Effect of five plant extracts on adhesion of *Candida albicans* onto human buccal epithelial cells: an *in-vitro* study

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Background
There is increased prevalence of both fluconazole-resistant *Candida albicans* and non-albicans *Candida* spp. isolated from oral candidiasis (OC) lesions. OC is the most common oral lesion, encountered in HIV infection. On the basis of this, the antiadherence approach to treat or prevent oropharyngeal candidiasis was studied using plant extracts.

Aim
The present study aimed to perform preliminary screening of five plant extracts, namely, aloe vera, Singapore cherries, tea tree, neem, and lemon grass, for their effect on adhesion of *C. albicans* to human buccal epithelial cells (HBEC) in an in-vitro condition.

Materials and methods
A set of 5 and 10 g of plant material, leading to a final concentration of 10 and 20%, respectively, was used. Both aqueous and ethanol extracts were tested. Both *C. albicans* and HBEC were treated with plant extracts under different in-vitro conditions.
An adhesion assay was carried out under an in-vitro condition. *C. albicans*, RL-24 and RL-112, isolated from OC lesions in HIV-seropositive individuals were analyzed for adhesion. The adhesion pattern of *C. albicans* to HBEC under test conditions was compared with the adhesion pattern observed under the control condition. The variation in adhesion was recorded.

Statistical analysis
Statistical analysis was carried out by two-way analysis of variance using IBM SPSS-version 20.

Results
Both aqueous and ethanol extracts of neem. followed by lemon grass were found to consistently inhibit adhesion, which was statistically significant.

Conclusion
This preliminary work has shown a trend that different plant extracts could efficiently inhibit the adherence of *C. albicans* to HBEC and can be explored for an antiadherence therapeutic approach. Development of antiadherent agents using plant extracts seems to be a promising approach in the treatment of OC.

Keywords:
adhesion, *Candida albicans*, lemon grass, neem, tea tree oil

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Introduction

Oral candidiasis (OC) is one of the most common oral lesions encountered in HIV-seropositive individuals [1]. The increased prevalence of both fluconazole-resistant *Candida albicans* and non-albicans *Candida* spp. causing oropharyngeal candidiasis in HIV-seropositive patients has increasingly been reported recently [2–5]. In the wake of these trends, an antiadherence approach to treat or prevent oropharyngeal candidiasis needs careful study.

Adhesion of *C. albicans* to the oral epithelial cells is a prerequisite for the colonization and production of OC infection by the organism [6]. *C. albicans* shows various virulence factors, among which the ability of this organism to adhere to various host cell surfaces has been studied extensively. Various cell wall proteins, lipids, secreted proteins, and carbohydrates of *C. albicans* act as adhesion molecules [7]. Studies have been carried out using various substances to assess their effect on adhesion, for example, the low doses of fluconazole, plant extracts, surfactants including biosurfactants, etc. [8–10].

In this study, we have screened plant products for their effect on adhesion of *C. albicans* to human buccal epithelial cells (HBEC). Plant extractions have been tested for antimicrobial activity since time immemorial. The anticandidal effect of various plants, for example, aloe vera, lemon grass, etc. has already been proved [11–13]. However, studies on the effect of plants on adhesion of *C. albicans* to HBEC are sparse. Therefore, as a preliminary screening test, in the present study, we have evaluated five different plant extracts to understand their action on adherence of *C. albicans* onto HBEC under an in-vitro condition.

Materials and methods

Due precaution was taken while collecting and selecting plant materials with respect to their origin, identification, and human usage.

Preparation of the plant extracts

The plant variety used and the material preferred are shown in Table 1. A set of 5 and 10 g each of plant material was used for extraction, leading to a final concentration of 10 and 20%, respectively. The fresh plant material required was washed in autoclaved tap water, dried, and ground in a sterile mortar and pestle to a fine paste. The paste was transferred to two sterile conical flasks containing 50 ml of distilled water and ethanol separately. The extraction was performed overnight at room temperature in a shaking incubator at 150 rpm. After extraction, to remove all the visible plant artifacts, the solution was filtered through a sterile muslin cloth into a sterile conical flask. Further, the solution was filter sterilized by passing through a membrane filter with a pore size of 0.1 μm (Sartorius Pvt. Ltd., India). The filtrate was lyophilized (Christ Alpha 1-2 LD Plus lyophilizer) and stored for future use. One gram of lyophilized powder was dissolved in 5 ml of sterile PBS, pH 7.2, to attain a final concentration of 200 mg/ml. The same procedure was followed for all the plant varieties used in this study. Each extraction was carried out in two sets. Because of the unavailability of tea tree in our locality, we have used tea tree oil (TTO) (Falcon, Bangalore, India).

