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Ayurvedic herbal extracts suppress Candidal biofilms *in vitro*

G K Wijesinghe[a,*], T A Jayarathna[b], K A A Dilhari[c], C Gunasekara[b], N Fernando[b], N Kottegoda[d,e,f], L P Samaranayake[g] & M M Weerasekera[b]

[a]Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, [b]Department of Microbiology, Faculty of Medical Sciences, [c]Department of Basic Sciences, Faculty of Allied Health Sciences, [d]Department of Chemistry, Faculty of Applied Sciences, [e]Center for Advanced Material Research, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

[f]Sri Lanka Institute of Nanotechnology, Nanoscience and Technology Park, Pitipana, Homagama, Sri Lanka

[g]Faculty of Dentistry, University of Hong Kong, Hong Kong

E-mail: gkwijesinghe1989gmail.com

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Plant derivatives have been used for centuries to treat various health disorders of the human including microbial infections. A vast majority of these infections are initiated and perpetuated by biofilms. This study investigated the biofilm suppressant effect of phytomedicinal preparations used widely in traditional Ayurvedic medicine. Triphala, a mixture of *Terminalia bellirica*, *Terminalia chebula* and *Emblica officinalis* and *Mimusops elengi* bark extract. Inhibitory effect of extracts was first investigated against the planktonic *C. albicans* and *C. tropicalis* using well diffusion method. Minimum biofilm inhibitory concentration for *in-vitro* biofilms was determined by MTT assay. The biofilm suppressant effect was determined by measuring biofilm viability at different time intervals, post-exposure to the two herbal extracts, using MTT. Scanning electron microscopy was performed to assess the post-exposure biofilm architecture. Triphala inhibited both species of the planktonic yeasts and only the biofilm phase of *C. tropicalis.* Further, biofilm inhibition was seen in the co-cultured mixed species *C. albicans* and *C. tropicalis* biofilms with Triphala treatment. Triphala did not have any inhibitory effect on *C. albicans. M. elengi* had no inhibitory effect on biofilms of either *Candida* species. Scanning electron microscopy (SEM) revealed increased cell density of *C. albicans* in the biofilm. However, Triphala treatment had an inhibitory effect against *C. tropicalis* biofilms. SEM imaging revealed altered cell morphology of *C. tropicalis* cells in the biofilm after exposure to Triphala. In conclusion Triphala, but not *M. elengi,* extracts exhibit selective and differential biofilm inhibitory activity against *Candida* species. *C. albicans* biofilms are more resistant to the anti-biofilm activity of Triphala.

Keywords: Antimicrobial, Biofilms, *Candida* species, *Mimusops elengi*, Triphala

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Candida species are opportunistic commensals residing in the human oral cavity. *Candida albicans* and *Candida tropicalis* are by far the most common and the virulent yeast species isolated from this eco-niche[1,2]. *Candida* species, just as many other organisms, exhibit two different lifestyles, either existing in suspensions of biological fluids in the planktonic phase, or living in communities attached to surface in the biofilm phase[3]. The latter, biofilm phase yeasts, in comparison to their planktonic counterparts, are highly resistant to antimicrobial compounds and thought to be due to the barrier of extracellular matrix in which they are embedded in the biofilm[4].

Mouthwashes and oral rinses in various guises are commonly used to minimize the microbial burden of the oral cavity. Some of these are artificially manufactured chemicals such as chlorhexidine gluconate and others are `green` plant or herbal extracts[5]. Such herbal plant extracts mouthwashes have been suggested as useful for combating oral *Candida* infections. In this context, there is evidence to show that the ayurvedic preparation known as "Triphala", a mixture of three dried fruits of *Terminalia belerica, Terminalia chebula* and *Emblica officinalis*, has an activity against *Candida* species[6-8]. Similarly, Gami *et al.*[9] and Satish *et al.*[10] and several other research studies[11,12] have reported that *Mimusopselengi* plant extracts are also antifungal in nature and have been recommended for use against fungal infections due to their minimal side effects and

