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Full Length Research Paper

Determination of the percentage inhibition of diameter growth (PIDG) of *Piper betle* crude aqueous extract against oral *Candida* species

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Species within the genus Candida have been implicated in many fungal diseases such as candidiasis or thrush. The increasing clinical and microbiological resistance of *Candida* species towards several commonly prescribed antifungal agents however, has lead to the search for new active antifungal compounds from natural resources. This study was carried out to screen the susceptibility of the aqueous extract of *Piper betle* towards seven species of oral Candida. It was found that *P. betle* extract exhibited high antifungal activities towards *Candida albicans, Candida tropicalis, Candida glabrata, Candida dubliniensis, Candida lusitaniae, Candida krusei* and *Candida parapsilosis*. The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) value of *P. betle* extract towards all *Candida* species was found to be similar (12 mg/ml) except towards *C. albicans* which has been shown to have MIC value of 12 mg/ml and slightly higher MFC value of 25 mg/ml. The recorded data on the growth responses of the species to various concentrations of the extract following a 24 h incubation period were analysed, using the percentage inhibition of diameter growth (PIDG) against chlorhexidine gluconate (CHX). The determination of PIDG values for *C. albicans*, *C. tropicalis*, *C. lusitaniae*, *C. dubliniensis* and *C. glabrata* has shown that the aqueous extract of *P. betle* outstrips the positive control used, that was 0.12% w/v chlorhexidine with PIDG values of more than 50% at *P. betle* concentration of 25 mg/ml. In contrast, PIDG for *C. krusei* and *C. parapsilosis* shows that at 25 mg/ml concentration of *P. betle* extract has little influence on growth inhibition compared to CHX. Thus, the results obtained have shown the potential use of *P. betle* extract as antifungal agent and thus significantly contribute to its antifungal development.

Key words: *Candida* sp., oral Candida, *Piper betle*, antifungal activity, percentage inhibition of diameter growth (PIDG).

INTRODUCTION

Candida species represents a very minor component of the normal oral flora. Despite its low carriage percentage, the population of *Candida* species has been known to increase in a condition known as oral candidiasis (Nguyen et al., 1996; Barchiesi et al., 1999; Kauffman, 2006), which is a prevalent problem among individuals wearing dentures (Bauer et al., 1966; Jorgensen et al., 1999; Barchiesi et al., 1999; Kauffman, 2006). This phenomenon is due to the acrylic dentures which act as a reservoir and plays an important role for *Candida* sp. colonisation. Besides this, the mucosa of the tongue dorsum may represent an attractive colonisation site for *Candida* sp. Many studies have shown that *Candida albicans* is the most pathogenic species among all other *Candida* sp. in the genus. *C. albicans* has also been frequently associated to be the main aetiology factor of oral candidiasis (Odds et al., 1990).

Being an opportunistic microorganism, *Candida* sp. is able to transit from a harmless commensal organism to a

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pathogen. However, in recent years, several other less pathogenic species of the same genus such as *Candida glabrata*, *Candida dubliniensis*, *Candida krusei*, *Candida parapsilosis*, *Candida lusitaniae* and *Candida tropicalis* known as the non-*Candida albicans* (NAC) species have also been implicated with increased incidence of infections (Powderly, 1992; Hazen, 1995; Pfaller, 1996; Coleman et al., 1997; He et al., 2006; Ramage et al., 2006; Bokor-Bratic, 2008). The emergence of higher resistance level of *C. albicans* and the non-*Candida albicans* (NAC) species towards many commonly prescribed antifungal agents (Nguyen et al., 1996) has become a great concern to society. This is said to be due to the consequence of the heavy usage of antifungal agents of such as amphotericin-B and fluconazole (Bokor-Bratic, 2008). Therefore, there is an increased need in obtaining alternative antifungal substances which can act effectively against oral *Candida* sp.

