

RESEARCH ARTICLE

The effect of *Piper betle* against *Candida albicans* adherence to denture acrylic surfaces

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Abstract: Antimicrobial activity of medicinal plants has been identified since ancient times, and has been used to cure bacterial and fungal diseases. *Piper betle* is an important medicinal plant with its medicinal properties extending to antimicrobial activity against several pathogenic fungi and bacteria. The objective of this study was to evaluate the effect of *P. betle* leaf extract on *Candida albicans* adhesion on denture acrylics. Young betel leaves (1st - 3rd leaf) were subjected to vacuum infiltration with ethanol. Anticandidal activity of a range of concentrations of the leaf extract (5,000 – 10,000 ppm) was assessed against *C. albicans* (ATCC 90028) and seven laboratory isolates using the agar well bioassay. Further, the same concentrations were used to test their ability to suppress the adhesion of *C. albicans* on denture acrylics, as compared to a commercial denture cleanser (positive control), and sterile distilled water (negative control) using an adhesion assay. The results indicated that sterile distilled water had the highest adhesion (23.75 ±5.3 cells per unit area) while both, the leaf extract of concentrations of 8,000 – 10,000 ppm and the commercial denture cleanser showed significant ($p < 0.05$) suppression of *C. albicans* adhesion on denture acrylics. There was no significant difference between adhesion in an 8000 ppm concentration extract of *P. betle* and the commercial denture cleanser of equal concentration, indicating that betel leaf extract is as efficient as the commercial denture cleanser, in suppressing the adhesion of *C. albicans* on denture acrylic surfaces.

Keywords: anticandidal, denture acrylic, *Piper betel*, adhesion.

INTRODUCTION

Piper betle (Piperaceae) is a popular vine, and its leaves are commonly used as a masticatory among Asians. It is reported to have various medicinal properties which could be attributed to secondary metabolites (Cowan, 1999). Hydroxychavicol, a major phenolic component of betel leaves isolated from chloroform extraction has been investigated by Ali *et al.* (2010), and reported to have activity against *Candida* spp. It can also act as an antioxidant, a chemopreventive agent, anti-inflammatory, antiplatelet and antithrombotic agent (Chang *et al.*, 2002; Chang *et al.*, 2007). Nanayakkara *et al.* (2014) reports that the ethanol extract of *P. betle* has a significant anticandidal effect against *C. albicans*, *Candida glabrata*, *Candida*

kurusei, *Candida parapsilosis* and *Candida tropicalis*.

Candida albicans is a common opportunistic fungal pathogen causing superficial infections. Most infections produced by *C. albicans* are associated with biofilm formation on body surfaces (Samaranayake and MacFarlane 1980; Melo *et al.*, 2004). *Candida* sp. forms biofilms on dentures and has been associated with denture related stomatitis. Oral candidiasis is the most common fungal infection in patients with human immune deficiency virus (HIV) infection (Samaranayake and MacFarlane 1980). Denture prostheses encourage the presence and growth of *Candida* species within the oral cavity (Arendorf and Walker, 1980). In denture wearers, the fitting surface of the denture is the main reservoir of *Candida* cells (Samaranayake and MacFarlane, 1980). *In vitro* studies indicate that the microbial contamination of denture acrylic resin occurs quite rapidly and the yeast cells adhere strongly to denture based material (Samaranayake and MacFarlane 1980). Therefore, denture cleansers are used to clean dentures to prevent the oral *Candida* infection on human oral cavity. Denture cleansers should be able to suppress the adherence of *Candida* cells as even one pathogenic cell is sufficient to cause the disease (Arendorf and Walker, 1980; Samaranayake *et al.*, 1980; Nikawa *et al.*, 1998, Ellepola & Samaranayake, 1998). Adhesion assays of *C. albicans* to denture acrylic resin have been researched by Samaranayake & MacFarlane (1980). Denture cleansers should be non-toxic to humans and be compatible with the denture material (Nikawa *et al.*, 1998a), while they should be able to control plaque formation of *C. albicans*, and other microorganisms' adhesion (Nikawa *et al.*, 1998b; Silva *et al.*, 2009; Nalbant *et al.*, 2008). According to the chemical composition, denture cleansers are classified as; neutral peroxide, enzymes, alkaline peroxides, alkaline hypochlorites, disinfectants and acids (Machado *et al.*, 2009). The most common commercial cleansers currently in use are alkaline peroxides which require immersion techniques (Keng & Lim, 1996.). Some of these denture cleansers have become ineffective while some denture cleanser tablets are expensive (Ali *et al.*, 2010). Therefore, the objective of the study was to evaluate the effect of *P. betle* leaf extract on *C. albicans* adhesion on denture acrylics.