*Corresponding author

toxicity. However, there is sparsityonanti-candidal effects of Triphala in literature and the current study was undertaken to bridge that gap. We evaluated the biofilm suppressant effect of Triphala and *Mimusops elengi* bark extract against two common opportunistic *Candida* pathogens: *Candida albicans* and *Candida tropicalis*

Materials and Methods

Candida strains and culture conditions

C. albicans (ATCC10231) and *C. tropicalis* (ATCC13803) strains were obtained from the culture collection of the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. Stock cultures were maintained on Sabouraud Dextrose Agar (SDA, Sigma-Aldrich, USA) slants and were sub-cultured on to SDA plates and then incubated at 35±2^{o}C for 48 h prior to use in all the experiments.

Herbal antifungal agents preparation

Air dried fruits of *Terminalia chebula* (Aralu), *Phyllanthus emblica* (Nelli), *Terminalia bellirica* (bulu) and *Mimusops elengi* (Munamal) bark were authenticated by Bandaranayake Memorial Ayurvedic Research Institute, Sri Lanka. The plant components were washed with running tap water and air dried. Triphalaaqueous extract and dried bark of *Mimusops elengi* (60.0 g) were prepared using the protocol published by Wijesinghe *et al.*[13].

The voucher specimens of the plant materials were stored in the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. Prepared decoctions were refrigerated at 4^{o}C until used. Chlorhexidine gluconate (0.2 mg/mL) (CHL, Sigma Aldrich, USA) was used as the positive control. Concentrations (mg/mL) of prepared aqueous extracts were determined by freeze drying (1 mL) of extracts. Prepared extracts were refrigerated at 4^{o}C up to 02 weeks until used.

Anti Candidal activity of triphala and *Mimusops elengi*- Agar well diffusion assay

The standard agar well diffusion method was used to evaluate the effect of two herbal extracts (triphala and *M. elengi*) and CHL on planktonic *Candida* cells as described by Magaldi *et al.*,[14] with a few modifications[13]. This was done as a screening test before moving to the biofilm study and results were published previously[13]. Briefly, standard suspensions (0.5 McFarland turbidity equivalent) of *C. albicans* and *C. tropicalis* were prepared in sterile distilled water and inoculated on sterile Mueller-Hinton agar (MHA) plates separately using a sterile cotton swab. Four wells were prepared on agar surface and the bottoms of the prepared wells were sealed with sterile bacteriological agar. Working solutions (200 µL) of CHL (0.2 mg/mL), Triphala (65.0 mg/mL) and *M. elengi* (12.0 mg/mL) were added separately into the wells and sterile distilled water was used as negative control. Agar plates were incubated overnight at 37±2^{o}C and the inhibition zones were measured after 24 h.

Determination of Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$)

Standard cell suspensions (10^{7} cells/mL) of *C. albicans and C. Tropicalis* and 1:1 mixed suspension were prepared in RPMI 1640. Sterile 96 wells micro titer plates were seeded with 100 µL of prepared yeast suspensions and incubated at 37±2^{o}C for 48 h. Culture medium was replenished at 24 h. The 48 h old mature biofilms in the wells were washed twice with sterile Phosphate Buffer Saline (PBS). CHL (0.2 mg/mL), Triphala (65.0 mg/mL) and *M. elengi* (12.0 mg/mL) were serially diluted separately in RPMI 1640 medium in order to obtain a series of doubling dilutions of antibiofilm treatments. A volume of 100 µL of diluted extracts was added to corresponding wells of the micro titer plate containing *C. albicans*, *C. tropicalis* and mixed species biofilms. Negative control contained *Candida* biofilms and 100 µL of RPMI 1640. Plates were then incubated at 37±2^{o}C for 48 h[15].