Exposure of cells to the plant extracts

Treating candida cells with plant extracts

The experiments were conducted using two isolates of *C. albicans*, RL-24 and RL-112, which were isolated from OC lesions in HIV-seropositive individuals and maintained in our laboratory. To perform the test, *C. albicans* cells were grown in yeast nitrogen broth with 500 mmol/l galactose up to the stationary phase. The cells were then washed thrice in PBS, pH 7.2, and adjusted to McFarland standard 0.5 (10^5 cells/ml). One milliliter of candida cells were incubated with 1 ml of concentrated extracts for 2 h in a shaking incubator at 37°C at 150 rpm. The candida cells pre-exposed to plant extracts were washed thrice in PBS (pH 7.2) to remove excess extract. Candida cells were again adjusted to McFarland standard 0.5 using PBS (pH 7.2) before subjecting them to adhesion.

Table 1 List of plants used for inhibition of adhesion with their scientific name

Plant name	Scientific name	Plant material used
Neem	*Azadirachta indica*	Leaf
Aloe vera	*Aloe barbadensis*	Gel present inside the leaf
Tea tree	*Melaleuca alternifolia*	Tea tree oil
Lemon grass	*Cymbopogon citratus*	Grass
Singapore cherries	*Muntingia calabura*	Ripened fruits

In addition, to determine the probable variation in the morphology and viability of candida cells treated with plant extracts, Gram staining and subculturing on Sabouraud dextrose agar plates were performed.

Treatment of human buccal epithelial cells with plant extracts

HBEC were collected from healthy volunteers by scraping the inner side of the cheeks using sterile wooden sticks. Cells attached to the wooden sticks were released and suspended in PBS (pH 7.2). This cell suspension was washed thrice with PBS (pH 7.2). The turbidity of HBEC was adjusted to around 150–200 cells/10 μl of the suspension using Neubaur's counting chamber. The aliquots of 500 μl each of HBEC were prepared and incubated with 500 μl of concentrated plant extracts for 2 h at 37°C at 150 rpm. The treated HBEC were then washed thrice in PBS (pH 7.2) and readjusted to the required turbidity.

Adhesion experiment

An adhesion experiment was carried out in under an in-vitro condition according to the method of Kimura and Pearsall [14].

Four different adhesion conditions were tested as explained below.

Condition A

Candida cells treated with plant extracts were exposed to untreated (plain) HBEC.

Condition B

Untreated candida cells exposed to HBEC pretreated with plant extract.

Condition C

Both candida and HBEC were pretreated with the plant extracts and adhesion was carried out in a specific plant extract instead of PBS (pH 7.2).

Condition D

Untreated candida cells exposed to untreated HBEC. This condition served as a control.

Under all the different experimental conditions, after pretreatment, cells were washed in PBS (pH 7.2) and adjusted to the required turbidity and a final ratio of 5 : 1 of candida to HBEC was maintained in the reaction mixture. In brief, an adherence test was carried out by

mixing 200 µl of PBS containing the candida cells with 200 µl PBS containing HBEC. However, in condition D, the suspensions of candida and HBEC were prepared in specific plant extracts instead of PBS (pH 7.2). The reaction mixture was incubated at 37°C for 1 h in a shaking incubator at 100 rpm. The mixture was then passed through a membrane filter of pore size 8 µm and the retenant was washed off into a sterile tube, using PBS, pH 7.2, and centrifuged. The sediment was subjected to Gram stain. The Gram-stained smear was observed under an oil immersion objective (×100) and a minimum of 100 epithelial cells were screened. The number of epithelial cells showing yeast cells that adhered onto them was noted and the percentage of adherence was calculated. The adhesion reaction was carried out for all the different sets of experimental conditions in triplicate, and the average of all the readings was calculated. The percentage of HBEC with adhered *C. albicans* cells under test conditions was compared with the results of the control condition and then, the percentage of variation, either reduction or enhancement in adhesion, was calculated.