Piper betle is a tropical creeper plant belonging to the pepper family. The leaves of *P. betle* plants have been reported to have various medicinal values, as it exhibits antimicrobial effect, antioxidant and contains antidiabetic properties (Fathilah et al., 2009). It is believed to be indigenous throughout the Indian Malay region (Chopra et al., 1956). The plants grow widely in South East Asia region, and are used in traditional remedy for the control of caries and periodontal diseases (Nalina and Rahim, 2007). In addition, treatment and prevention of halitosis, headache, joint pain and itches (Chopra et al., 1956; Ong and Nordiana, 1999; Ramji et al., 2002) has also been implicated with *P. betle* plant. Besides having stimulatory influence on intestinal digestive enzymes activities, *P. betle* has also been used for the treatment of alcoholism (Prabhu et al., 1995; Varier, 1997). Recently, it has been reported by Fathilah et al. (2009) that crude aqueous extract of *P. betle* leaves exhibits antibacterial activity which acts against an important group of early dental plaque colonizers, that is of *Streptococcus mitis*, *Streptococcus sanguis* and *Actinomyces viscosus*.

Thus, in this study, the crude aqueous extract of *P. betle* leaves has been screened for the potential of inhibitory activity and the growth profile against seven important strains of oral *Candida* sp. that are *C. albicans, C. glabrata, C. dubliniensis*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae* and *C. tropicalis.* Therefore, the objectives of the study were to determine the antifungal effects of *P. betle* crude aqueous extract, and to obtain the percentage inhibition of diameter growth (PIDG) of *P. betle* extract towards seven strains of oral *Candida* species.

MATERIALS AND METHODS

Plant specimen

Leaves of *P. betle* were collected from a local farm in Selangor, Malaysia. The fresh leaves were washed and oven-dried at 65°C over a period of two days. The dried specimen was then ground into powder and sealed in a plastic bag and kept at room temperature for further use.

Preparation of *P. betle* crude aqueous extract

The dried ground specimen was weighed and 100 g of sample was boiled at low heat for 5 to 6 h in distilled water with a ratio of *P. betle*:water at 1:10. The decoction of *P. betle* leaves was filtered through a filter paper (Whatman No.1) to remove the presence of debris particles. The filtrate was further boiled to a final volume of 100 ml. The decoction was concentrated by freeze drying in a freeze dryer (EYELA FDU-1200, Tokyo), and prepared into stocks of 20 mg/ml in sterile microfuge vials.

Yeast cultures and growth

Seven strains of *Candida* species in this study were purchased from The American Type Culture Collection (ATCC), USA. The species included *C. albicans* ATCC 14053, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 13803, *C. krusei* ATCC14243, *C. lusitaniae* ATCC 64125, *C. parapsilosis* ATCC 22019 and *C. dubliniensis* ATCC MYA-2975. These lyophilised *Candida* sp. were rehydrated in sterile distilled water and inoculated onto Yeast Peptone Dextrose (YPD) agar media (BD Difco, USA). Following incubation at 37°C for 24 h the colonies were subcultured on fresh YPD agar slants and stored at 4°C for further use in the experiment. Stocks for long term storage was also prepared in 20% glycerol and kept at -70°C.

Throughout the experiment period, the cultures were maintained at 4°C and sub-culturing was done regularly to maintain fresh cultures for the experiments. Before use, each culture was also checked and confirmed the purity using the API Yeast Identification System (API 20C Aux, BioMerieux, France). The grown colonies were harvested and dispersed in YPD broth, and the turbidity of the suspension was adjusted to an optical density (OD_{550nm}) of 0.144 which is equivalent to $1x10^6$ cells/ml.

Antifungal activity screening

The antifungal activity of *P. betle* aqueous extract was carried out according to the disc diffusion concept of Kirby-Bauer sensitivity test (Bauer et al., 1966). Sterile blank discs of 6 mm diameter were impregnated with the prepared *P. betle* crude aqueous extracts to give a final concentration of 200, 150, 100, 50, 25 mg/ml respectively. The discs were then placed firmly on the agar surface which has been seeded with the candidal strains suspension. The same steps were repeated for the other six types of *Candida* sp.

All plates were incubated overnight at 37°C, except for *C. parapsilosis* which was incubated at 35°C. Throughout this experiment, a blank disc impregnated with sterile distilled water represented as negative control while a disc impregnated with 100 µl of 0.12% (w/v) chlorhexidine (CHX) represented as the positive control. The susceptibility of each *Candida* sp. was determined by the diameter of growth inhibited zone surrounding the disc. The experiment was done in duplicate and carried out three times to ensure reproducibility of results.

