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RESEARCH ARTICLE

Evaluation of *in vitro* antifungal effects of synthetic and herbal mouth rinses on oral *Candida albicans* and *Candida glabrata*

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ABSTRACT

Mouth rinses which function as breath fresheners, medicaments, and antiseptics can also deliver oral therapeutic agents. This study evaluated and compared the antifungal effects of synthetic and herbal mouth rinses on oral *C. albicans* and *C. glabrata* via disk diffusion, minimal inhibition concentration (MIC), minimum fungicidal concentration (MFC), time-kill assay, and growth profile tests. The four chemical mouth rinses, namely Brand O (A), Brand M (B), Brand H (C), and Brand B (D) used in the study showed positive antifungal activity in these two species. The average diameter of the inhibition zones obtained from the disk diffusion test was higher in mouth rinse B (*C. albicans* = 12.0 ± 0.9 mm, *C. glabrata* = 13.5 ± 0.8 mm) compared to those in C, A and D. Both *Candida* species exhibited similar MIC and MFC values, ranging from 1.63 ± 0.5 to 18.75 ± 0.0 µg/mL and 6.51 ± 2.01 to 50.00 ± 9.36 µg/mL, respectively. These synthetic mouth rinses had efficient killing activity eliminating 50% of the growing population of both *Candida* spp. following 15 seconds exposure time. Analyses of the growth profile curves showed that mouth rinses B and A resulted in rapid growth depletion of both *Candida* spp. Meanwhile, three herbal mouth rinses, namely Brand S (E), Brand C (F), and Brand P (G), were less effective against *C. albicans* and *C. glabrata*. Mouth rinses B and A contained cetylpyridinium chloride and chlorhexidine, respectively, and could be an effective alternative for controlling and preventing oral candidiasis.

Keywords: Antifungal effects; synthetic mouth rinses; herbal mouth rinses; *Candida albicans*; *Candida glabrata*.

INTRODUCTION

Oral candidiasis is an opportunistic infection of the oral cavity resulting in various clinical manifestations depending on its type (Rajendra Santosh *et al*., 2021). Types of oral candidiasis include the acute (pseudomembranous and erythematous) and chronic (pseudomembranous, erythematous, and hyperplastic) forms as well as *Candida*-associated lesions (angular cheilitis, denture-associated erythematous and median rhomboid glossitis) (Garcia-Cuesta *et al*., 2014). The disease can develop regardless of age, including in infants (Patil *et al*., 2015), HIV/AIDS-infected individuals (Hosain Pour *et al*., 2018), cancer patients (Alnuaimi *et al*., 2015), denture wearing patients (Lakshmi *et al*., 2015) as well as diabetics (Obradovic *et al*., 2011).

Oral candidiasis is caused by an overgrowth or infection in the oral cavity by a fungus from the genus *Candida* (Guida, 1988; Aggarwal *et al*., 2018). *Candida* spp. is predominantly found as a common commensal organism in the oral cavities of 53% of the general population. Of the 150 species of this genus isolated in the oral cavity, 80% are *Candida albicans*, which remain the most common and essential causative agent of the disease (Coronado-Castellote & Jimenez-Soriano, 2013). *C. albicans* possesses pathogenicity factors that allow for more frequent disease development than other *Candida* species. It has also been identified as the most prevalent human fungal pathogen responsible for mucosal and systemic fungal infections (Tsui *et al*., 2016). *C. tropicalis*, *C. glabrata*, *C. pseudotropicalis*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. stellatoidea* are among *Candida spp*. isolated in the oral cavity (Premanathan *et al*., 2011). Of these species, *C. glabrata* is ranked the second most common *Candida* spp. causing candidiasis after *C. albicans*, depending on the site of infection (Mota *et al*., 2015).

Patients infected with oral candidiasis could suffer various symptoms such as oral mucosal inflammation that produces uncomfortable feelings, pain, erosion, swallowing problems, taste abnormalities, and hyperplasia of the oral mucosa (Yamamoto, 2010; Vila *et al*., 2020). Overgrowths of *Candida* spp. in the oral cavity may also spread to other tissues or organs. The application of antifungal

or antimicrobial treatments remains the best option to effectively control and avoid the sequelae of oral candidiasis (de Oliveira Santos *et al*., 2018). Antifungal medications (i.e., mouth rinses) can deliver therapeutic ingredients to accessible interproximal hard and soft tissues (Akca *et al*., 2016). It also has a generally lower incidence of adverse events, with little potential for resistance development, and is safe for routine use (Tartaglia *et al*., 2019).

Mouth rinses (mouthwashes) are solutions used to destroy or eliminate bacteria in the mouth, serve as an astringent, conceal unpleasant smells, relieve infections, and avoid dental caries (Akande *et al*., 2004; Amit, 2015). Mouth rinses are usually safe and have active ingredients to significantly reduce or remove plaque accumulation (Haydari *et al*., 2017). Since most individuals do not adequately brush their teeth, mouth rinses can be considered an alternative tool for teeth cleaning and routine application (Pradeep Kumar & Athiban Raj, 2017).

Different mouth rinses have been manufactured to treat or control oral candidiasis. Most of the commercialized mouth rinses available are synthetic-based and contain chlorhexidine gluconate, triclosan, ethanol, dyes, astringent components such as zinc chloride or acetate, and aluminium potassium sulphate (Dar-Odeh *et al*., 2011; Jeddy *et al*., 2018). Chlorhexidine is an antiseptic agent that works against bacteria, viruses, and fungi (Brookes *et al*., 2020) and prevents oral complications such as chronic or opportunistic infections (Lanzos *et al*., 2010; Ahmad, 2021). Triclosan is a non-phenolic, broad-spectrum antimicrobial and antiplaque agent. It enables the mouth rinse to coat the oral mucosa (Phan & Marquis, 2006). Nevertheless, these chemicals can generate various side effects, ranging from taste disturbances to allergic contact stomatitis (Jeddy *et al*., 2018).

To overcome these side effects, non-toxic and natural mouth rinses using various plants and herbal extracts have been introduced (Bhat *et al*., 2013; Jeddy *et al*., 2018). These mouth rinses are effective against *Candida* spp. In Iran, Talebi *et al*. (2014) tested the effects of herbal ingredients in mouth rinses, such as *Salvadora persica* and *Matricaria chamomilla,* against *C. albicans*. Meanwhile, in India, an *in vitro* study was conducted to evaluate the antifungal effects of mouth rinses comprising seven natural ingredients, specifically *S. persica*, *Terminalia bellerica*, *Piper betle*, *Gossia fragrantissima*, *Elettaria cardamomum*, *Mentha* spp. and *Trachyspermum* against *C. albicans* (Ravikumar *et al*., 2016). The effectiveness of herbal mouth rinses containing red ginseng extracts in reducing oral bacterial count has also been reported in India (Jeddy *et al*., 2018). To the best of our knowledge, no study has been conducted in Malaysia on the differences between synthetic and herbal mouth rinses containing antifungal properties for inhibiting the growth of *C. albicans* and *C. glabrata*. This study thus aimed to evaluate and compare the antifungal effects of synthetic and herbal mouth rinses on oral *C. albicans* and *C. glabrata*.

MATERIALS AND METHODS

Synthetic and herbal mouth rinses

Seven mouth rinses were tested in this study, four of which namely Brands O, M, H, and B, were synthetic-based while Brands S, C, and P were herbal-based. These mouth rinses are commercially available in local pharmacies. The synthetic- and herbal-based mouth rinses with their respective active ingredients are presented in Table 1.