Statistical analysis

Statistical analysis was carried out by two-way analysis of variance using IBM SPSS-version 20 (IBMM Company, Manufactured in USA, Licence received by SDM College of Medical Sciences, Dharwad). The significance of conditions and the effects of both aqueous and ethanol plant extracts were evaluated separately for their effect on the adhesion.

Results

Effect of various plant extracts on the adhesion pattern of *Candida albicans* onto human buccal epithelial cells

Details of the conditions followed for each extract and abbreviations used for ease of explanation of the results are presented in Tables 2 and 3. Results of adhesion reaction and percentage of reduction in adhesion under each experimental condition are presented in Table 4. Figures 1 and 2 show the results obtained for RL-24 and RL-112, respectively.

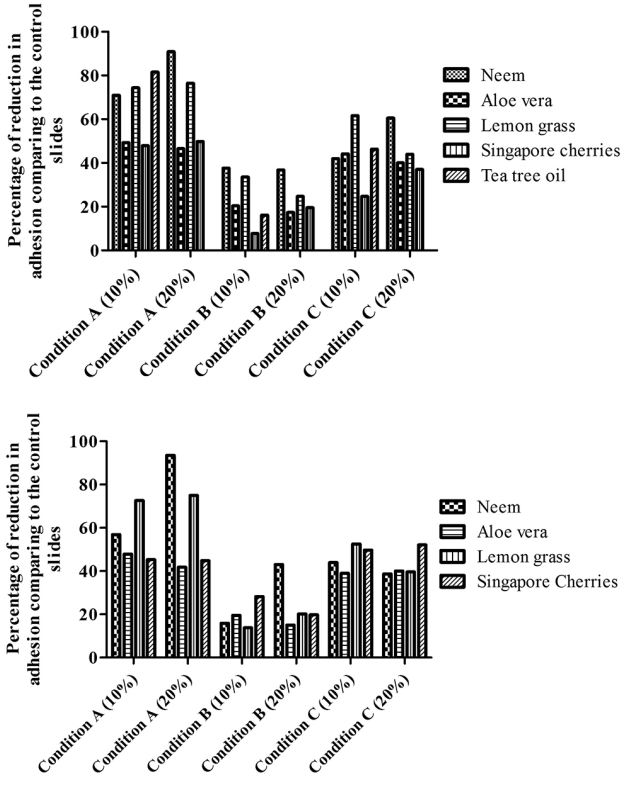
Gram staining of the candida cells treated with plant extract did not show any morphological variations, and the candida isolates showed viability.

Results with RL-24

Condition A

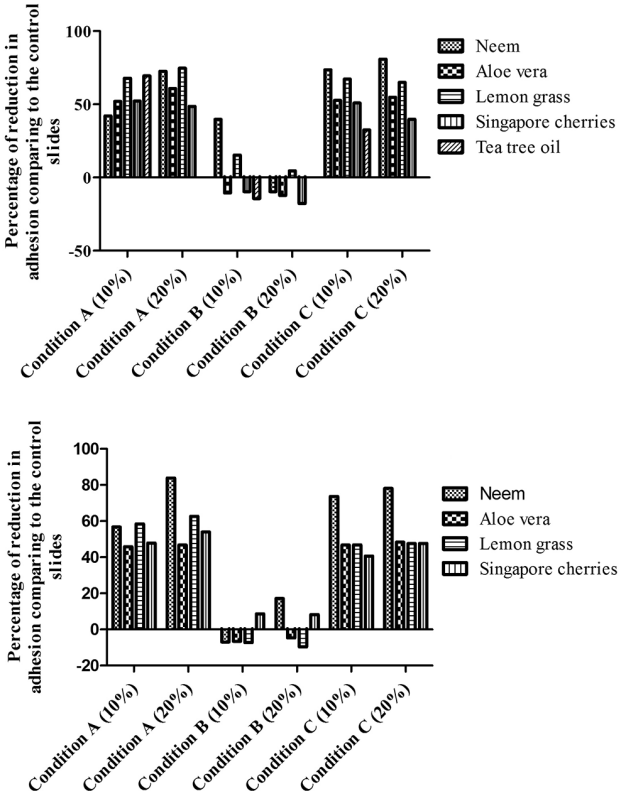
(A least 41.78% to a maximum of a 93.53% reduction in candida adherence was observed. We observed a marked inhibition of candida adhesion with neem 10 g aqueous and ethanol extracts. Neem (20%, ethanol extraction)

Figure 1



Adhesion pattern of RL-24 under different experimental conditions compared with the control condition (CN in the graph).

