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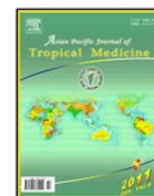

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Effect of *Phyllanthus emblica* Linn. on candida adhesion to oral epithelium and denture acrylic

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ABSTRACT

Objective: To investigate the effect of *Phyllanthus emblica (P. emblica)* Linn. ethanolic extract on the adhesion of *Candida albicans (C. albicans)* to human buccal epithelial cells (BECs) and denture acrylic surfaces. **Methods:** Human BECs and transparent acrylic strips were pretreated with ethanolic extract solution of *P. emblica* fruits at concentration ranged from 18.7 to 300 mg/mL. After washing BECs and the strips were inoculated with three strains of *C. albicans* (ATCC 10281 and two clinical isolates) (10^7 cells/mL). Normal saline solution (NSS) and 0.2% chlorhexidine gluconate were used as negative and positive controls, respectively. BECs were harvested on 12 μ m-polycarbonate filters (Millipore, USA). The membrane filters and the strips were stained with Gram stain. Adherent yeast cells on 100 randomly selected epithelial cells and 20 randomly selected fields on each strip were counted under microscope. The statistical significance was calculated by Kruskal-Wallis and Tukey tests at a significant level of $P< 0.05$. **Results:** Significant lower numbers of all strains of yeasts adhering to BECs and acrylic strips were observed after exposure to 75-300 mg/mL of plant extract compared with NSS. **Conclusions:** The present study demonstrates that *P. emblica* ethanolic extract interferes with the adhesion of *C. albicans* to BECs and denture acrylic surfaces *in vitro*.

1. Introduction

Candida albicans (C. albicans) is commensal yeast generally present in the oral cavity as normal flora. However, in some individuals especially those with diabetes, AIDS, leukemia and cancer *C. albicans* can act as an opportunistic pathogen, causing infections range from superficial to systemic and potentially life-threatening diseases. In addition, among the elderly, particularly those who wear dentures and in many cases is avoidable with good oral hygiene, candida infection or candidosis is common. In general population, carrier rates have been reported to range from 20% to 75% without any symptoms[1]. However, overgrowth of candida can lead to local discomfort, an altered taste sensation and dysphagia. In immunocompromised patients, infection can spread through the bloodstream leading to severe infection with significant morbidity and mortality[2].

The persistence of candida on oral surfaces exposed to the flushing action of saliva requires fungal adhesion to oral epithelial cells or to denture acrylic surfaces. The ability of such attachment is an important prerequisite for the successful colonization or a crucial step in the pathogenesis of infection[3]. *C. albicans* adhesion to oral surfaces is reported to be enhanced by several factors such as germ tube formation, protease, phospholipase, other extracellular enzymatic activities, carbohydrate, pH and temperature[3-5]. Adhesion involves interactions between the yeast cells and host surfaces. The *C. albicans* molecules that mediate binding of cells to other host cells, microbial cells or inert polymers are called adhesins. Several *C. albicans* adhesins have been identified. Most are glycoproteins present in the cell wall of the yeast[3].

There is considerable interest in the possible use of alternative medicinal plants either to delay the growth of pathogens or to prevent the colonization or infection. A wide variety of plant extracts have been demonstrated to

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have antimicrobial properties[6-8]. *Phyllanthus emblica (P. emblica)* Linn.(*Emblica officinalis* Gaertn.) or Indian gooseberry is a medium to large deciduous tree widely distributed in tropical and subtropical areas of India, Pakistan, China, Malaysia and Thailand. Its fruits have been mentioned by many authors to have medicinal properties such as antioxidant, hypoglycemic, diuretic and laxative effects, and useful in the treatment of digestive disorder, jaundice and coughs[9-12]. Fresh fruit is light greenish yellow on appearance and the taste is sour, bitter and astringent. The therapeutic potential of the fruits was attributed to their high ascorbic acid content. According to Scartezzini *et al* (2006)[10], processed fruit contains as high as 1.28 g/100 mL of vitamin C. In pharmacological point of view, the fruit extract has many activities including inhibition of micronuclei formation, sister chromatid exchanges, clastogenicity, and mutagenicity induced by heavy metals[13] as well as cytoprotective and immunomodulating activities[14]. Aqueous and ethanolic extracts of this plant exhibited potent antibacterial activities against several gram negative and gram positive bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus*[15,16]. Furthermore, antifungal activity of essential oils from *P. emblica* was demonstrated against food spoilage yeasts including *C. albicans*[17].