Table 1. The active ingredients of synthetic- and herbal-based mouth rinses used in the study

Mouth rinses		Active ingredients
Commercial names	Designation	
Synthetic-based		
Brand O	A	Chlorhexidine (CHX) at 0.12% (w/v)
Brand M	B	Cetylpiridinium chloride (CPC) at 0.053% (w/w)
Brand H	C	Chlorhexidine + Cetylpiridinium chloride (CHX + CPC) at 0.12% w/v CHX
Brand B	D	Hexetidine (HEX) at 0.1% (w/v)
Herbal-based		
Brand S	E	*Piper betle* at 100% (w/v)
Brand C	F	*Eugenia caryophyllus* at 100% (v/v)
Brand P	G	Consist of equal concentration of *Calendula officinalis, Plantago major, Fragaria vesca, Matricaria chamomilla,* at 100% (v/v)

Preparation of *Candida* spp. suspension

The clinical isolates of *C. albicans* (C467 IMR) and *C. glabrata* (C466 IMR) were obtained as pure cultures from the Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. The stocks were thawed and revived in Sabouraud Dextrose Agar (SDA) broth media (Becton Dickinson, Franklin Lakes, NJ, USA). An inoculum of the growth suspension was spread on the SDA agar plates and incubated for 18 to 24 hours at 37°C. Several colony-forming units were then transferred and suspended in fresh SDA broth. The optical density (OD) was adjusted to 0.144 using a spectrophotometer (Shimadzu UV160A, Kyoto, Japan) and read at 550 nm wavelength. This concentration corresponded visually to the 0.5 of the McFarland standards. At an OD of 0.144, the cell concentration was standardized to 10^6 CFU/mL (Fathilah *et al.*, 2009).

Morphological characterization of *C. albicans* and *C. glabrata* colonies and cells

Fifty µL of *C. albicans* and *C. glabrata* suspension were pipetted and inoculated on SDA plates. After 18 to 24 hours incubation, the morphological colonies of both species were observed and recorded. A Gram-staining procedure was carried out using a single colony and identified under a light microscope at 100X magnification. The characteristics and morphology of the cells were visualized using an image analyzer.

Disk diffusion (Kirby-Bauer) test

Sterile paper disks of 6 mm diameter (Oxoid, Basingstoke, Hampshire, UK) were impregnated with 40 µL of mouth rinse at a neat concentration. A similar disk was impregnated with 40 µL of distilled water as a negative control. As positive control, another disk was impregnated with 40 µL of nystatin (Oxoid, Basingstoke, Hampshire, UK). These disks were placed on Mueller-Hinton agar plates (Becton Dickinson, Franklin Lakes, NJ, USA), previously streaked with *C. albicans* and *C. glabrata* suspension. After incubating for 24-48 hours at 37°C, the plates were observed for colony growth. The susceptibility of the mouth rinses to such growth was determined by the inhibition growth zones of *C. albicans* and *C. glabrata* surrounding the disks. The absence of inhibition zones indicated the negative growth inhibition capacity of the mouth rinses towards *Candida* spp. (Smith *et al.*, 1985; Bhattacharjee, 2015). The diameter of the inhibition growth zones was measured in millimeters using a digital caliper.

Minimal inhibition concentration

A broth dilution method was used to determine the MIC of mouth rinses in inhibiting the growth of *C. albicans* and *C. glabrata* (Cappuccino & Sherman, 2007). Ten sterile test tubes (T_1 to T_{10}) containing 5 mL of SDA broth were prepared for each mouth rinse. Five mL of mouth rinse with a neat concentration was added to T_1 and T_2 and mixed thoroughly. Subsequently, 5 mL from T_2 was pipetted and transferred into T_3. These steps were serially repeated to T_9 to obtain the descending order of concentration in the tubes (Table 2). Five mL of candida suspension (10^6 CFU/mL) was added from T_2 to T_{10}. The positive control was T_1, which contained a mix of the SDA broth and the highest concentration of mouth rinse without candida suspension. Meanwhile, the negative control was T_{10} containing a mix of the SDA broth and the candida suspension without mouth rinse. Similar steps were performed for nystatin as the control group. After 18 to 24 hours incubation at 37°C, the MIC of the mouth rinses on *C. albicans* and *C. glabrata* growth was measured by comparing the turbidity levels among all tubes (T_1 to T_{10}) and the control tube (nystatin).

Minimal fungicidal concentration

A 100 µL suspension was aliquoted from three tubes with no visible growth of *C. albicans* and *C. glabrata* and subsequently sub-cultured onto three separate SDA plates. The three tubes used to determine the MFC were T_6, T_7, and T_8. Following incubation of 18 to 24 hours at 37°C, the MFC of each mouth rinse was determined based on the absence of growth of *C. albicans* and *C. glabrata* colonies on the SDA plates.

Time-kill assay

Each mouth rinse was diluted to 50% using saline. A 100 µL of candida suspension (i.e., *C. albicans* and *C. glabrata*) (10^6 cell/ml) was inoculated into a test tube containing 5 mL of mouth rinse (50% dilution) and mixed thoroughly. Subsequently, 20 µL of the suspension was aliquoted and streaked onto an SDA plate at 15-second intervals (i.e., t_0 to t_{180}). In total, 13 plates representing 13 intervals (t_0, t_{15}, t_{30}, t_{45}, t_{60}, t_{75}, t_{90}, t_{105}, t_{120}, t_{135}, t_{150}, t_{165} and t_{180}) were each incubated for 24 hours at 37°C before the reductions in CFU (colony-forming units) were recorded. The time-kill curves of each mouth rinse were constructed by plotting the percentage reduction in a CFU at each time interval. The killing-efficacy rate for each mouth rinse in this study was based on the following formula:

Table 2. The concentration of active ingredients in a respective mouth rinse used in the study

Mouth rinses	Concentration of active ingredients (ug/mL)									
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
Synthetic (w/v)										
A	600.00	300.00	150.00	75.00	37.50	18.75	9.37	4.68	2.34	–
B	250.00	125.00	62.50	31.25	15.62	7.81	3.91	1.95	0.98	–
C	600.00	300.00	150.00	75.00	37.50	18.75	9.37	4.68	2.34	–
D	500.00	250.00	125.00	62.50	31.25	15.62	7.81	3.91	1.95	–
Herbal (v/v)										
E	50.00	25.00	12.50	6.25	3.12	1.56	0.78	0.39	0.19	–
F	50.00	25.00	12.50	6.25	3.12	1.56	0.78	0.39	0.19	–
G	50.00	25.00	12.50	6.25	3.12	1.56	0.78	0.39	0.19	–
Control (v/v)										
N	50.00	25.00	12.50	6.25	3.12	1.56	0.78	0.39	0.19	–

A: Brand O; B: Brand M; C: Brand H, D: Brand B; E: Brand S; F: Brand C; G: Brand P; N: nystatin.

$$\text{Colony count} = \frac{\Sigma \text{ CFU } (1-6)}{6}$$

$$= X \pm SD$$

$$\text{Killing-efficacy percentage} = 100 - \frac{X \pm SD}{C_0} \times 100$$

Where C_0 represents CFU at t_0 and X ± SD is the mean of six determinations and standard deviation at time intervals.