Figure 2



Adhesion pattern of RL-112 under different experimental conditions compared with the control condition (CN in the graph).

Table 2 Effect of plant extracts on RL-24 under different experimental conditions

Extraction condition (%)	Neem		Aloe vera		Lemon grass		Singapore cherries		Tea tree oil	
	A	B	A	B	A	B	A	B	A	B
Condition A										
10 (aqueous)	13.475	70.93	23.52	49.27	11.89	74.35	24.16	47.89	8.57	81.51
20 (aqueous)	4.24	90.85	24.78	46.55	10.9	76.49	23.27	49.81		
10 (ethanol)	20	56.86	24.15	47.91	12.68	72.65	25.38	45.25		
20 (ethanol)	3	93.56	26.99	41.78	11.6	74.98	25.6	44.78		
Condition B										
10 (aqueous)	28.9	37.66	36.9	20.41	30.8	33.56	42.8	7.68	38.9	16.09
20 (aqueous)	29.3	36.8	38.3	17.39	34.9	24.72	37.3	19.54		
10 (Ethanol)	39	15.88	37.3	19.54	39.98	13.76	33.3	28.17		
20 (ethanol)	26.4	43.05	39.4	15.01	37	20.19	37.2	19.76		
Condition C										
10 (aqueous)	26.9	41.98	25.9	44.13	17.8	61.6	34.8	24.74	24.9	46.25
20 (aqueous)	18.3	60.53	27.8	40.03	26	43.92	29.2	37.01		
10 (ethanol)	26	43.92	18.3	38.96	22	52.55	23.3	49.74		
20 (ethanol)	22	38.74	26	40.03	28	39.6	22.2	52.11		

A indicates the actual percentage of HBEC that adhered to candida cells, B indicates the percentage of reduction in adhesion in a particular condition compared with the results of control experiments (control D slides).

Table 3 Effect of plant extracts on RL-112 under different experimental conditions

Extraction condition (%)	Neem		Aloe vera		Lemon grass		Singapore cherries		Tea tree oil	
	A	B	A	B	A	B	A	B	A	B
Condition A										
10 aqueous	26.4	42.11	21.93	51.91	14.7	67.76	21.78	52.24	14	69.39
20 aqueous	12.5	72.59	17.88	60.79	11.56	74.65	23.47	48.53		
10 ethanol	19.73	56.73	24.7	45.83	18.97	58.4	23.79	47.83		
20 ethanol	7.35	83.88	24.3	46.71	17.03	62.65	20.98	53.99		
Condition B										
10 aqueous	27.5	39.69	50.4	−10.53	38.6	15.35	50	−9.65	52.2	−14.46
20 aqueous	50	−9.65	51.2	−12.28	43.5	4.61	53.7	−17.76		
10 ethanol	48.76	−6.93	48.6	−6.58	48.9	−7.24	41.66	8.64		
20 ethanol	37.76	17.19	47.7	−4.61	50	−9.65	41.86	8.2		
Condition C										
10 aqueous	12.1	73.62	21.7	52.68	15.07	67.14	22.5	50.94	31	32.4
20 aqueous	8.8	80.81	20.7	54.86	16.09	64.91	27.6	39.82		
10 ethanol	12.1	73.62	24.4	46.79	24.39	46.82	27.2	40.69		
20 ethanol	10	78.14	23.7	48.32	24.04	47.58	24.01	47.65		

A indicates the actual percentage of HBEC that adhered to candida cells, B indicates the percentage of reduction in adhesion in a particular condition compared with the results of control experiments (control D slides).

yielded the best result, with a 93.53% reduction in candida adhesion compared with the control. Neem (20%) aqueous extraction also showed good results, with a 90.85% reduction in adhesion. Similarly, candida cells treated with lemon grass extracts (aqueous and ethanol extracts) also showed a reduction in adhesion to HBEC, ranging from 72.65 to 79.49%. TTO showed the second best effect, with an 81.51% reduction in adhesion. Aloe vera, both aqueous and ethanol extracts, and Singapore cherries (aqueous and ethanol extracts) exerted lower inhibition effect on adhesion of candida, ranging from 41.78 to 49.27%, compared with the control condition (Table 2 and Fig. 1).