The aim of the present study was to investigate the effect of *P. emblica* Linn. ethanolic extract on the adhesion of *C. albicans* to human buccal epithelial cells (BECs) and denture acrylic.

2. Materials and methods

2.1. Plant extract preparation

Fresh fruits of *P. emblica* Linn. were air-dried, crushed and extracted with 95% ethanol with a ratio of 1:10 g/mL at room temperature for 72 h. Then the extract was collected, filtered and freeze dried. The extract (12 g) was dissolved in distilled water (5 mL) and further diluted two-fold with phosphate buffer saline (PBS) to the desired concentration.

2.2. Microorganisms

C. albicans ATCC 10281 and two clinical isolates obtained from oral lesions of patients with candidosis in Dental Hospital, Faculty of Dentistry, Mahidol University. Identification of these clinical isolates was established by germ tube test and the use of API 20C system (bioMerieux, UK). All the strains were cultured in Sabouraud dextrose broth (Difco Laboratories, Detroit, USA) at 37 ℃ for 18 h, harvested by centrifugation, and washed three times in PBS (0.01 M, pH 7.2). Yeast cells were counted with a hemocytometer and resuspended in PBS at 10^7 cells/mL.

2.3. Epithelial cells preparation

The method of BECs preparation was modified from Kimura and Pearsall (1978)[18]. BECs were collected from three healthy volunteers (age 25-32 years), by gently scraping the inner aspect of the right and left buccal mucosa with sterile wooden spatula and suspending in 5 ml of PBS. The pooled BEC suspension was washed four times in PBS to remove the attached organisms by centrifugation at 1 700g for 20 min. Absence of any microbial attach to the washed epithelial cells was checked under the microscope. The BECs were then resuspended to a concentration of 10^5 cells/mL by hemocytometer counting and used immediately. Epithelial cell suspension was pretreated with extract solutions (at concentrations 18.7, 37.5, 75, 150 and 300 mg/mL) for 5 min at 37 ℃. Cells were washed twice and resuspended in PBS. Extract-treated BECs were further tested for their adhesion abilities.

2.4. Epithelial cells adhesion assay

Equal volume (0.25 ml) of BEC (10^5 cells/mL) and yeast suspensions (10^7 cells/mL) were mixed and incubated at 37 ℃ for 1 h with gentle agitation (120 rpm/min). The yeast/BEC suspensions were then harvested on a 12 μm pore size polycarbonate filter (Millipore, USA). The filters were then washed four times with 20 mL of PBS to remove unattached yeasts. Epithelial cells retained on the membrane filters were then air-dried, methanol fixed and stained with crystal violet. The adherent yeast cells per 100 randomly selected epithelial cells were counted by direct microscopic examination. The procedure was repeated in triplicate on four separate occasions by a single observer.

2.5. Denture acrylic preparation

The acrylic strips were prepared based on the method described by Samaranayake and MacFarlane (1980)[19]. Self-polymerized acrylic powder and monomer liquid were mixed as recommended by manufacturer instructions (Takilon, Rodent, s.r.l., Milano, Italy). The mixture was placed between two glass slides firmly secured at both ends with two binder clips. The acrylic was then polymerized in a hydroflask at 50 ℃ for 5 min. Subsequently, the firmed transparent acrylic sheet was cut into strips (5×5 mm square and 0.3 mm thick). The strips were immersed in distilled water for 1 week to leach excess monomer, disinfected by dipping in 70% alcohol for 1 min, and washed with sterile distilled water. The strips were then ultrasonicated (VWR B9500E-DTH, VWR International, USA) for 20 min, washed again in sterile distilled water, dried and checked for sterility. They were pretreated with extract solutions (at concentrations 18.7, 37.5, 75, 150 and 300 mg/mL) for 30 min at room temperature and used in the adhesion assay.

2.6. Denture acrylic adhesion assay

The adhesion assay was modified from the procedure of Samaranayake and MacFarlane (1980)[19]. Briefly, the pretreated acrylic strips were placed vertically in the wells of 12-well plates (Nunclon, Denmark). Approximately 400 μL of yeast suspension (10^7cells/mL) was added to each well, which completely soaked the acrylic strips. The strips were then placed in a shaker incubator for 1 h at 37 ℃ with gentle agitation at 120 rpm/min. After incubation, the acrylic strips were washed three times with PBS to remove the unattached yeast cells. Then, the strips were stained with crystal violet. The adherent yeast cells per 20 fields were randomly quantified under microscope. The experiments were repeated on four separate occasions with triplicate determinations on each occasion.