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of *P. betle* leaves

Table 1. Antifungal activity of *P.betle* aqueous extracts against oral *Candida* species

Microorganisms	Diameter of inhibition zones (mm^2)					
	P. betle extract (mg/ml)					CHX
	25	50	100	150	200	0.12%
Candida albicans (ATCC 14053)	20	25	29	30	31	13
Candida tropicalis (ATCC 13803)	16	21	28	30	30	9
Candida krusei (ATCC 14243)	9	14	21	21	26	20
Candida lusitaniae (ATCC 64125)	19	23	28	32	36	12
Candida dubliniensis (ATCC MYA-2975)	17	18	32	34	38	11
Candida glabrata (ATCC 90030)	11	13	17	17	21	9
Candida parapsilosis (ATCC 22019)	9	11	11	16	20	25

* : All data were the means obtained from three sets of tests carried out in duplicates. - : absence of inhibition zones.

extract against the seven strains tested was determined using the microdilution method as described by Jorgensen et al. (1999). Different concentrations of *P. betle* L. extracts were prepared in a microtiter plates with the final concentration of 100, 50, 25, 12.5, 6.25 and 3.13 mg/ml. Duplicate wells were also prepared for each concentration of plant extracts. *Candida* sp. suspension of 10^6 cells/ml was pipetted into each well. The same procedures were also carried out for the testing of the other six oral *Candida* sp. All plates were incubated overnight at 37°C, except for *C. parapsilosis*, which required incubation temperature of 35°C. Following this, the turbidity was observed and recorded. The lowest concentration of extracts that inhibited the visible growth of the *Candida* sp. was recorded as the MIC value. The negative and positive controls were performed using sterile distilled water and 0.12% (w/v) CHX respectively.

Determination of the minimum fungicidal concentration

The minimum fungicidal concentration (MFC) of *P. betle* extract was determined according to a standard procedure as described by Espinel-Ingroff et al. (2002). Following an overnight incubation for the MIC determination, 50 µl from each well which indicate no growth of all respective candidal yeast, were subcultured onto fresh YPD agar plates. The plates were incubated at 35°C for 24 to 48 h until the visible growth was observed. The MFC value was the concentration where no growth or fewer than three colonies were obtained to give approximately 99 to 99.5% killing activity (Espinel-Ingroff et al., 2002).

Determination of the percentage inhibition of diameter growth (PIDG)

Following the observation for MFC, the percentage inhibition of diameter growth (PIDG) values were determined according to the equation as below:

$$\text{PIDG (\%)} = \frac{\text{Diameter of sample} - \text{Diameter of control}}{\text{Diameter of control}} \times 100$$

RESULTS

Based on the result of the disc diffusion test, all seven strains of oral *Candida* sp. showed varying susceptibilities towards the aqueous extract of *P. betle*. The diameters of the growth inhibition zones for all strains were determined to range between 9.0 to 38.0 mm (Table 1). For all strains, the inhibition zones were increased as the higher concentrations of the extract-impregnated discs used. At the lowest concentration of 25 mg/ml, *C. albicans* produced the largest zone of inhibition, followed in a descending order by *C. lusitaniae*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata, C. krusei* and *C. parapsiolosis*.

Subsequently, the antifungal activities of *P. betle* aqueous extract against all the seven strains of oral *Candida* sp. were evaluated further by performing the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). The MIC of *P. betle* aqueous extract effectively inhibited the growth of all seven oral *Candida* species at concentration of 12.5 mg/ml (Table 2). The MFC for *Piper betle* aqueous extract was found to be similar to MIC value of 12.5 mg/ml, except for *C. albicans*, which has higher MFC of 25.0 mg/ml (Table 2).

The determination of the (PIDG) for all strains have shown that *P. betle* aqueous extract exhibited a higher inhibition ability (Table 3) compared to chlorhexidine, a positive control used in the study, which is a common antimicrobial agents in commercialized oral rinses. The percentage of inhibition of various concentration of *P. betle* extract towards the seven selected oral *Candida* species are plotted in Figure 1.

DISCUSSION

Candidal yeasts constitute a part of the normal oral flora and are considered as harmless commensal microbes in the oral cavity. However, this opportunistic yeast species could induce oral infections when the ecological balance is disturbed causing changes in host defense

Table 2. MICs and MFCs of *P. betle* aqueous extracts against oral *Candida* species.