Growth profile curve study

To determine the average growth profile for both *Candida* species, 1 mL of candida suspension (*C. albicans* and *C. glabrata*) was inoculated in a conical flask containing 100 mL of SDA media and subsequently incubated in a shaking water bath (SWB 27, Marshal Scientific, Hampton, NH, USA) at 37°C. One mL of each mouth rinse at a neat concentration was added to determine the effect of the mouth rinse on the growth profile of *C. albicans* and *C. glabrata*. The growth profile was recorded at the beginning of the log phase (A_0). Both untreated and treated growth profiles of the *Candida* species with mouth rinses were monitored for 24 hours by recording the OD readings. Growth profile curves were generated by plotting the changes in OD against time. Similar protocols were carried out using nystatin as a positive control.

Data analysis

Data were analyzed using Microsoft Excel and Statistical Package for the Social Sciences (SPSS) for Windows version 20.0 (SPSS, Chicago, IL, USA). In this study, the values of the growth inhibition zones in the disk diffusion tests, the MIC and MFC in the susceptibility tests, and percentage reductions in the growth profiles were presented as mean ± standard deviation (Mean ± SD).

RESULTS

Morphological characteristics of *C. albicans* and *C. glabrata* colonies and cells

Similar characteristics of *C. albicans* and *C. glabrata* colonies and cells were observed on the SDA plates. The colonies of both species were circular, creamy white colored, and had a soft mucoid texture. They were approximately 1 mm in diameter. Both *C. albicans* and *C. glabrata* cells reacted positively to the Gram-staining procedure, and large purple and grape-like cells were observed (Figure 1).

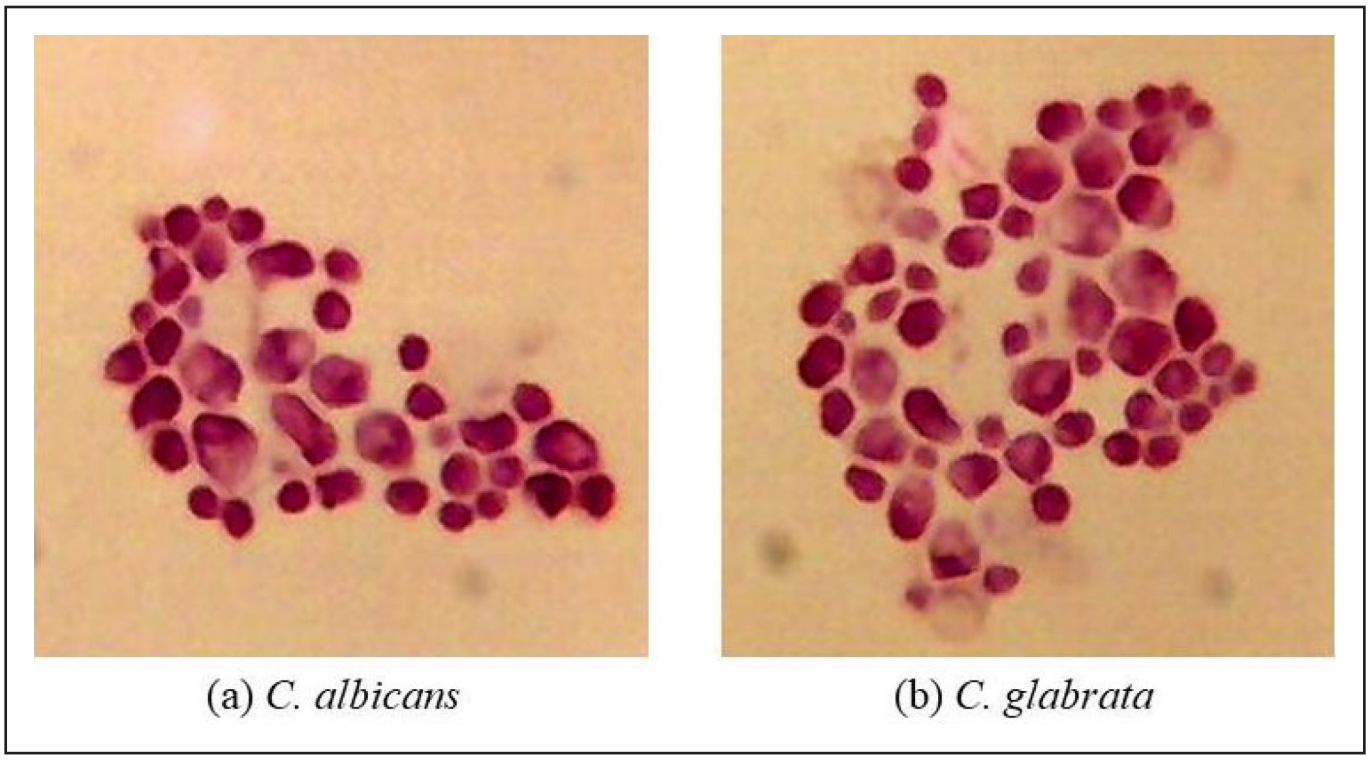

Figure 1. Morphological characteristics of (a) *C. albicans* and (b) *C. glabrata* cells.

Sensitivity of mouth rinses towards *Candida* spp. growth

Overall, *C. albicans* and *C. glabrata* were sensitive to the active ingredients in each synthetic mouth rinse. Based on the diameter of the growth inhibition zone (Table 3), both *Candida* species were most sensitive to mouth rinse B (*C. albicans* = 12.0 ± 0.9, *C. glabrata* = 13.5 ± 0.8), followed by C (*C. albicans* = 11.8 ± 0.9, *C. glabrata* = 11.5 ± 0.5) and A (*C. albicans* = 11.3 ± 0.4, *C. glabrata* = 11.2 ± 0.4) and least sensitive to mouth rinse D (*C. albicans* = 7.3 ± 0.3, *C. glabrata* = 7.3 ± 0.4). The study showed that both *Candida* species were not sensitive (resistant) to all herbal mouth rinses, as no growth inhibition zones were detected from the disk diffusion test.

Table 3. The inhibition growth zones of *C. albicans* and *C. glabrata* recorded in the disk diffusion test

Mouth rinses	Active ingredient(s)	Diameter of growth inhibition zone (mm)	
		C. albicans	*C. glabrata*
Synthetic			
A	CHX	11.3 ± 0.4	11.2 ± 0.4
B	CPC	12.0 ± 0.9	13.5 ± 0.8
C	CHX + CPC	11.8 ± 0.9	11.5 ± 0.5
D	HEX	7.3 ± 0.3	7.3 ± 0.4
Herbal			
E	*P. betle*	resistant	resistant
F	*E. caryophyllus*	resistant	resistant
G	*C. officinalis, P. major, F. vesca, M. chamomilla*	resistant	resistant
Control			
N	Nystatin	13.8 ± 0.6	14.7 ± 0.5
D	dH_2O	resistant	resistant

A: Brand O; B: Brand M; C: Brand H, D: Brand B; E: Brand S; F: Brand C; G: Brand P; N: nystatin; D: distilled water.

Minimal inhibition concentration and minimal fungicidal concentration

All four synthetic-based mouth rinses showed positive antifungal activity against the growth of *C. albicans* and *C. glabrata*, with MIC values ranging between 1.63 ± 0.5 and 18.75 ± 0.0 µg/mL (Table 4). The MIC values for four mouth rinses in inhibiting the growth of both *Candida* species were similar. Mouth rinse B had the lowest MIC (1.63 ± 0.5), followed by mouth rinse A (9.37 ± 0.0), B (15.62 ± 0.0), and C (18.75 ± 0.0). The MIC of the herbal-based mouth rinses (E and F) in inhibiting growth of *C. albicans* and *C. glabrata* were similar (25.00 ± 0.0) except for mouth rinse G, which was not detected. The MFC values of synthetic-based mouth rinses were more than 50% of the MIC values in inhibiting the growth of both *Candida* species. Mouth rinse B (6.51 ± 2.01) had the lowest MFC value followed by mouth rinse A (25.00 ± 9.68), D (31.25 ± 0.0), and C (50.00 ± 9.36). In contrast, no MFC of herbal mouth rinses was detected in the study.