Normal saline solution (NSS) and 0.2% chlorhexidine gluconate were used as negative and positive controls for both of the adhesion assay, respectively.

2.7. Statistical analysis

The effects of each concentration of plant extract on candida adhesion were analysed using Kruskal-Wallis test followed by Tukey test to evaluate the differences in adhesion between the test and control groups. A P-value of <0.05 was considered to be significant. The analysis was achieved in the software SPSS version 11.5 and Sigma Stat 3.5.

3. Results

The mean numbers of yeasts adhered to BECs treated with various concentrations of plant extract are presented in Table 1. Significant lower numbers of all strains of yeasts adhering to BECs were observed after exposure to 75-300 mg/mL of plant extract compared to NSS. A 85% reduction was achieved after the ATCC strain was exposed to 300 mg/mL, while both strains of clinical isolates exhibited 84% and 72% reduction. These reductions of adhesion were concentration-dependent, since higher concentrations resulted in higher adhesion blockage. Exposure to 37.5 mg/mL of plant extract resulted in a decrease in yeast adhesion with no significant difference. At 18.7 mg/mL concentration, no suppressive effect was observed.

The effect of *P. emblica* on candida was also investigated on denture acrylic surfaces. As shown in Table 2, the inhibition of adhesion were found at the concentration > 75 mg/mL with the percentage of reduction varies from 36% to 80%. Though the effect was not concentration-dependent, highest reduction was observed at the concentration of 300 mg/mL. Chlorhexidine gluconate which was used as a positive control exhibited more than 90% inhibitory effect on both epithelial cells and denture acrylic surfaces.

Table 1

Adhesion of *C. albicans* to human BECs in the presence of *P. emblica* Linn. ethanolic extract at various concentrations.

Group		No. of yeasts/100 BEC			Reduction of adhesion (%)		
		C. albicans ATCC 13801	*C. albicans* clinical isolate I	*C. albicans* clinical isolate II	*C. albicans* ATCC 13801	*C. albicans* clinical isolate I	*C. albicans* clinical isolate II
Chlorhexidine		10.2±0.4	8.6±0.1	10.4±0.6	91	92	92
NSS		112.2±5.8	116.0±14.7	125.0±8.9	–	–	–
Extract of *P. emblica*	18.7 mg/mL	114.2±9.2	115.2±9.5	112.4±7.5	–	–	–
	37.5 mg/mL	99.8±8.6	85.2±6.4	97.2±9.4	11	27	22
	75 mg/mL	73.0±4.9*	61.8±11.3*	53.2±4.5*	35	47	57
	150 mg/mL	33.0±6.2*	32.2±3.1*	34.6±3.7*	70	72	72
	300 mg/mL	16.4±1.6*	19.0±2.0*	28.2±1.3*	85	84	75

* significant different from NSS (P< 0.05).

Table 2

Adhesion of *C. albicans* to denture acrylic in the presence of *P. emblica* Linn. ethanolic extract at various concentrations.

Group		No. of yeasts/100 BEC			Reduction of adhesion (%)		
		C. albicans ATCC 13801	*C. albicans* clinical isolate I	*C. albicans* clinical isolate II	*C. albicans* ATCC 13801	*C. albicans* clinical isolate I	*C. albicans* clinical isolate II
Chlorhexidine		0.86±0.06	0.81±0.08	0.88±0.04	92	94	94
NSS		11.02±1.16	13.45±0.67	14.47±1.08	–	–	–
Extract of *P. emblica*	18.7 mg/mL	11.50±1.72	11.97±1.69	13.87±1.23	–	–	–
	37.5 mg/mL	12.03±1.08	11.77±6.40*	12.75±1.96	–	12	–
	75 mg/mL	7.03±4.90*	8.62±1.47*	7.31±0.67*	36	40	50
	150 mg/mL	3.63±0.80*	3.16±0.56*	3.90±0.30*	67	76	73
	300 mg/mL	2.21±0.42*	3.05±0.19*	3.24±0.56*	80	77	78

* significant different from NSS (P<0.05).