Microorganisms	Concentration of *P. betle* extract (mg/ml)						MIC	MFC
	100.0	50.0	25.0	12.5	6.25	3.13	MIC range MIC value (mg/ml)	MFC range MFC value (mg/ml)
C. albicans (ATCC 14053)	-	-	-	-	++	++	3.13-100.0 (12.5)	12.5-100.0 (25.0)
C. tropicalis (ATCC 13803)	-	-	-	-	++	++	3.13-100.0 (12.5)	12.5-100.0 (12.5)
C. krusei (ATCC 14243)	-	-	-	-	++	++	3.13-100.0 (12.5)	12.5-100.0 (12.5)
C. lusitaniae (ATCC 64125)	-	-	-	-	++	++	3.13-100.0 (12.5)	12.5-100.0 (12.5)
C. dubliniensis (ATCC MYA-2975)	-	-	-	-	+	++	3.13-100.0 (12.5)	12.5-100.0 (12.5)
C. glabrata (ATCC 90030)	-	-	-	-	++	++	3.13-100.0 (12.5)	12.5-100.0 (12.5)
C. parapsilosis (ATCC 22019)	-	-	-	-	+	+	3.13-100.0 (12.5)	12.5-100.0 (12.5)

- : no growth; + : turbid (strong growth); ++: highly turbid (dense growth).

Table 3. PIDGs of oral *Candida* sp. towards different concentrations of *P. betle* aqueous extract.

Microorganisms	*P. betle* extract (mg/ml)			
	25 (%)	50 (%)	100 (%)	200 (%)
Candida albicans (ATCC 14053)	53.8	92.3	123.1	138.5
Candida tropicalis (ATCC 13803)	77.8	133.3	211.1	233.3
Candida krusei (ATCC 14243)	-55.0	-30.0	5.0	30.0
Candida lusitaniae (ATCC 64125)	58.3	91.7	133.3	200.0
Candida dubliniensis (ATCC MYA-2975)	54.5	63.6	190.9	245.5
Candida glabrata (ATCC 90030)	22.2	44.4	88.9	133.3
Candida parapsilosis (ATCC 22019)	-64.0	56.0	-56.0	-20.0

- : no growth.

mechanisms (Gonzales, 1987; Walker, 1988; Slots, 1988, 1990; Henderson, 1989; Ciancio, 1995). During the last decade, oral candidiasis caused by *Candida* sp. constituted a major clinical problem. This is due to the oral mucosa and periodontal tissues in the oral cavity which represent as an important entrance for systemic fungal infections (Heimdahl et al., 1986; Odden et al., 1994).

The emergence of resistant candidal strains towards antifungal agents has increased great concern among scientists, medical and dental practitioners, and has lead to the search of new antifungal agents from natural resources.

Natural products are currently in great demand for research purposes due to the huge and extensive biological properties which has medicinal and commercialisation values. Traditional medicinal plants have long been implicated based on folklore information. The antimicrobial properties of certain medicinal plant have inhibitory activity against certain pathogenic fungi. A major phenolic compound called hydroxychavicol from aqueous extract of the *P. betle* leaves, was reported to

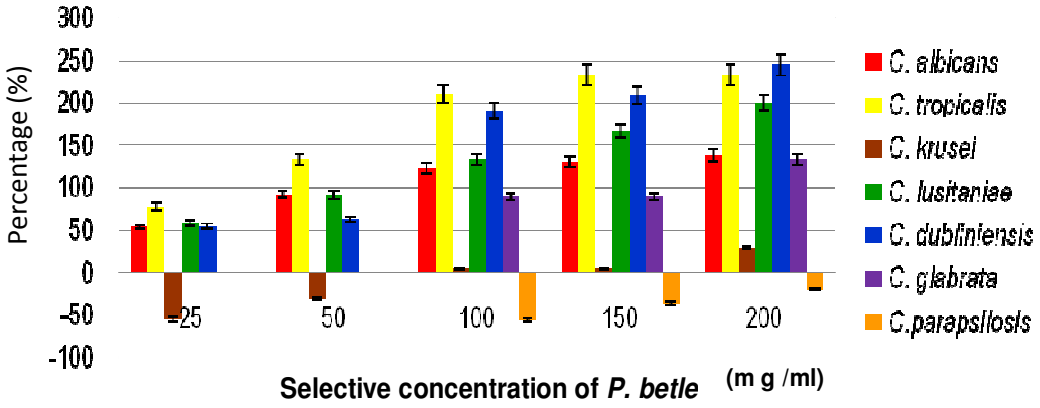

Figure 1. The PIDGs evaluation which represents the percentage of inhibition of oral *Candida* species upon exposure with *P. betle* aqueous extract.