Table 4. The MIC and MFC of *C. albicans* and *C. glabrata,* when tested by mouth rinses

Mouth rinses	MIC (Mean ± SD)		MFC (Mean ± SD)	
	C. albicans	*C. glabrata*	*C. albicans*	*C. glabrata*
Synthetic				
A	9.37 ± 0.0	9.37 ± 0.0	25.00 ± 9.68	25.00 ± 9.68
B	1.63 ± 0.5	1.63 ± 0.5	6.51 ± 2.01	6.51 ± 2.01
C	18.75 ± 0.0	18.75 ± 0.0	50.00 ± 9.36	50.00 ± 9.36
D	15.62 ± 0.0	15.62 ± 0.0	31.25 ± 0.0	31.25 ± 0.0
Herbal				
E	25.00 ± 0.0	25.00 ± 0.0	not detected	not detected
F	25.00 ± 0.0	25.00 ± 0.0	not detected	not detected
G	not detected	not detected	not detected	not detected
Control				
N	0.195 ± 0.0	0.195 ± 0.0	0.39 ± 0.0	0.32 ± 0.1

A: Brand O; B: Brand M; C: Brand H, D: Brand B; E: Brand S; F: Brand C; G: Brand P; N: nystatin.

Efficacy of mouth rinses as antifungals based on time-kill assay

The synthetic-based mouth rinses showed active fungal activity against *C. albicans* (Figure 2a) and *C. glabrata* (Figure 2b). Mouth rinse A produced the most effective antifungal activity, followed by mouth rinse D, B, and C, registering reductions in growth population of 85%, 65%, 45%, and 38%, respectively following 15-second exposures. At 60-second exposure, all four synthetic-based mouth rinses had more than 50% reduction in growth populations. The herbal-based mouth rinses were less effective in killing *C. albicans* and *C. glabrata* than the synthetic-based one. At 60-second exposure, about 20% of *C. albicans* and *C. glabrata* populations were reduced. No changes (percentage reduction) were observed throughout the 180-second exposure time (data not shown).

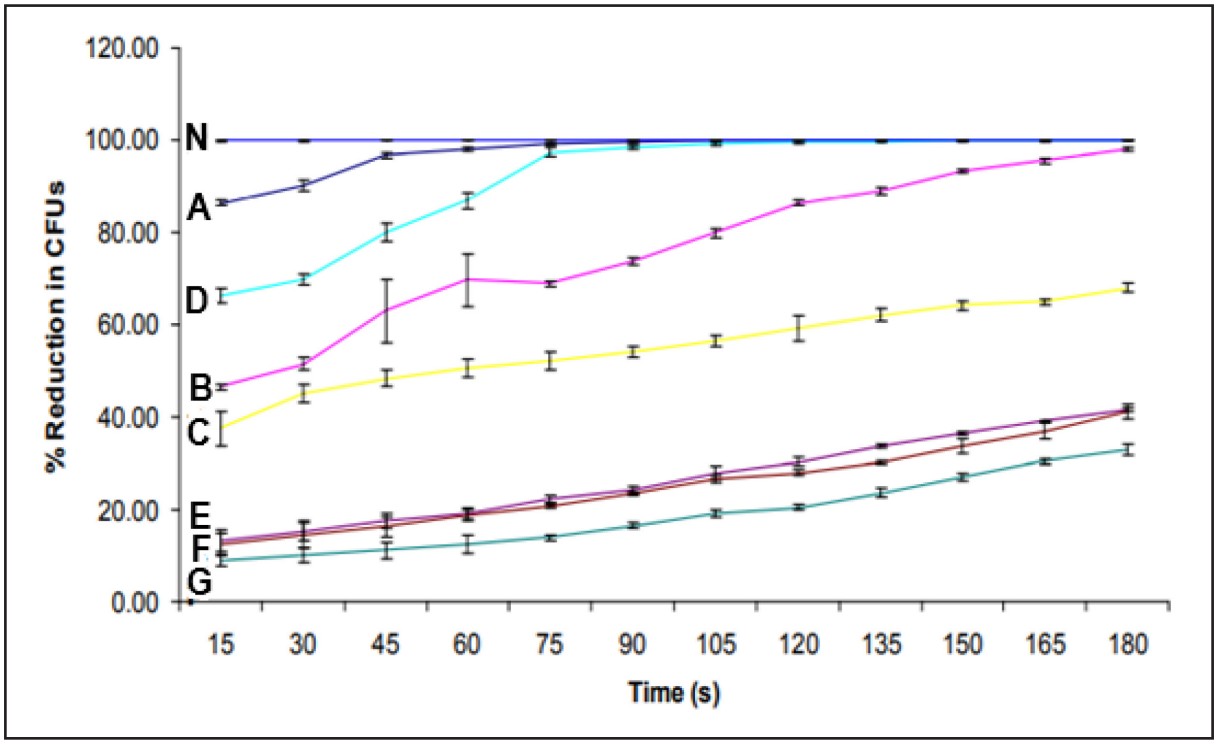

Figure 2a. Time-kill curves showing the percentage reduction in the colony-forming units (CFUs) of *C. albicans* tested by synthetic-based mouth rinses (A: Brand O; B: Brand M; C: Brand H, D: Brand B; E: Brand S; F: Brand C; G: Brand P; N: nystatin).