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MATERIALS AND METHODS

Preparation of crude ethanol extract from leaves of *P. betle*

Young *P. betle* leaves (1st -3rd leaf) were collected, as previously proved to be more effective than mature leaves (Nanayakkara *et al.*, 2014), and washed, cleaned and air dried at room temperature. The cleaned and dried *P. betle* leaves were cut into pieces ($\sim 0.5 \times 0.5$ cm) and 20 g of sample was measured. Subsequently it was soaked for 10 min in 200 ml of 99% ethanol at room temperature ($27 \pm 1^\circ\text{C}$). Using vacuum infiltration technique where soluble compounds are extracted to the solvent under a vacuum, the plant parts were stirred with the same aliquot of alcohol in a magnetic stirrer at a speed of 3 revolutions per second, at room temperature for one hour. Subsequently, the extract obtained was filtered through sterile Whatman no. 1 filter paper and the filtrate was collected. The ethanol extract was concentrated at 40°C using a rotary evaporator (Stuart RE 300) at a speed of 25 rpm (Rangama *et al.*, 2009). Subsequently 1 g of crude extract was dissolved in 10 ml of Dimethyl sulfoxide (DMSO).

Identification of *C. albicans* isolates

A standard strain of *C. albicans* (ATCC 90028) and seven laboratory isolates of *C. albicans* initially isolated from the oral cavity were obtained from a culture collection of the Division of Microbiology, of Faculty of Dental Science, University of Peradeniya, and sub cultured on sterile Sabouraud Dextrose Agar (SDA) plates. The plates were incubated at 37°C for 24 hours and the resultant colonies were maintained at 4°C for short-term storage. Identity was reconfirmed by Gram staining and germ tube formation as follows.

A loop full of the standard strain and the laboratory isolates were inoculated into 0.5 ml of blood serum separated from human blood, in a separate eppendorf tube, and incubated at 37°C (Mackenzie, 1962; Kim *et al.*, 2002). After incubation, the cultures were observed under the oil immersion lens of the light microscope ($\times 10 \times 100$).

Anticandidal activity of *P. betle* extract using agar well diffusion assay

Separate cell suspensions were prepared using sterile distilled water from the standard strain of *C. albicans* (ATCC 90028) and seven laboratory isolates of *C. albicans*, and the turbidity was made similar to that of McFarland 0.5 standard.

A dilution series was prepared between 5000 – 10000 ppm, with the stock solution of *P. betle* extract according to Andrews (2001). One ml of DMSO with 9 ml of water served as the negative control, and a commercial denture cleanser tablet containing cyclohexane served as the positive control. Agar well diffusion assay was carried out with the standard isolate and seven laboratory isolates of *C. albicans*.

One ml of *C. albicans* suspension was flood inoculated

on to solidified Sabouraud Dextrose Agar (SDA) plates and spread evenly by swirling the plate. The plates were dried at 44°C for 15 minutes. Using a sterile cork borer, wells were cut and base of the wells were sealed by adding a drop of molten SDA into each well. Subsequently, wells were loaded with 200 μl of previously prepared concentrations of *P. betle* extract, negative control and positive control. The plates were incubated for 24 hours at 37°C , and the diameter of zone of inhibition was measured. This procedure was repeated for all *Candida* isolates and the experiment was carried out in triplicate.

Evaluation of the effect of *P. betle* extract on *C. albicans* adherence to denture acrylic surfaces

The adherence to acrylic surfaces of *C. albicans* standard strain (ATCC 90028) and seven laboratory isolates with the addition of *P. betle* extract was evaluated. Acrylic strips for the assay were made using a modified method of Samaranyake and Macfarlane (1980) as follows. Under sterile conditions transparent self-polymerizing acrylic powder was spread on an aluminium foil-covered glass slide. Monomer liquid (1 ml) was poured onto the surface of the slide with acrylic powder, and immediately a second slide was placed on top of the polymerizing mixture and the slides were firmly secured at both ends with two binder clips. After bench curing, the slides were separated. The acrylic strips were then carefully stripped off the aluminium foil. Then they were immersed in distilled water for one week to leach excess monomer. Subsequently, they were washed in running water for three hours. Then the strips were cut aseptically into 5x5 mm squares and autoclaved for 20 min.