Condition B

Reduction in adhesion was not high when HBEC were pretreated with plant extracts. The highest reduction was observed with neem (ethanol, 20%) (43.05%). Neem 10% (aqueous) and 20% (aqueous) extracts reduced the adhesion to 37.66 and 36.80%, respectively. All other extracts showed a nearly similar pattern of reduction in adhesion. The least reduction was observed with Singapore cherry (10%) aqueous (i.e. 7.68%).

Condition C

Under this experimental condition, the highest reduction of 61.60% was observed. Singapore cherries 20% in ethanol led to a good reduction (52.11%) compared with its aqueous (20%) counterpart (37.01%). Lemon grass 5 g, aqueous, led to the best inhibition, with a 61.60% reduction in adhesion, followed by neem 10 g aqueous extract, which led to a 60.53% reduction. Besides this, conditions with ethanol extracts of both lemon grass 10%

and Singapore cherries 20% led to 52.55 and 52.11% reductions in adhesion. All other plant extracts yielded similar results, the least reduction observed with Singapore cherry, 10% aqueous extract (i.e. 24.74% reduction).

Results of RL-112

Condition A

Neem, 10 g ethanol extract, led to a reduction of 83.88%, followed by a 74.65% reduction by lemon grass (20%, aqueous). Similarly, neem 20%, aqueous extract, led to a 72.59% reduction, TTO led to a 69.39% reduction, and aloe vera (20%) aqueous led to a 60.79% reduction in adhesion. The remaining plant extracts showed similar results (Table 3 and Fig. 2).

Table 4 Description for the abbreviations used in the graphs

Abbreviation used (%)	Description for the plant extract type
SC 10, aq	Singapore cherries, 5 g extracted using 50 ml distilled water
SC 20 aq	Singapore cherries, 10 g extracted using 50 ml distilled water
SC 10, eth	Singapore cherries, 5 g extracted using 50 ml ethanol
SC 20 eth	Singapore cherries, 10 g extracted using 50 ml ethanol
AL 10, aq	Aloe vera, 5 g extracted using 50 ml distilled water
AL 20 aq	Aloe vera, 10 g extracted using 50 ml distilled water
AL 10, eth	Aloe vera, 5 g extracted using 50 ml ethanol
AL 20 eth	Aloe vera, 10 g extracted using 50 ml ethanol
NM 10, aq	Neem, 5 g extracted using 50 ml distilled water
NM 20 aq	Neem, 10 g extracted using 50 ml distilled water
NM 10, eth	Neem, 5 g extracted using 50 ml ethanol
NM 20 eth	Neem, 10 g extracted using 50 ml ethanol
LG 10, aq	Lemon grass, 5 g extracted using 50 ml distilled water
LG 20 aq	Lemon grass, 10 g extracted using 50 ml distilled water
LG 10, eth	Lemon grass, 5 g extracted using 50 ml ethanol
LG 20 eth	Lemon grass, 10 g extracted using 50 ml ethanol
T0	Tea tree oil
CN	Control slide (condition D)

Condition B

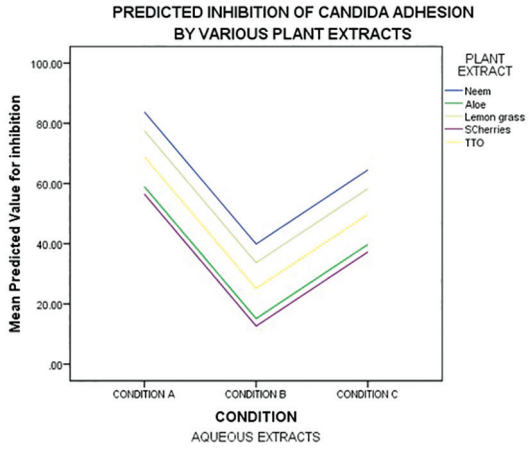
Under this experimental condition, candida cells showed increased adhesion unlike that observed in condition A. Singapore cherries aqueous extracts (10 and 20%) (-9.65 and -17.76%), Aloe vera, both aqueous (10 and 20%) (-10.53 and -12.28%) and ethanol extracts (10 and 20%) (-6.58 and -4.61%), lemon grass ethanol extract (10 and 20%) (-7.24 and -9.65%), and tea leaf oil (-14.46 %) extracts favored adhesion. Even the aqueous extracts of neem 20% and ethanol extracts led to 10% increased (-9.65 and -6.93%) adhesion of candida cells to pretreated HBEC compared with the control. Aqueous extracts of lemon grass and ethanol extracts of Singapore cherries showed a slight reduction in the percentage of adhesion with pretreated HBEC. However, with neem 10% aqueous extraction, there was a 39.69% reduction in adhesion compared with the control.