After 48 h incubation, micro titer plate was washed twice with sterile PBS and the biofilm viability was determined by using MTT (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 1 mg/mL MTT working solution was prepared by diluting the stock solution (5 mg/mL). To quantify the cell viability, 50 µL of MTT working solution was added to each well and incubated at 37±2^{o}C for 4 h. After incubation, remaining MTT solution was aspirated. Dimethyl sulfoxide (Sigma-Aldrich, USA) 100 µL was added to each well and absorbance was measured at 570 nm and 630 nm, respectively using a micro titer plate reader (SPECTRAmaxPLUS384 Molecular Devices, Inc, USA)[16].

Determination of killing time of mature biofilms

Biofilms of *C. albicans, C. tropicalis* and 1:1 mixed species co-biofilm were prepared on a sterile 96 well flat bottom micro titer plate as explained previously.

Prepared biofilms were washed with 100 µL sterile PBS. After washing, the biofilms were immersed in working concentrations of antifungal agents (0.2 mg/mL CHL, Triphala aqueous extract (65.0 mg/mL) and *M. elengi* aqueous extract (12.0 mg/mL)) at room temperature for 30, 60, 90 and 120 seconds[5]. Biofilm viable cell mass was quantified using MTT assay at each time point as explained previously.

Scanning Electron Microscopy (SEM)

For ultra-structural examination of biofilms exposed to the herbal agents, *C. albicans* and *C. tropicalis* biofilms were grown on sterile cover slips, processed and examined using SEM as per the method of Pu *et al*.[17]. Briefly fully developed 48 h old mature biofilms were treated with working concentration (65.0 mg/mL) of Triphala for another 48 h, fixed with 2.5% glutaraldehyde for 4 h and dehydrated with ethyl alcohol. After overnight drying in a desiccator, biofilms were gold coated and examined using the SEM (Hitachi SU 6600)[18].

Statistical analysis

All experiments were performed in triplicates on different occasions. The statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS) version 16. Multiple means were compared using one way and two ways ANOVA. The level of significance was taken at 5% ($p<0.05$).

Results

Agar well diffusion

When the effect of CHL and ayurvedic preparations on planktonic *Candida* cells were taken into consideration, any zone of growth inhibition in the agar well diffusion was considered as sensitive to the relevant ayurvedic treatment for each organism tested. The results for antifungal properties of CHL (0.2 mg/mL), Triphala (65.0 mg/mL) and *M. elengi* (12.0 mg/mL) aqueous extracts against two planktonic *Candida* species were published by Wijesinghe *et al*.[13].

Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$)

Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$) is defined as the minimum concentration of antifungal agent required to reduce the MTT metabolic activity of the test biofilm by 50% compared to negative control (Control biofilm formed by same *Candida* species without antifungal agent).

Fig. 1a,b,c represent the mean MTT activity of 48 h *Candida* biofilms after the exposure to the antifungal agent for 24 h. $MBIC_{50}$ for *Candida* biofilms was