The adhesion assay was carried out under strict aseptic conditions as recorded by Samaranyake and Macfarlane (1980) with modifications. Acrylic strips were placed vertically in the wells of a sterile 96 well microtiter plate. A dilution series between 5000 – 10000 ppm of *P. betle* leaf extract was prepared, and 200 μl of each dilution and 200 μl of each broth culture, were added into wells of the above microtiter plate containing acrylic strip. The prepared microtiter plate was incubated in a shaker incubator for one h at 37°C with gentle agitation. The strips were taken out and washed with sterile distilled water to wash out loosely adhered cells. The strips were air-dried and stained using modified Gram stain without counter staining. After drying at room temperature, the strips were mounted on glass slides with glycerol and adherent yeast cells were quantified using an optical microscope. The presence of *Candida* cells in 30 microscopic fields were randomly counted in each strip at $\times 10 \times 40$ magnification. The experiment was triplicated. The same procedure was carried out for positive and negative controls. The final value of the number of cells adhered to denture acrylic strips were taken as the average value of all replicates.

Statistical analysis

The data were subjected to normality testing, followed by GLM Procedure and LS means-pdiff mean separation procedure using statistical package, SAS 9.1.3 (SAS Institute, NC, Cary, USA).

RESULTS AND DISCUSSION

Preparation of crude ethanol extract from young leaves of *P. betle*

The dark brownish green sticky crude extract of *P. betle* which was obtained after rotary evaporation was partially soluble in sterile distilled water, while it dissolved completely in DMSO.

Identification of *C. albicans*

Hyaline germ tubes were observed, confirming the identity of *C. albicans*, in the standard culture and laboratory isolates after incubating cultures at 37 °C in fresh blood serum. Seven strains were identified as *C. albicans* (Figure 1).

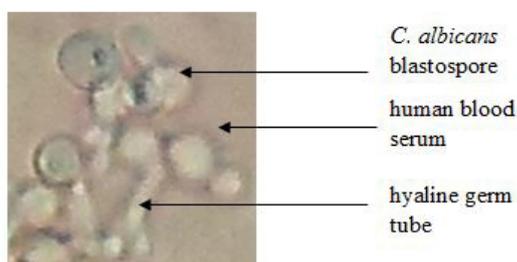


Figure 1: Germ tube formation of *C. albicans* in blood serum (magnification: $2 \times 10 \times 40$).

Evaluating the activity of *P. betle* extract against *C. albicans* by the agar well diffusion assay

Zone of inhibitions were observed with different concentrations of *P. betle* and with the solution of commercial denture tablet, while there was no inhibition zone observed with the SDW control.

The highest zone of inhibition was shown by the solution of commercial denture cleanser tablet, which was higher than 4 mm in radius, followed by 10000 ppm concentration ethanol extract of *P. betle* which exhibited an inhibitory zone of more than 3 mm in radius (Table 1).

Evaluation of the effect of *P. betle* extract on *C. albicans* adherence to denture acrylic surfaces

The highest mean adhesion was shown by 5000 ppm concentration of ethanol extract of *P. betle*, which had 2.733 ± 0.32 *C. albicans* cells per unit area of the field of vision under the high power of the light microscope. The lowest adhesion was shown by 10,000 ppm concentration of ethanol extract of *P. betle* 0.002 ± 0.003 cells per unit area. To prevent infections, mean adhesion of *C. albicans* should be below one cell per unit area (Panagoda *et al*, 1998). Mean adhesion of cells were below one cell per unit area beginning from 7,500 ppm concentration of *P. betle* solution. However, since there was no significant difference in the adhesion of cells at 7,000 ppm and 7,500 ppm concentrations, 8,000 ppm was concluded to be more effective than the 7,500 ppm concentration, in preventing adhesion of *C. albicans* cells on denture acrylic surfaces. Therefore 8,000 ppm could be taken as the concentration to be used in the preparation of a denture cleanser. Table 2 shows the effect of different concentrations of *P. betle* leaf ethanol extract on *C. albicans* adhesion to denture acrylic surfaces.

When comparing the mean inhibition zones and the mean adhesion with respect to *P. betle* ethanol extract concentrations, inhibition zones increased with increasing concentration. Adhesion of *C. albicans* (ATCC 90028) on denture acrylic decreased with increase in concentration of *P. betle* extract (Figure 3). This same trend was observed with all seven laboratory isolates.

Table 1: Zone of inhibition of a standard isolate of *C. albicans* (ATCC 90028) against a solution of denture cleanser tablet and different concentrations of *P. betle*.