Condition C

Under this condition, the adhesion behavior of RL-112 varied slightly compared with RL-24. Here, both 10 and 20% of the aqueous and ethanol extracts of neem reduced the adhesion to 73.62, 80.81, 73.62, and 78.19 %, respectively. When both the cells were pretreated with plant extracts and later subjected to adhesion, a reduction was observed in the following order: neem 20%, aqueous 80.81%>neem 20%, ethanol, 781.9%>neem 10% ethanol and 10% aqueous, 73.62%>lemon grass 10%, aqueous 67.14%>lemon grass, and 20% aqueous, 64.91%. Aloe vera, Singapore cherries, lemon grass ethanol extracts, and TTO did not differ much in their inhibition pattern.

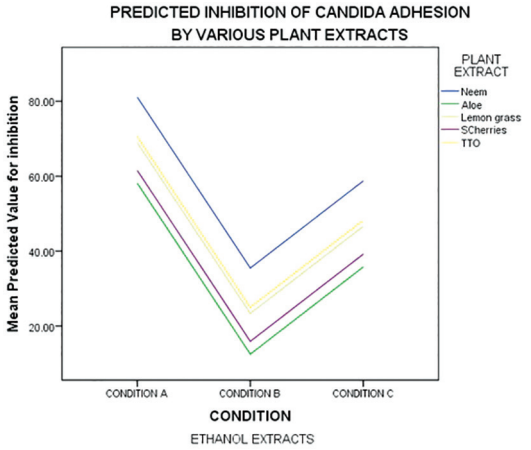
Statistical analysis of these results showed in Figures 3 and 4 confirms that condition 1 is superior to condition 2 and condition 3. In the inhibition of adherence

Figure 3



Comparison of mean predicted value for inhibition of *Candida* on HBEC by aqueous extracts plants under different conditions

Figure 4



Comparison of mean predicted value for inhibition of *Candida* on HBEC by ethanol extracts of plants under different conditions

of candida to HBEC, neem and lemon grass, both aqueous and ethanol extracts, and TTO, scored the first, second, and third positions, respectively.

Discussion

In view of emerging infectious diseases and the alarming increase in drug resistance, studies on antiadherence treatment would be more promising. We selected two *C. albicans* isolates RL-24 and RL-112 to conduct an antiadhesion experiment. Both the strains were isolated from the OC lesions presented by HIV-seropositive individuals. OC is one of most prevalent oral lesions encountered among HIV-seropositive individuals. Fluconazole is the drug of choice for the treatment of OC [1]. Recently, studies from India and other countries have shown an increased prevalence of fluconazole-resistant strains of *C. albicans* in HIV-seropositive individuals with OC [2–5]. In this context, to develop an antiadherence therapeutic approach, screening of plant extracts for their antiadherence activity was attempted in the present study.

Receptor analogs, adhesin analogs, and surface-modifying agents were used to inhibit the adhesion of microbes onto host cells. The number of organisms developing resistance to antiadhesive substances seems to be less compared with antimicrobial agents. However, the limitation of this kind of therapy is the requirement of multiple antiadhesion agents to counter each type of adhesin of infecting pathogens [15].

