ABSTRACT

Candida species is an opportunistic microorganism residing in the human oral cavity. There is increasing prevalence of candidal infections in the oral cavity largely because of the increasing size of the population at risk. The rise in diagnosed cases of oral candidiasis is also due to the immunosuppressive effect of prescribed antifungal agents on resistant hosts. The ability of *Candida* species to adhere on mucosal tissues and/or denture surfaces, and the production of hydrolytic enzymes along with defective host immunity are among the known key factors of invasions and pathogenesis of oral candida. Although *Candida albicans* remains as the most pathogenic organism, the emergence of non-*Candida albicans Candida* (NCAC) species with reduced susceptibility to prescribed antifungal agents has prompted efforts to study antifungal agents from natural sources. Plants have long been known to possess medicinal values and are rich in chemical constituents that can be used in the development of antifungal products. Initially, seven local plants were systematically screened for their antifungal activity and two of them - *Brucea javanica* L. and *Piper betle* L. were selected based on their positive antifungal activities on seven *Candida* species tested. The specific objectives of this study were; (i) to determine the minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) values of the extracts, (ii) to investigate the growth inhibitory effect of the extracts based on changes in the pattern of growth profile of each *Candida* species, (iii) to investigate the influence of extracts on adherence mechanisms which include the non-specific and specific bindings, (iv) to investigate any morphological changes of the cells following treatment with the extracts, and (v) to explore the differential expressions of multigene family of secreted aspartyl proteinases (*SAPs*) and hyphal cell wall protein 1 (*HWP1*).

Candida species purchased from the American Types Culture Collection (ATCC) were *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. Growth inhibitory responses of the *Candida* species to the extracts include determination of the MIC and MFC, while effect on the growth curve was determined based on spectrophotometric assay. Deviations in the doubling time (g) and specific growth rates (μ) were computed as percentage to extract-treated cells relative to that of the total cells in the absence of extracts. In addition, the anti-adherence effect of the *B. javanica* and *P. betle* extracts which included study on the cell-surface hydrophobicity (CSH) and specific bindings on pellicles, ultrastructure and the regulations of *SAP1-10* and *HWP1* were also analysed. 0.12% w/v chlorhexidine gluconate-containing mouthrinse was used as a reference.

In preliminary screening, the diameter of inhibition zone (DIZ) values showed that *B. javanica* and *P. betle* aqueous extracts exhibited a wide range of antifungal activities over the seven *Candida* species with *C. dubliniensis* identified as the most sensitive. Of the seven *Candida* species, *C. tropicalis* showed the highest growth rates (0.319 ± 0.002 h^{-1}) while the others were in the range of 0.141 ± 0.001 to 0.265 ± 0.005 h^{-1}. This indicated that different species of candida reproduce at different rate. In the presence of extracts, the lag and log phases were extended and shifted to the right. This resulted in the deviations of the g- and μ-values, indicating that the extracts may have exerted fungistatic activity towards the candidal cells. Growth kinetics of the candidal species was also elucidated based on colony forming unit (CFU) enumeration. Different *Candida* species have shown different CSH values and adhering capacity to the pellicle.

In view of the CSH, *C. krusei*, *C. dubliniensis* and *C. tropicalis* showed the highest adsorption to hexadecane at 30.23%, 26.19% and 19.70%, respectively, while the others were much lower within the range of 7% to 10%. The CSH of all *Candida* species were significantly affected by these two extracts ($P < 0.05$), with *B. javanica* exhibiting more than 60% reduction of CSH than *P. betle.* Specific bindings of the candidal cells on the pellicles were also shown to be affected by the treatment of extracts. Exposure to *P. betle*-treated pellicle drastically reduced the adhering capacity of three out of seven candidal species by more than 50% (*C. tropicalis* 86.02%, *C. albicans* 61.41% and *C. krusei* 56.34%). Pellicles treated with *B. javanica* exhibited similar effect on *C. tropicalis* (89.86%), *C. lusitaniae* (89.66%), *C. albicans* (79.74%), *C. glabrata* (76.85%) and *C. krusei* (67.61%). Comparatively, the adherence interference effect of *B. javanica* towards the candidal cells was slightly higher than *P. betle*. In addition to the growth inhibitory and anti-adherence effects, physical changes in the cell walls of *Candida* species were also demonstrated following treatment of the candidal cells with the extracts. The expressions of *SAP1-10* and *HWP1* were affected by the extracts treatment, suggesting that the extracts have successfully penetrated and disrupted the intrinsic environment of the cells. The genes seemed to be suppressed and this may then revoke the pathogenesis of oral *Candida*.