4. Discussion

Although the development and use of antibiotics have decreased public health hazards from microbial infections, an increase in resistance and dose-limiting toxic effects to these chemotherapeutic agents have been reported due to their indiscriminate use. In addition, antibiotics are occasionally associated with adverse effects to the host, including hypersensitivity, depletion of beneficial gut and mucosal microflora, immune-suppression and allergic reactions. These have created immense clinical problem in the treatment of infectious diseases.

According to the World Health Organization (WHO, 1993)[20], 80% of the world population rely chiefly on traditional medicine and a major plant of the traditional therapies involve the use of plant extracts or their active constituents. Many investigators have searched for new compounds with some antifungal action in natural products. Some plant extracts have been shown to possess activity against several pathogens and may be a good source of new active agents. Extracts from fruits of *P. emblica* have been assessed for various kinds of biological activity. In the present study, we investigated the potential on candida adhesion to BECs and denture acrylic surfaces.

Adhesion to host surfaces, such as the BEC or denture acrylic surfaces is considered an important factor influencing the balance among oral clearance mechanisms, colonization and development of clinical signs of candidosis. The degree of adhesion of candida to biological surfaces may indicate their pathogenic potential. *C. albicans* isolated from candidosis patients were found to be more adherent than the other isolates[21]. Binding of candida to BECs depends on specific interactions of lectin-like proteins, adhesins, of the yeast cells with terminal sugars of cell surface glycoproteins of epithelial cell surfaces[22]. This binding could be interfered by altering the expression of adhesins on yeast cells or ligands on host cells. Many host factors have been proposed to influence the attachment such as cigarette smoking, malnutrition, antibiotic therapy and hormonal effects[23]. According to the previous studies, there are several molecules contribute to the binding interactions. Fibronectin was one to be suggested as a ligand recognized by candida[24]. Pre-treatment yeast cells with fibronectin showed reduced binding to BECs[25]. Additionally, the complement fragment iC3b and epithelial cell glycosphingolipids have also been reported as adherence targets for candida adherence[26]. Apart from BECs, candida can adhere to a variety of materials used in medical devices including oral prostheses. Attachment of candida to denture acrylic or to salivary macromolecules adsorbed on its surface is believed to be a critical event in the development of denture stomatitis. *C. albicans* is able to form biofilm on the surfaces of these materials and adherent yeasts are less susceptible to antifungal drugs. Several factors implicated in the adhesion have been reported from *in vitro* studies[27,28]. Surface structure, properties and composition of materials, hydrophobicity and roughness influence the adhesion. The inhibition of adhesion to these oral surfaces is regarded as a promising strategy to control oral candida colonization and subsequent infection.

The results from the present study shows that epithelial and denture acrylic adhesion of *C. albicans* were significantly reduced after treatment with *P. emblica* extract at the concentration of 75, 150 and 300 mg/mL. It is worth noting that inhibitory effect on candida adhesion to epithelial cells and denture acrylic was similar. The mechanisms responsible for inhibition of adhesion are still to be determined, but these could be the antimicrobial properties of tannins, lignans, flavonoids, alkaloids and other phenolic compounds presented in this kind of plant[29,30]. Many phenolic compounds have been isolated from *P. emblica* extract through the use of high performance liquid chromatography (HPLC) such as geraniin, quercetin 3-β-D-glucopyranoside, kaempferol 3-β-D-glucopyranoside, isocorilagin, quercetin and kaempferol[31]. Moreover, these constituents may alter cell surface structures and integrity that could mask the adhesins present on the yeast or on the receptors present on the epithelial cell or denture acrylic surfaces.

In the present study, adhesion was evaluated with the aid of the *in vitro* assay system using BECs as a substrate for yeast attachment in the presence of *P. emblica* extract and denture acrylic surface after exposure to extract. This system, however, does not take into account the dynamics of the oral environment. It is not known to what extent the relative *P. emblica* extract contributes to colonization *in vivo* as other factors such as saliva and the presence of bacteria on these surfaces may confound its effect. A clinical study, therefore, is required to confirm the validity of the results obtained. Further investigations of the toxic and irritant properties are imperative when considering its use in humans. Additionally, the use would depend on cost considerations, odor and flavor.

In conclusion, the present study demonstrates that *P. emblica* extract interferes with the adhesion of *C. albicans* to BEC and denture acrylic *in vitro*.

Conflict of interest statement

We declare that we have no conflict of interest.

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