Plant extracts and surfactants were evaluated for their anti-candidal-adhesive properties both on HBEC and on inert surfaces by earlier researchers [16–20]. Patel *et al.* [21]showed that *Dodonaea viscosa var. angustifolia* (Sand Olive plant) crude extract could inhibit adhesion of *C. albicans* isolated from HIV-seropositive and HIV-seronegative individuals to oral epithelial cells. Mouth rinses with date extract have also been proven to decrease the adhesion of three different species of candida to HBEC [9]. In the present study, we screened crude extracts of aloe vera gel, lemon grass, neem leaf, ripened fruits of Singapore cherries, and TTO for their antiadhesive action. All these plants were available in our locality, except TTO. In the present study, we used distilled water and ethanol as extraction solvents [17,22]. A few workers have used oil forms of plant materials Taweechaisupapong *et al.* [23].

In our study, we found that among the five plant extracts evaluated, neem and lemon grass were more effective in reducing adhesion, followed by TTO, whereas aloe vera and Singapore cherry extracts were not very effective. However, in one set of experiments (condition A in RL-112), aloe vera was also found to be effective in inhibiting adhesion. However, the persistent antiadhesion activity of neem extract was not observed with other plant extracts screened.

In general, neem is known to have antimicrobial activity against a broad range of organisms [13]. In the present study, the neem extract showed maximum antiadhesive activity. A similar observation was made by Polaquini *et al.* [17] although composite resins were used as the adhesion matrix.

In the present study, lemon grass extract was found to be only the second best plant material in inhibiting adhesion. Taweechaisupapong *et al.* [23] reported that lemon grass oil (1.7 mg/ml) showed 80% candidicidal activity toward the cells in preformed biofilms.

Another promising candidate for inhibition of candidal adhesion observed in the present study was tea tree (*Melaleuca alternifolia*) oil. TTO was shown to exert an anticandidal effect. TTO shows in-vivo and in-vitro anticandidal activity against vaginal candidiasis produced both by azole-resistant and susceptible strains of *C. albicans* [20]. TTO and terpinen-4-ol administered 3 and 24 h after candida infection in mice with fluconazole-sensitive and fluconazole-resistant *C. albicans* strains separately reduced the symptoms of OC and viable candida cell numbers in their oral cavity [24].

Terpinen-rich TTO at a sub inhibitory concentration (0.016–0.25%) has been shown to inhibit biofilm formation and adhesion of *C. albicans* on polystyrene, HBEC, and HeLa cells [25]. We also observed that under condition A, TTO effectively inhibited the adhesion of candida cells to HBEC and can be considered as a promising antiadherent drug.

Aloe vera also showed a good result in inhibiting the adhesion in condition A, where only candida cells were pretreated with plant extracts, and in condition C, where both types of cells were pretreated with the plant extracts. In case of aloe vera, leaf without gel and only the gel were used separately to study the antimicrobial activity against both bacteria and fungi [26]. In the present study, we used a gel of aloe vera. Gel from aloe vera was shown to exert better antimicrobial effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Trichophyton metagrophyte* than leaf extracts of aloe vera. However, gel did not exert any inhibitory effect on *C. albicans* [13]. We suggest here that other preparations of aloe vera might show different and/or improved results toward adhesion inhibition, which can be further elucidated.

Singapore cherry plant (*Muntingia calabura*) was shown to be antiseptic, antispasmodic, hypotensive,

cardioprotective, and anticarcinogenic by previous researchers [27,28]. However, reports on its antimicrobial or antiadherent nature are sparse. The ripened fruits, used in the present study, showed a reducing effect on adhesion, but less than that of the other extracts.

All the plant materials used here can also be recommended for *in vivo* use; therefore, the effect of these extracts in-vivo conditions can also be evaluated. The specific effect of these extracts on adhesins and cell surface properties such as cell surface hydrophobicity and ultrastructural modification needs to be elucidated as shown in other references [23,29].

Interestingly, we observed that pretreatment of only HBEC with plant extracts and did not yield a promising result. This could be because of the effect of these plant extracts mainly on candidal cells rather than on HBEC. Molecular elucidation of surface receptors/cell membrane factors of both candida and HBEC after exposure to these plant extracts can provide us information on the possible basis for this kind of variations and can also shed light on the probable mechanism involved in adhesion.

Conclusion

In the present study, both aqueous and ethanol extracts of neem, followed by lemon grass were found to consistently inhibit adhesion. As there is an increased prevalence of fluconazole-resistant *C. albicans* from OC in HIV-seropositive individuals, development of antiadherent agents seems to be a promising approach in the treatment of OC that should be explored further.

Acknowledgements

Conflicts of interest

None declared.

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