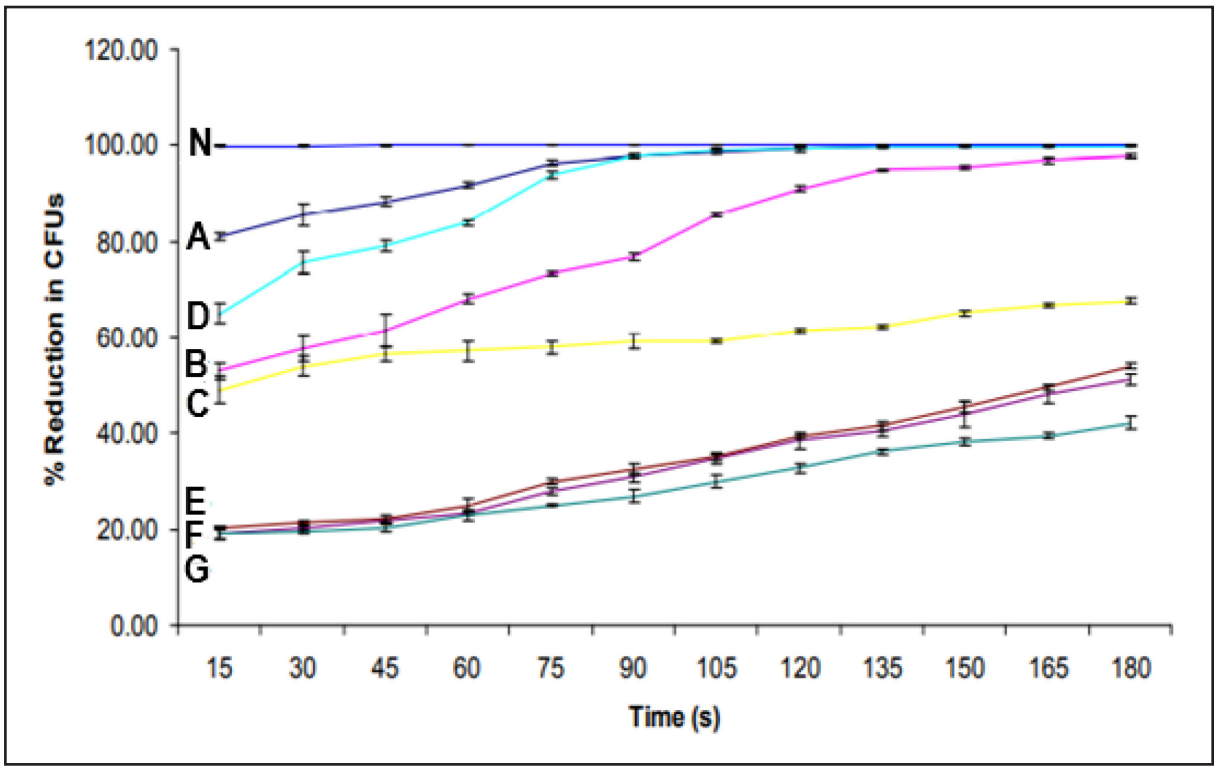

Figure 2b. Time-kill curves showing the percentage reduction in the colony-forming units (CFUs) of *C. glabrata* tested by synthetic-based mouth rinses (A: Brand O; B: Brand M; C: Brand H, D: Brand B; E: Brand S; F: Brand C; G: Brand P; N: nystatin).

Effect of mouth rinses on *C. albicans* and *C. glabrata* evaluated by growth profile curve patterns.

Both *C. albicans* and *C. glabrata* produced similar sigmoidal-shaped growth curves under the untreated growth condition. A 7-8 hours lag phase was followed by a stable logarithmic phase from the 12th to 17th hour. Stationary growth occurred about 20 hours after inoculation (Figure 3). The curve patterns deviated when *C. albicans* (Figure 4a) and *C. glabrata* (Figure 4b) were treated with synthetic-based mouth rinses. Meanwhile, there was no deviation in the sigmoidal patterns of the growth profile curves of *C. albicans* and *C. glabrata* when treated with herbal-based mouth rinses E, F, and G (data not shown). Further analysis showed that mouth rinse B had the highest percentage of reduced growth of *C. albicans* (99.11 ± 0.88) and *C. glabrata* (99.02 ± 0.27) during the A_2 to A_8 of the logarithmic phase (Table 5). The percentage reduction in *C. glabrata* growth (98.84 ± 0.58) was slightly higher compared to *C. albicans* (94.42 ± 3.42) when tested using a mouth rinse A. Likewise, mouth rinses C and D recorded a higher percentage reduction in *C. glabrata* growth (H = 87.92 ± 2.08, B = 64.43 ± 6.34) compared to *C. albicans* (H = 51.39 ± 4.65, B = 47.70 ± 9.74). Meanwhile, the lowest reduced growth percentage of both *Candida* species was tested with all three herbal-based mouth rinses (Table 6). Mouth rinse E recorded a slightly greater reduction in the growth of *C. albicans* (11.09 ± 0.722) than *C. glabrata* (8.61 ± 6.89). A similar pattern was observed with mouth rinses F (*C. albicans* = 8.48 ± 4.06, *C. glabrata* = 5.73 ± 5.04) and G (*C. albicans* = 5.54 ± 1.84, *C. glabrata* = 3.17 ± 2.71).

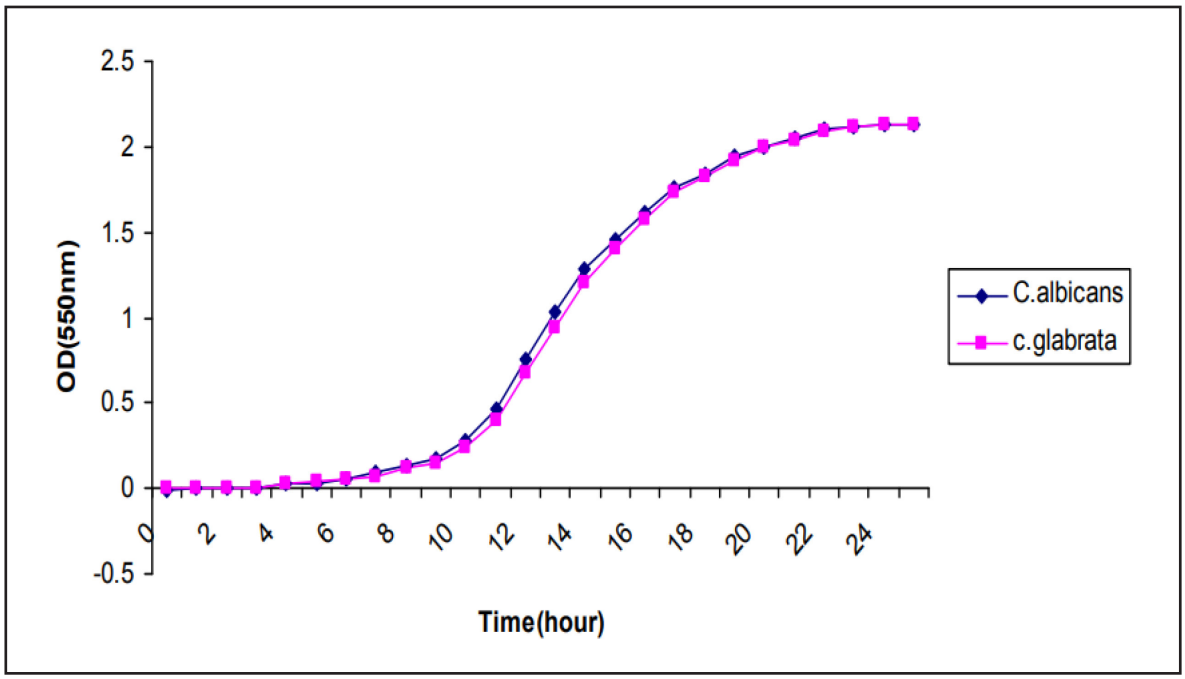

Figure 3. The growth curve pattern of *C. albicans* and *C. glabrata* was plotted under normal growth conditions.