have antimicrobial activity, antioxidant and anticancer properties in several studies conducted by various researchers (Chang et al., 2002; Ramji et al., 2002; Dasgupta and Be, 2004; Sharma et al., 2009). These properties may cause the reduction in growth and the adhesion ability in colonising host tissues or even to interfere and disrupt the integrity of bacterial cell wall, that leads to the disruption of the cytoplasmic membrane and coagulation of the bacterial content (Sikkema et al., 1995; Friedman et al., 2002). It has also been reported that *P. betle* is rich in chemical constituents such as campene, carvacrol, methyl chavibetol, eugenol, limonene, pinene, safrole and 1,8-cineole (Rimando, 1986). Among these, the common terpene compounds such as carvacrol, linalool and eugenol have been known to exhibit antifungal activity towards several strains of microorganisms (Juven et al., 1994; Kim et al., 1995; Hirasa and Takemasa, 1998; Friedman et al., 2002). In addition, there are many secondary metabolites and chemical constituents produced in *P. betle* extracts that may contribute to the observed positive antifungal effects.

The determination for antifungal activity of *P. betle* in this study was performed by disc diffusion method. It was found that *P. betle* extract exhibited antifungal activity towards all oral *Candida* sp. with various degree of inhibition. MIC value is the lowest concentration of *P. betle* extract that inhibit the growth, whereby MFC value is the lowest concentration of extract which will kill 99 to 99.5% of the tested microorganisms. The MIC and MFC values obtained in the study explains that *P. betle* extract acts as bacteriostatic and bactericidal agent against oral *Candida* species.

Except for *C. albicans*, *P. betle* extract was found to be bacteriostatic and bactericidal at low concentration (12 mg/ml). Bactericidal effect exhibited could probably be due to the limitation of the fungal growth by interfering with the production of fungal protein, the replication of DNA, or other various cellular metabolism of the organism (Pandima et al., 2010).

In addition, another possible mechanism of the bacteriostatic and bactericidal activity of *P. betle* extract was on the cell membrane of *Candida* cells which causes damage to the membrane, following which it could lead to the death of the fungal cells. In contrast to the six oral *Candida* sp., the effect of *P. betle* extract towards C. *albicans* has shown that the MIC value (12 mg/ml) was lower than MFC value (25 mg/ml), which suggest that at lower concentration, *P. betle* extract is bacteriostatic and acts as a bactericidal at a slightly higher concentration towards *C. albicans*.

The percentage inhibition of radial growth (PIRG) (Skidmore and Dickson, 1976) was employed in the study, with some modification. In the present study, the results were incorporated into the formula for PIDG, as the diameters were measured instead of the zone radius. The determination of PIDG values for *C. albicans*, *C. tropicalis*, *C. lusitaniae*, *C. dubliniensis* and *C. glabrata* has shown that, the aqueous extract of *P. betle* outstrips the positive control used, that was 0.12% w/v chlorhexidine with PIDG values of more than 50% at *P. betle* concentration of 25 mg/ml. In contrast, PIDG for *C. krusei* and *C. parapsilosis* shows that, at 25 mg/ml concentration, *P. betle* extract has little influence on growth inhibition compared to CHX. This was indicated by the negative values of PIDG calculated against CHX.

The results of this study has elucidated that *P. betle* extract exhibited better antifungal activity towards oral *Candida* species compared to chlorhexidine. Furthermore, additional information from further studies on the mechanism of action of this extract, might contribute to its usage as an alternative to the conventional antifungal drugs for the management of candidiasis. Therefore, *P. betle* extract has a great potential to be considered in the development of oral

health care products.

Conclusion

Aqueous extract of *P. betle* possesses strong antifungal activity which is very effective in inhibiting the growth of a variety of species of *Candida*. *C. krusei* and *C. parapsilosis* showed that *P. betle* extract has little influence on the PIDG values compared to CHX. In contrast, the extract was found to exhibit high PIDG values on the other five species of *Candida*, and is summarised in the following order: *C. tropicalis* > *C. dubliniensis* > *C. lusitaniae* > *C. albicans* > *C. glabrata*. Thus, the *P. betle* extract has shown to have strong antifungal properties against *Candida* sp. and may contribute as an alternative antifungal agent.

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