Solution (mg/ml)	Inhibition zone (mm)
	<i>C. albicans</i>
10,000 (commercial denture cleanser)	4.40 ± 0.57
10,000	3.25 ± 0.35
9,500	3.00 ± 0.41
9,000	2.25 ± 0.49
8,500	1.75 ± 0.26
8,000	1.50 ± 0.33
7,500	1.55 ± 0.37
7,000	1.25 ± 0.35
6,500	1.20 ± 0.26
6,000	0.55 ± 0.55
5,500	0.60 ± 0.57
5,000	0.30 ± 0.42

All values are expressed as mean \pm standard error (n=10).

Table 2: Mean adhesions of *C. albicans* (standard isolate + seven laboratory isolates) against different concentrations of *P. betle* leaf ethanol extract

Solution (ppm)	Mean adhesion of no. of cells
5,000	2.733±0.32 ^a
5,500	2.208±0.36 ^b
6,000	1.607±0.40 ^c
6,500	1.531±0.37 ^c
7,000	1.116±0.22 ^d
7,500	0.876±0.22 ^d
8,000	0.387±0.18 ^e
8,500	0.215±0.23 ^e
9,000	0.021±0.0 ^e
9,500	0.013±0.02 ^e
10,000	0.002±0.003 ^e

Means denoted by the same letters within the column are not significantly different at $P < 0.05$.

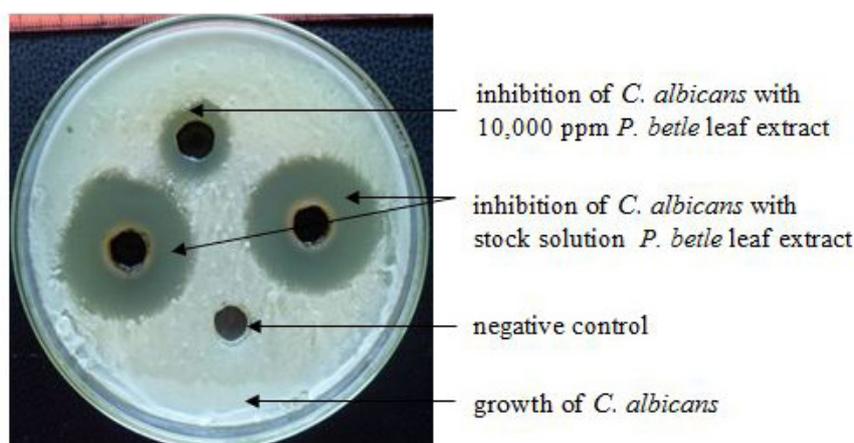


Figure 2: Zones of inhibition of *C. albicans* with different concentrations of *P. betle* leaf extract.

Statistical analysis

Considering the standard isolate and the seven laboratory isolates, there was a significant difference at ($P < 0.0001$) of the adherence of cells to denture acrylics between Sterile Distilled Water (SDW- negative control) and the commercial denture cleanser (positive control). Similarly there was a significant difference at ($P < 0.0001$) of the adherence of cells to denture acrylics between SDW and the *P. betle* extract at a concentration of 8000 ppm. It could be concluded that the ethanolic leaf extract of *P. betle* has an effect on the adherence of *C. albicans* to denture acrylic surfaces, similar to the commercial denture cleanser ($R^2 = 93.66\%$). There was no significant difference ($P < 0.05$) of the adherence of cells to denture acrylics between the commercial denture cleanser and the *P. betle* leaf extract (8,000 ppm) (Table 3).

The results from the present study showed that denture acrylic adhesion of *C. albicans* was significantly reduced after exposure to ethanol extract of *P. betle* leaves. Ten thousand ppm of the leaf extract had the lowest adhesion on denture acrylic surface, while a concentration of 5,000 ppm had the highest adhesion on the denture acrylic surface. To

be effective, these concentrations should be able to suppress *Candida* adhesion below one cell per unit area. According to the results obtained, 8,000 ppm can be taken as the ideal concentration to develop a denture cleansing solution with an adhesion mean value of 0.387 ± 0.18 cells per unit area while the commercial denture cleanser tablet adhesion mean value was 0.110 ± 0.08 cells per unit area, with no significant difference between these two values (Table 3). In previous studies it has been found that several plant extracts can suppress the adhesion of *C. albicans* on denture acrylic strips (Taweekaisupapong *et al.*, 2006, Thaweboon and Thaweboon 2011). According to the experiment done by Taweekaisupapong *et al.*, (2006) 4-h exposure of *C. albicans* to 31.25 mg/ml of *Streblus asper* leaf ethanolic extract resulted in a reduction in the ability of the yeasts to adhere to denture acrylic, possibly preventing denture stomatitis. As reported by Thaweboon and Thaweboon (2011) a reduction of *Candida* cells adhering to acrylic strips were observed after exposure to 75-300 mg/ml ethanolic extract of *Phyllanthus emblica*. It is recorded that *P. emblica* ethanolic extract interferes with the adhesion of *C. albicans* to denture acrylic surfaces *in vitro*. The results of the current study revealed that 8 mg/ml (8,000 ppm) of