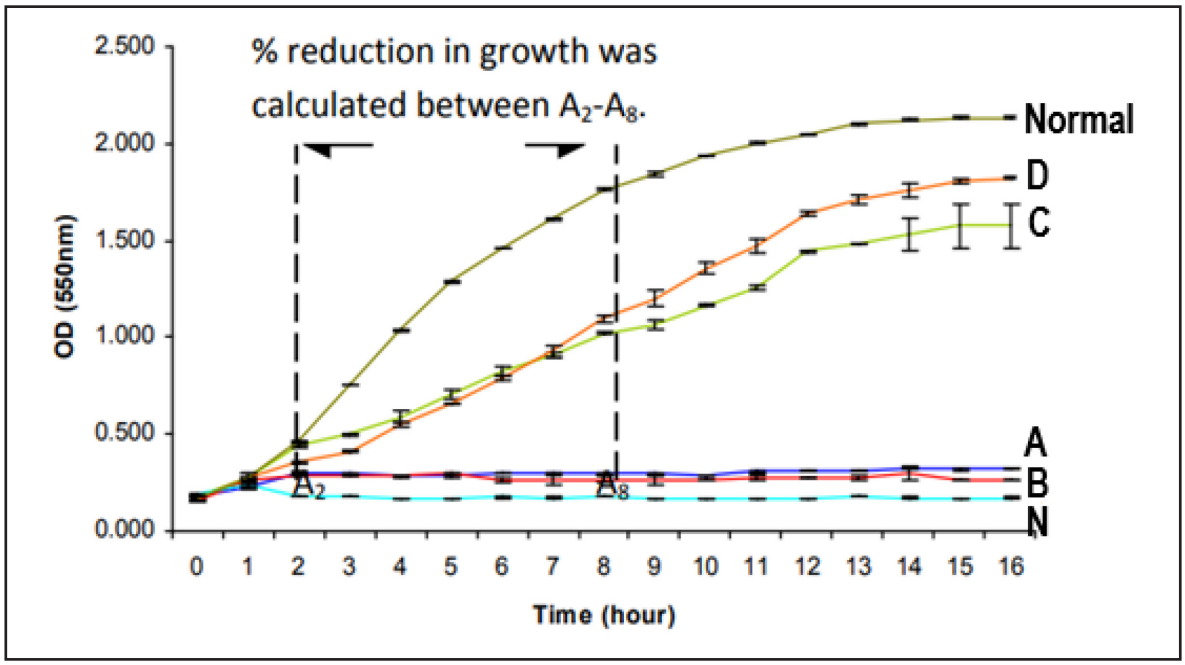

Figure 4a. The growth curve pattern of *C. albicans* plotted under the normal growth condition following treatment by synthetic-based mouth rinses (A: Brand O; B: Brand M; C: Brand H, D: Brand B; N: nystatin).

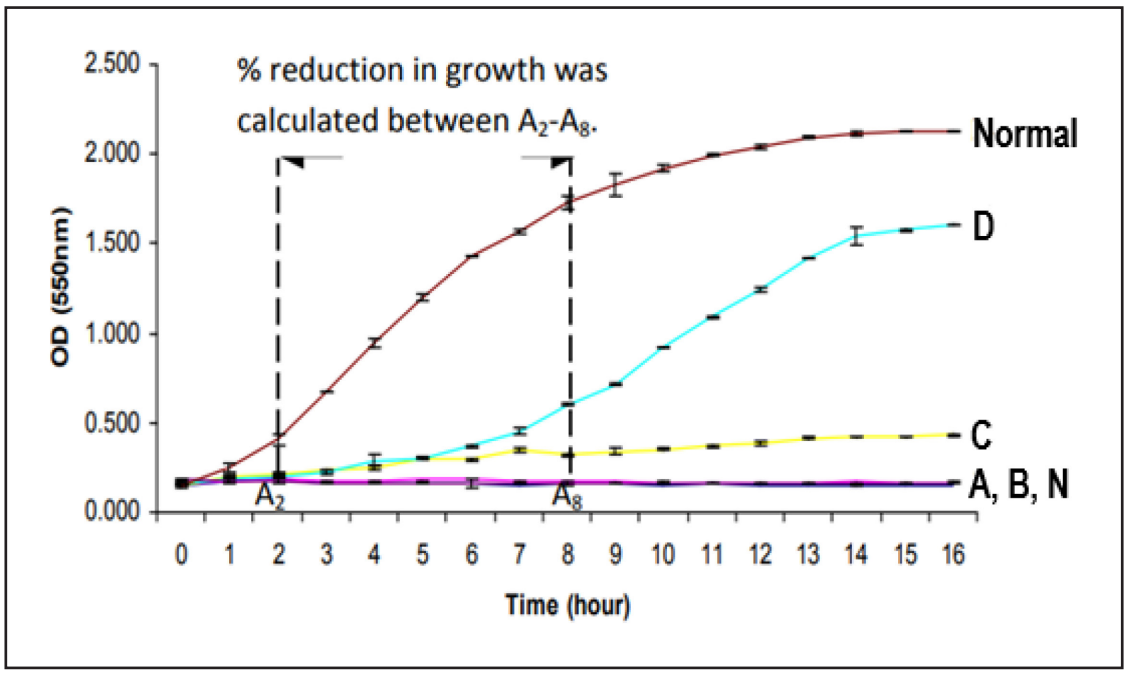

Figure 4b. The growth curve pattern of *C. glabrata* plotted under the normal growth condition following treatment by synthetic-based mouth rinses (A: Brand O; B: Brand M; C: Brand H, D: Brand B; N: nystatin).

Table 5. The percentage of reduced growth of *C. albicans* and *C. glabrata* during the logarithmic phase of A_2 to A_8 when tested with synthetic-based mouth rinses

Mouth rinses	*C. albicans*			
	Percentage of reduction growth from A_2 to A_8			
	A_2 ± SD	A_8 ± SD	A_2-A_8 ± SD	% reduction ± SD
A	0.273 ± 0.034	0.350 ± 0.082	0.077 ± 0.054	94.42 ± 3.42
B	0.262 ± 0.005	0.274 ± 0.008	0.012 ± 0.011	99.11 ± 0.88
C	0.338 ± 0.039	0.984 ± 0.096	0.647 ± 0.081	51.39 ± 4.65
D	0.247 ± 0.047	0.948 ± 0.316	0.701 ± 0.272	47.70 ± 9.74
Normal	0.430 ± 0.640	1.765 ± 0.192	1.336 ± 0.156	0.00 ± 0.00
N	0	0	0	100
Mouth rinses	*C. glabrata*			
	Percentage of reduction growth from A_2 to A_8			
	A_2 ± SD	A_8 ± SD	A_2-A_8 ± SD	% reduction ± SD
A	0.156 ± 0.005	0.172 ± 0.006	0.016 ± 0.008	98.84 ± 0.58
B	0.167 ± 0.004	0.180 ± 0.004	0.013 ± 0.004	99.02 ± 0.27
C	0.214 ± 0.015	0.377 ± 0.171	0.163 ± 0.167	87.92 ± 2.08
D	0.181 ± 0.067	0.654 ± 0.043	0.472 ± 0.099	64.43 ± 6.34
Normal	0.448 ± 0.050	1.671 ± 0.243	1.223 ± 0.242	0.00 ± 0.00
N	0	0	0	100

A: Brand O; B: Brand M; C: Brand H, D: Brand B; N: nystatin.

Table 6. The percentage of reduced growth of *C. albicans* and *C. glabrata* during the logarithmic phase of A_2 to A_8 when tested with herbal-based mouth rinses

Mouth rinses	*C. albicans*			
	Percentage of reduction growth from A_2 to A_8			
	A_2 ± SD	A_8 ± SD	A_2-A_8 ± SD	% reduction ± SD
E	0.439 ± 0.076	1.616 ± 0.096	1.177 ± 0.098	11.09 ± 0.722
F	0.419 ± 0.068	1.630 ± 0.112	1.211 ± 0.076	8.48 ± 4.06
G	0.414 ± 0.048	1.664 ± 0.075	1.250 ± 0.056	5.54 ± 1.84
Normal	0.430 ± 0.064	1.765 ± 0.192	1.336 ± 0.156	0.00 ± 0.00
N	0	0	0	100
Mouth rinses	*C. glabrata*			
	Percentage of reduction growth from A_2 to A_8			
	A_2 ± SD	A_8 ± SD	A_2-A_8 ± SD	% reduction ± SD
E	0.411 ± 0.041	1.624 ± 0.082	1.213 ± 0.080	8.61 ± 6.89
F	0.403 ± 0.037	1.659 ± 0.106	1.253 ± 0.086	5.73 ± 5.04
G	0.431 ± 0.078	1.735 ± 0.164	1.304 ± 0.124	3.17 ± 2.71
Normal	0.448 ± 0.050	1.671 ± 0.243	1.223 ± 0.242	0.00 ± 0.00
N	0	0	0	100

E: Brand S; F: Brand C; G: Brand P; N: nystatin.