As a conclusion, *B. javanica* and *P. betle* exhibited antifungal activities towards the seven oral *Candida* species tested. Data from this study strongly suggest the fungistatic and growth inhibitory effects of the extracts. Thus, *B. javanica* and *P. betle* extracts may be considered as promising adjuncts in oral health products.

ABSTRAK

Spesies *Candida* merupakan mikroorganisma oportunis yang menetap di dalam mulut manusia. Jangkitan *Candida* dalam mulut (oral kandidiasis) biasanya berlaku kepada golongan yang berisiko masalah kesihatan. Peningkatan kes kandidiasis juga berpunca daripada kesan imunosupresif agen antikulat yang diberikan kepada hos secara konsistan. Keupayaan spesies *Candida* untuk melekat ke atas tisu mukosa dan/atau permukaan dentur, dan penghasilan enzim-enzim hidrolitik ketika sistem imunisasi tidak sempurna antara faktor utama kolonisasi dan patogenesis bagi spesies *Candida*. Walaupun *Candida albicans* masih kekal sebagai organisma patogen, tetapi kemunculan spesies *Candida* selain *C. albicans* yang rintang terhadap agen antikulat telah mendorong usaha untuk mengkaji agen antikulat yang baru daripada sumber semulajadi. Tumbuh-tumbuhan telah lama diketahui mempunyai nilai perubatan dan kaya dengan juzuk kimia yang boleh digunakan dalam pembangunan produk-produk antikulat. Dalam penyelidikan ini, tujuh spesies tumbuhan tempatan telah disaring secara sistematik bagi mengetahui aktiviti antikulatnya, dan dua daripadanya iaitu *Brucea javanica* L. dan *Piper betle* L. telah dipilih untuk kajian seterusnya kerana menunjukkan potensi aktiviti antikulat ke atas tujuh spesies *Candida* yang diuji. Objektif khusus penyelidikan ini adalah untuk (i) menentukan kepekatan perencatan minimum (MIC) dan kepekatan fungisidal minimum (MFC) bagi ekstrak yang diuji, (ii) mengkaji kesan perencatan ekstrak terhadap pertumbuhan sel *Candida*, (iii) mengkaji pengaruh ekstrak ke atas mekanisma perlekatan spesies *Candida* meliputi pengikatan khusus dan bukan khusus, (iv) mengkaji sebarang perubahan struktur permukaan sel *Candida* selepas rawatan ekstrak diberikan, dan (v) meneroka perbezaan ekspresi gen *SAP* (*SAP1-10*) dan *HWP1* di bawah pengaruh ekstrak.

Tujuh spesies *Candida* yang telah dibeli daripada American Types Culture Collection (ATCC) adalah *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 dan *Candida tropicalis* ATCC 13803. Tindak balas perencatan pertumbuhan tujuh spesies *Candida* terhadap ekstrak merangkumi penentuan MIC and MFC, manakala kesan ke atas lengkok pertumbuhan ditentukan dengan kaedah asai spektrofotometri. Perubahan bagi peratusan masa ganda dua (g) dan kadar pertumbuhan spesifik (μ) selepas rawatan ekstrak berbanding jumlah sel sebelum rawatan ekstrak ditentukan. Kesan ekstrak *B. javanica* dan *P. betle* terhadap mekanisma perlekatan, struktur morfologi sel, dan aturan expresi gen *SAP1-10* dan *HWP1* turut dikaji. 0.12% w/v chlorhexidine digluconate diguna sebagai kawalan positif.

Dalam ujian penyaringan awal, diameter zon perencatan menunjukkan ekstrak *B. javanica* dan *P. betle* mempunyai kepelbagaian aktiviti antikulat ke atas tujuh spesies *Candida* yang diuji, di mana *C. dubliniensis* merupakan spesies yang paling sensitif berbanding yang lain. *C. tropicalis* merupakan spesies yang mempunyai kadar pertumbuhan yang tinggi ($0.319 \pm 0.002\ h^{-1}$), manakala spesies *Candida* lain sekitar julat 0.141 ± 0.001 to $0.265 \pm 0.005\ h^{-1}$. Ini menunjukkan bahawa spesies yang berbeza dalam genus *Candida* mempunyai kebolehulangan yang berbeza. Dengan kehadiran ekstrak, fasa-fasa lag dan log telah diperluas dan beralih ke kanan. Ini menunjukkan ekstrak mempunyai aktiviti fungistatik. Kesan ekstrak ke atas kinetik pertumbuhan spesies *Candida* juga dikaji berdasarkan jumlah pembentukan koloni unit (CFU). *C. krusei, C. dubliniensis* dan *C. tropicalis* mempunyai kapasiti penyerapan hexadecane yang tinggi masing-masing pada 30.23%, 26.19% dan 19.70%, berbanding yang lain