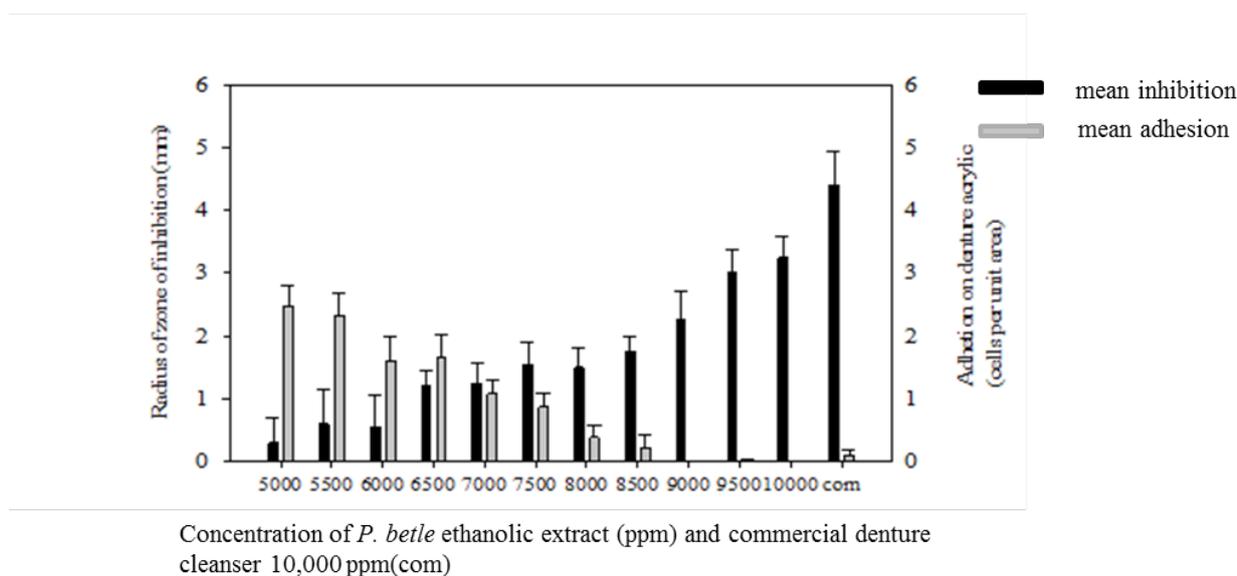


Figure 3: Effect of *P. betle* leaf ethanol extract and commercial denture cleanser on *C. albicans* (ATCC 90028) inhibition and adhesion.

Table 3: Mean *C. albicans* (standard isolate + seven laboratory isolates) cell adhesion to denture acrylic surface with exposure to *P. betle* leaf ethanol extract (8,000 ppm), commercial denture cleanser (COM) and sterile distilled water (SDW).

Solution	Mean adhesion of no. of cells
8,000 ppm	0.387±0.18 ^b
COM	0.110±0.08 ^b
SDW	23.750±5.33 ^a

Means denoted by the same letters within the column are not significantly different at $P < 0.05$.

P. betle leaf ethanol extract, which is a much lower value, exhibited a clear suppression of *C. albicans* adhesion to denture acrylic surface. This shows that betel extract (8,000 ppm) is as efficient as the commercial denture cleanser and results indicate that short-term exposure (one hour) of oral *C. albicans* isolates to specific concentrations of betel leaf ethanol extract significantly reduces their ability to adhere to denture acrylic surfaces. As normally a denture is removed at night and kept for at least a period of 6-8 hours in a denture cleanser, further research could be carried out by increasing the incubation period of *C. albicans* on acrylic strips, with lower concentrations of *P. betle* extracts.

CONCLUSION

The *P. betle* leaf extract possesses an anti Candidal effect and it can be used to suppress the adhesion of *C. albicans* on denture acrylic surfaces. Since *P. betle* is commonly found in Sri Lanka and other Asian countries, using a leaf extract as a denture cleanser solution is economically feasible. Therefore, there is a potential for *P. betle* leaf extract to be developed as an alternative to commercial denture cleansers.

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