DISCUSSION

Candida species residing in the oral cavity are opportunistic pathogens and perform synergistic and mutual interactions with other components of the oral ecosystem for their survival (Vazquez-Munoz & Dongari-Bagtzoglou, 2021). The species flourish among humans with low immunity levels and when oral ecosystems are disrupted (Akpan & Morgan, 2002; Patil *et al*., 2015). The increased resistance by pathogenic microorganisms to currently used antibiotics and chemotherapeutics call for alternative prevention and treatment options and health care products. Products such as mouth rinses containing antifungal agents such as CHX and CPC have been more effective in this regard than systemic azole agents like fluconazole and voriconazole (Ramage *et al*., 2011).

The study sought to determine the antifungal effects of synthetic and herbal mouth rinses on *C. albicans* and *C. glabrata* via the designated tests mentioned above. The disk diffusion test results showed that mouth rinse B, which contains CPC as its main active ingredient, was the most effective in inhibiting the growth activities of both *Candida* species. CPC is a cationic surface-active agent with a wide range of antimicrobial properties having immediate antiseptic effects on gram-positive pathogens (Haps *et al*., 2008). The interaction between CPC and bacteria disrupts membrane function, causing cytoplasmic components leakage and eventually breaking down the intra-cellular equilibrium (Scheie, 1989; Pitten & Kramer, 2001). This mechanism's molecular, biochemical, or physiological events interfere with the cell's respiration process, making it more susceptible to CPC (Fathilah *et al*., 2012). A similar action mechanism may work on the *Candida* cells despite it not being a bacterium. CPC's effectiveness in inhibiting *Candida* growth relies on the concentration or amounts utilized in the formulation (Franco Neto *et al*., 2008). For instance, the 0.053% CPC used in this study resulted in a larger average diameter of the inhibition zones of both *Candida* species compared to other active compounds tested. However, a different study found that a higher concentration of CPC (0.07%) recorded smaller average inhibition zone diameters when tested on two oral bacteria, *Enterococcus faecalis* and *Staphylococcus aureus* (de Sousa *et al*., 2021). In a separate study by Evans *et al*. (2015), 0.05% CPC produced a larger average diameter of the inhibition zone for *S. mutans* and *S. sanguinis*.

Previous studies reported that a combined formulation of CHX and CPC (0.12% CHX+CPC) as used in mouth rinse C, provides clinical and microbiological benefits (Quirynen *et al*., 2001; Herrera *et al*., 2003). Quirynen *et al*. (2001) noted that the efficiency of a combined formulation of CHX+CPC was similar to a formulation of a single active compound CHX to prevent *de novo* plaque formation. Results obtained from disk diffusion show that the average diameter of the inhibition zones of both *Candida* species was slightly higher when tested using mouth rinse C, which contained CPC+CHX, compared with mouth rinse A, which had CHX. However, this finding differs from an earlier study by Abdulrahman *et al*. (2016) which noted a significantly smaller average diameter of the inhibition zone of *C. albicans* for the CHX+CPC-mouth rinse compared to the CHX-mouth rinse. In addition, mouth rinse A, which with its CHX ingredient, showed good antifungal activity against both *Candida* species, as the mean diameter of the inhibition zone was slightly less than for mouth rinses B and C.

The susceptibility test demonstrated that mouth rinse B containing CPC produced more significant antifungal effects than the other mouth rinses. The MIC value recorded by mouth rinse B (1.63 ± 0.5 µg/mL) was sufficient to inhibit the growth of *C. albicans* and *C. glabrata* to a minimal population. This finding agrees with several studies that reported the effectiveness of CPC-mouth rinses compared to CHX, CHX+CPC, and HEX-mouth rinses in inhibiting the growth of oral microorganisms. A study in Malaysia by Fathilah *et al*. (2012) reported that CPC-mouth rinses were more efficient against *C. krusei*, recording the lowest MIC value (33.0 ± 0.0 µg/mL) compared to the CHX (150.0 ± 0.0 µg/mL) and CHX+CPC (75.0 ± 0.0 µg/mL) mouth rinses. An earlier investigation in the US reported that CPC-mouth rinse's lowest concentration (MIC) exhibited a more significant antimicrobial activity against several periodontal organisms, including *Campylobacter rectus*, *Prevotella intermedia, Actinomyces meyeri,* and *Eikenella corrodens* (Sreenivasan *et al*., 2013). In Pakistan, Shafiq *et al*. (2018) reported that mouth rinses with CPC exhibited lower MIC values than those with CHX against *S. mutans* and *S. intermedius.*

This study's MFC values for the CPC, CHX, CHX+CPC, and HEX-mouth rinses were 2 to 4-folds higher than their MIC values. In addition, the concentration MFC of 6.51 ± 2.01 µg/mL for the CPC-mouth rinse was adequate to kill all cell populations, followed

by 25.00 ± 9.68 µg/mL and 31.25 ± 0.0 µg/mL for CHX and HEX-mouth rinses, respectively. Surprisingly, the CHX+CPC-mouth rinse produced the slightest antifungal activity effect against both *Candida* species, recording the highest MFC value (50.00 ± 9.36 µg/mL). However, this contradicts a previous finding by Fathilah *et al*. (2012) who reported that CPC incorporation potentially enhances the effects of CHX on antifungal activity.

This study shows that all three herbal mouth rinses (E, F, and G) exhibited lower antifungal activity against *C. albicans* and *C. glabrata* than chemical mouth rinses based on the disk diffusion and susceptibility tests. The absence of inhibition growth zones for both *Candida* species suggests their resistance to the active ingredients tested. Both species showed no MFC values when tested with herbal mouth rinses. Mouth rinse S contained *P. betle*, or betel vine, as an active ingredient. It is a propitious plant cultivated in Asian countries and used during auspicious functions, ceremonies, and sacred rituals (Rai *et al*., 2011). Although the *P. betle*-mouth rinses in this study exhibited no or lesser antifungal activity against oral *Candida*, the ethyl-acetate fractionated extract of *P. betle* possessed a potent inhibitory activity against *C. albicans* as reported by Phumat *et al*. (2017). Their study also noted that this extract produced the highest average diameter of the inhibition zone of *C. albicans* compared to *S. mutants* and *S. gordonii* (Phumat *et al*., 2017). A significant antifungal effect of *P. betle* against other *Candida* species including *C. glabrata*, *C. krusei*, *C. parapsilosis,* and *C. tropicalis* has also been confirmed in Nanayakkara *et al*. (2014).

Mouth rinse F contained *E. caryophyllus* or eugenol, an active ingredient naturally found in the clove bud or the scientifically named *S. aromaticum* and clove oil (Pavithra, 2014). Although no or less antifungal activity of *E. caryophyllus* was found in the first two experiments, large spectrums of clove oil and eugenol against *Candida* spp. and other oral bacteria were observed in past research. For example, Himratul-Aznita *et al*. (2009) reported that a drastic reduction in dental plaque populations was possibly due to the natural extracts of *E. caryophyllus* that killed bacteria such as *Candida* spp., *Streptococcus* spp., *Lactobacillus* spp. as well as *Staphylococcus* spp. Other than that, Pinto *et al*. (2009) concluded that eugenol from *S. aromaticum* is potentially used as a therapeutic medication against fungi infections. The study also suggested that clove oil might be helpful in the clinical management of candidiasis, especially mucocutaneous candidiasis, which is known for its fungicidal activity and its inhibition of germ tube formation of *Candida* cells (Pinto *et al*., 2009).

