of the candida, whereas non-specific adhesion involves the surface proteins of the candidal cells and the acquired pellicle.

The surface proteins of a candidal cell comprised of hydrophobic domain that consists of non-polar amino acids which represent a measurable physiochemical variable to estimate the adhesion potential of candida to surfaces. The involvement of CSH in the adhesion of other microorganisms has been reported in many studies. In the experiment, hexadecane was used to mimic the hydrophobic surfaces of the teeth or dentures. Hydrophobic interaction is fundamentally based on the tendency of water molecules (polar) to

![Fig. 2](image-url) – The cell surface hydrophobicity of seven \(Candida\) species measured by their binding affinities to hexadecane. Each bar represents the mean ± SD of three independent experiments performed in triplicate \((n = 9)\).
exclude non-polar molecules, leading to segregation of polar and non-polar substances. Thus, any deviation to the hydrophobic affinity may influence the adherence mechanism of candida cells.

In this study, all seven species of Candida had demonstrated some degree of CSH. C. krusei, C. dubliniensis and C. tropicalis were found to be significantly more hydrophobic compared to the other four species (P < 0.05). It was reported that the distinct surface proteins encompassing the hydrophobic area that confer the CSH status in each organism can differ significantly as a result of physiological adaptation to its growth environment. Differences in chemical composition in

Fig. 3 – Comparison of the effect of P. betle (■) and B. javanica (▲) extracts on the CSH of seven Candida species. The percentages were means (SD) of three independent experiments performed in triplicate (n = 9) (P < 0.05). 0.12% CHX ( anomal) as a positive control.
the cell wall of each of the species may also contribute to the varying CSH.\textsuperscript{22,23} Data obtained from this study showed that the extracts of both \textit{P. betle} and \textit{B. javanica} were able to influence the binding affinity of the seven \textit{Candida} to hexadecane (Fig. 3). The CSH of \textit{C. albicans}, \textit{C. krusei}, \textit{C. lusitaniae}, \textit{C. parapsilosis} and \textit{C. tropicalis} was remarkably reduced by the extract of \textit{B. javanica} while \textit{C. dubliniensis} and \textit{C. glabrata} were found to be more susceptible to \textit{P. betle} (Fig. 3). The CSH was also observed to diminish uniformly with increased concentrations of the extract demonstrating a concentration dependent effect of plant extracts.

\textit{Candida} has been reported to possess numerous surface adhesins including one that acted as receptor for immobilised proteins of the salivary pellicle that assist adherence.\textsuperscript{24,25} In this case the adhesin proteins may act as ligands that mediate attachment of an organism to specific receptors on the salivary pellicles. This specific interaction which was in accordance with earlier reports\textsuperscript{26,27} was clearly displayed by the varying adhering ability of the seven \textit{Candida} species to the saliva-coated glass surface.

\textit{C. parapsilosis} exhibited a high degree of intra-species heterogeneity in adherence compared to other NCAC.\textsuperscript{28,29} This may explain for its high prevalence in oral candidiasis and its pathogenic potential. \textit{C. dubliniensis} and \textit{C. krusei} in contrast exhibited the lowest adhering ability. The adherence of the former to oral surfaces was not influenced by the presence of saliva and produce hyphal at much lower rate compared to the others\textsuperscript{30,31} which may suggest for its low adherence ability. The adherence of the later to salivary pellicle through specific bindings was also low. In addition, \textit{C. krusei} has been reported to be less invasive on superficial epithelium\textsuperscript{32} which may explain for its lower virulence when compared to \textit{C. albicans}.

In this study, \textit{P. betle} and \textit{B. javanica} extracts were found to show anti-adherence effects on the specific adhesion of oral \textit{Candida} to the experimental pellicle. A brief treatment of the experimental pellicle for 2–3 min with the extracts was able to significantly reduce the adhering capacity of five out of the seven \textit{Candida} species to the pellicle ($P < 0.05$). The constituents of the extracts may have altered some receptors on the pellicle, thereby disturbs the recognition of adhesins on the candidal cells, hence diverting specific interactions between the two surfaces.

Throughout the study, CHX was used in both the non-specific and specific adhesion assays as positive control. Responses of the cells to CHX were used as reference to compare the effectiveness of the extracts on inhibiting the adhesion and reducing the hydrophobic interaction of candida cells. Although \textit{C. krusei} was found to be most hydrophobic, this was not reflected in the population of cells adhering to the experimental pellicle in the specific interaction. Similarly, the adherence of \textit{C. parapsilosis} to the experimental pellicle was significantly higher than the others, its cell surface proteins showed low hydrophobicity. Findings suggested that CSH is not a major contributing role in facilitating the adherence. This can be supported by Gibbons and Etherden\textsuperscript{33} who reported that saliva does not contain unique macromolecules to serve as receptors for the hydrophobic microorganisms to adhere.

In conclusion, the extracts of \textit{B. javanica} and \textit{P. betle} were able to divert the CSH of all seven oral \textit{Candida} tested with \textit{B. javanica} exhibiting a greater effect in making the cells less adherent. The extracts were also able to reduce the adherence of oral candida via specific adhesion to the experimental pellicle. The extracts may have modified the salivary receptors, which in turn disturb the adherence of the \textit{Candida} to the experimental pellicle.

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\section*{Competing interests}

No conflict of interest for this study with any person or institution.
Ethical approval

Not required.

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References

30. Elguezabal N, Maza JL, Ponton J. Inhibition of adherence of Candida albicans and Candida dubliniensis to a resin composite