dalam julat 7% hingga 10%. Kehidrofobian permukaan sel bagi semua spesies *Candida* telah terjejas berikutan rawatan ekstrak yang diberi ($P < 0.05$), di mana ekstrak *B. javanica* lebih berkesan dengan 60% pengurangan kehidrofobian berbanding ekstrak *P. betle*. Kapasiti pengikatan spesifik setiap spesies *Candida* ke atas pelikel turut terjejas dengan rawatan kedua-dua ekstrak yang diberikan. Pendedahan sel ke atas pelikel yang dirawat ektrak *P. betle* telah mengurangkan kapasiti pelekatan tiga daripada tujuh spesies *Candida* lebih daripada 50% (*C. tropicalis* 86.02%, *C. albicans* 61.41% dan *C. krusei* 56.34%). Pelikel yang dirawat dengan ekstrak *B. javanica* menunjukkan kesan yang sama ke atas *C. tropicalis* (89.86%), *C. lusitaniae* (89.66%), *C. albicans* (79.74%), *C. glabrata* (76.85%) dan *C. krusei* (67.61%). Secara perbandingan, kesan gangguan pelekatan yang dikenakan ekstrak *B. javanica* terhadap sel *Candida* sedikit tinggi daripada ekstrak *P. betle*. Di samping kesan perencatan pertumbuhan dan anti-pelekatan, perubahan fizikal pada dinding sel spesies *Candida* juga kelihatan selepas rawatan ekstrak diberi. Ekpresi gen *SAP1-10* dan *HWP1* juga terjejas menunjukkan bahawa ekstrak berjaya menembusi membran sel dan membeku persekitaran intrinsik sel-sel. Gen-gen ini kelihatannya ditindas dan menafikan proses patogenesis spesies *Candida*.

Kesimpulannya, ekstrak *B. javanica* dan *P. betle* mempunyai kesan anti-kulat terhadap tujuh spesies *Candida* yang diuji. Data yang diperoleh daripada kajian ini juga membuktikan bahawa ekstrak *B. javanica* dan *P. betle* mempunyai aktiviti fungistatik dan kesan perencatan pada kepekatan yang lebih tinggi. Justeru, kedua-dua tumbuhan tempatan ini berpotensi untuk pembangunan sebagai agen antikulat khususnya dalam penghasilan produk-produk kesihatan mulut.

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LIST OF ABBREVIATIONS

Abbreviation	Description
bp	Base pair
cm	Centimetre
CSH	Cell surface hydrophobicity
CWP	Cell wall protein
CHX	Chlorhexidine gluconate
CFU/mL	Colony forming units per millilitre
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
dH_2O	Distilled water
EtBr	Ethidium bromide
GPI	Glycophophatidylinositol
g	Gravity
g	Gram
g/mL	Gram per millilitre
g/L	Gram per litre
h	Hour(s)
L	Litre
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microlitre
mL	Millilitre
mL/min	Millilitre per minute

LIST OF ABBREVIATIONS

Abbreviation	Description
µL	Microlitre
µm	Micromolar
µmole/ml	Micromoles per millilitre
mg	Milligram
mg/mL	Milligram per millilitre
mm	Millimetre
mM	Millimolar
min	Minute
M	Molar
MW	Molecular weight
nm	Nanometer
OD	Optical density
pp.	Pages
%	Percentage
RT-PCR	Reverse transcription-polymerase chain reaction
rDNA	Ribosomal deoxynucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
sec	Seconds
NaCl	Sodium chloride
sp.	Species
i.e.	That is
TBE	Tris-Borate-EDTA

LIST OF ABBREVIATIONS

Abbreviation	Description
U/mL	Unit per millilitre
V	Voltage
v/v	Volume per volume
w/v	Weight per volume
YPD	Yeast peptone dextrose