Without exception, mouth rinse G (Brand P) was found to exert no or less antifungal effects on the growth of *C. albicans* and *C. glabrata* in all laboratory tests. The mouth rinse G in this study consisted of an equal concentration of four active ingredients, namely *C. officinalis, P. major, F. vesca,* and *M. chamomilla*. *C. officinalis* is a medicinal plant with yellow to orange flowers found in the Mediterranean region. It contains several active compounds, including sesquiterpenes glycosides, saponins, xanthophylls, triol triterpenes, flavonoids, and volatiles (Gazim *et al*., 2008). Unlike *C. officinalis*, *P. major* is a perennial herb originating in Europe and Asia. It contains five classes of eleven biologically active compounds, namely benzoic, flavonoids, iridoid glycoside, phenolic, and triterpenes, with the majority of its medicinal properties attributed to iridoid glycoside (aucubin) and flavonoids (baicalein) (Shirley *et al*., 2017). Compared to them, *F. vesca* generally refers to berries and strawberries that are largely cultivated worldwide. It contains a rich source of biologically active phenolic compounds such as tannins, anthocyanins, flavonoids, and phenolic acids (Ivanov *et al*., 2015). Another active ingredient of mouth rinse G is *M. chamomilla*, commonly known as chamomile, an annual plant mainly found in Europe and Asia but widely introduced in North America and Australia (Singh *et al*., 2011).

Many studies and reviews on the health benefits provided by the respective active ingredients of mouth rinse G i.e., *C. officinalis* (Atai *et al*., 2007), *P. major* (Sharma *et al*., 2016), *F. vesca* (Vakil *et al*., 2019) and *M. chamomilla* (Cairone *et al*., 2021), underline the effectiveness of antifungal properties against *Candida* spp. Nevertheless, no reports on antifungal activity against *C. albicans* and *C. glabrata* have been attributed to mixtures comprising the four active ingredients. Besides, the lower antifungal activity found in herbal mouth rinses might be due to uncertainty on the effectiveness of active ingredients and their properties on two *Candida* species. In addition, it could be assumed that some of the components associated with the active ingredients may present no effects (medical or pharmaceutical) and are used only for marketing purposes (Radzki *et al*., 2022). Furthermore, there is also a possibility of differences between the declared and actual composition of a product, which may alter the characteristics and functions of the substances in the herbal mouth rinses tested in the study.

TKA showed that all four synthetic mouth rinses efficiently produced antifungal activity on *C. albicans* and *C. glabrata*. More than 50% of the *C. glabrata* cell populations were killed within 15 seconds of exposure to CHX, HEX, CPC, and CHX+CPC-mouth rinses. A full 100% killing of *C. glabrata* was recorded at 120-second exposure time for mouth rinses A and D. Meanwhile, all synthetic mouth rinses showed 50% killing effects on *C. albicans* at 60-second exposure time, except mouth rinse C containing CHX+CPC. In comparison with *C. glabrata*, a full 100% killing of *C. albicans* for mouth rinses A and D was recorded in the shorter 90-second exposure time, suggesting slightly higher efficacy of both the mouth rinses' antifungal agents in *C. albicans* than *C. glabrata*.

Furthermore, the killing efficiency of mouth rinses B and C on both *Candida* species was less than for A and D, as a longer exposure time (150 seconds) was needed to achieve a 100% of killing effect. Since TKA-associated with bacteria is regularly used to assess the bacterial activity of antibiotics containing a single or combined formulation (Pankey *et al*., 2014), some researchers believe that this assay is more relevant in a clinical setting (Eliopoulos & Eliopoulos, 1988; Pillai *et al*., 2005). Nevertheless, Pankey *et al*. (2014) described TKA as expensive, time-consuming, and requiring specific equipment and expertise. Therefore, it is impractical for most clinical microbiology laboratory settings. Due to the lack of standardized Clinical and Laboratory Standards Institute (CLSI) guidelines, only a few antifungal time-kill studies have been performed (Pankey *et al*., 2014).

A typical sigmoidal curve pattern obtained in a growth profile study indicates the generation cycle of an organism with all the cells performing optimal physiological and biochemical activities to grow (Cappuccino & Sherman, 2007). According to researchers, the normal biological functions of oral bacteria cells are affected by the active ingredients in the growth environment, preventing bacteria cells from propagating and increasing their population (Fathilah *et al*., 2007, 2009). In this study, all four synthetic-based mouth rinses exhibited significant effects on the growth profiles of *C. albicans* and *C. glabrata*. The growth suppression of mouth rinses B and A containing CPC and CHX, respectively was immediately observed, generating no growth curve. This finding is supported by an earlier study by Fathilah *et al*. (2012), which reported that no growth curves were generated for *C. krusei* and *C. tropicalis* when treated with CHX and CPC-mouth rinses. The log phases of mouth rinses B and A were more linear, with the total cell population attained at a lower optical density indicating the suppression of cell activity affecting the growth of *Candida* cells. In a past investigation, Hellstein *et al*. (1993) explained that the affected *Candida* could exhibit abnormal colonies and cell morphology under a suppression phase. The presence of uncommon colonies and cells, especially in pathogenic oral bacteria, will increase the frequency of phenotypic switching by cells that cause the survival of *Candida* cells in a stressed growth environment (Fathilah *et al*., 2012; Balagopal & Arjunkumar, 2013). On the other hand, the results of this study show that none of the herbal mouth rinses produced antifungal effects on the *Candida*

species. A similar pattern of growth curves was obtained as the those under average conditions.

In summary, mouth rinse B, which contained CPC, was the most effective in all tests, except TKA, compared to mouth rinses A (CHX), C (CHX+CPC), and D (HEX). In most situations, CHX remains the gold standard antiplaque agent, and this active compound can be found in most commercialized mouth rinses worldwide (Balagopal & Arjunkumar, 2013). The long-term use of CHX-mouth rinses can cause teeth and tongue discoloration, parageusia, irritation, and hypersensitivity reactions to the oral mucosa (James *et al*., 2017). Recent studies suggest that CPC is a better and more effective alternative for CHX as an antiplaque agent (Retamal-Valdes *et al*., 2017; Nasila *et al*., 2021). Although CPC is relatively less effective than CHX in controlling dental plaque development, it is more convenient for long-term use due to its less severe side effects. It is also responsible for reducing the bacterial count that causes periodontal disease and in preventing gingivitis and halitosis (Lee *et al*., 2017).

CONCLUSION

All synthetic mouth rinses tested in this study possessed antifungal properties against *C. albicans* than *C. glabrata*. Mouth rinse B, which contained CPC, was the most effective followed by mouth rinses A, C, and D which had CHX, CHX+CPC, and HEX, respectively. Meanwhile, lesser antifungal activity against both *Candida* species was observed when evaluated using all three herbal mouth rinses. Therefore, further research should investigate antifungal activity on the growth of *C. albicans* and *C. glabrata* using different concentrations of the active ingredients for mouth rinses E (*P. betle*), F (*E. caryophyllus*) and G (*C. officinalis, P. major, F. vesca, M. chamomilla*).

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Conflict of interest

All authors have no conflict of interest concerning the work reported in this paper.

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