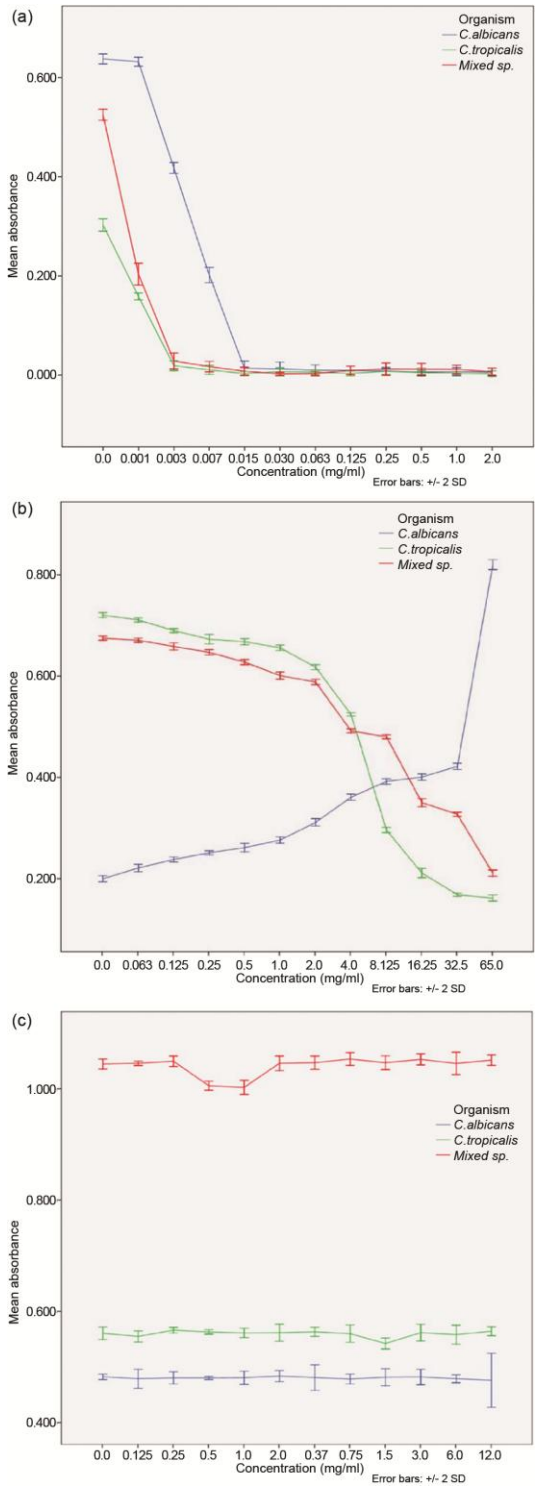

Fig. 1 — (a) Mean biofilm metabolic activity (evaluated by MTT assay) after 48 h exposure to the different concentrations of chlorhexidine gluconate. Data are mean±SD. (b) Biofilm MTT activity after exposure of 48 h old biofilm of the two *Candida* species to varying concentrations of Triphala. Data are mean ± SD & (c) Biofilm MTT activity after exposure of 48 h old biofilm of the two *Candida* species to varying concentrations of *Mimusosps elengi* extract. Data are mean±SD.

calculated using growth curves and is represented in Table 1.

Biofilm killing time assay

To determine the time required by antifungal agents for a 50% reduction in the viability of 48 h biofilms, the latter were exposed to 65.0 mg/mL Triphala, 12.0 mg/mL *M. elengi* and 0.2 mg/mL CHL for 30, 60, 90 and 120 seconds, respectively. CHL (0.2 mg/mL) was the most effective agents, producing >95% viability reduction in biofilm viability within 30s whereas Triphala and *M. elengi* did not cause any MTT metabolic activity reduction of 48h mature *Candida* biofilms (mono-species and mixed species) after 120 sec exposure (Table 2).

Scanning Electron Microscopy (SEM)

Based on the results obtained for biofilm susceptibility testing, SEM was performed on 48 h mature *C. albicans* and *C. tropicalis* biofilms treated with Triphala to determine the structural changes of *Candida* biofilms after the exposure to Triphala and *M. elengi*.

There was an increased cell mass with abundant hyphae formation in *C. albicans* biofilm when compared to control biofilm (Fig. 2a and 2b). Significant reduction of biofilm cell mass was observed with *C. tropicalis* mature biofilm after treating with Triphala for 48 h. Further, cellular shrinkage, deformities of cell walls and intracellular components leakages were clearly observed (Fig. 2c and 2d). These observations indicate chemical stress of Triphala treatment on sessile cells of *C. tropicalis*.

Table 1 — $MBIC_{50}$ of 48 h *Candida* biofilms*

Organism	Treatment	$MBIC_{50}$
C. albicans (ATCC 10231)	Chlorhexidine gluconate	0.008 mg/mL
	Triphala	No $MBIC_{50}$ point
	Mimusops elengi extract	No $MBIC_{50}$ point
C. tropicalis (ATCC13803)	Chlorhexidine gluconate	0.002 mg/mL
	Triphala	32.5 mg/mL
	Mimusops elengi extract	-
1:1 Mixed Species	Chlorhexidine gluconate	0.002 mg/mL
	Triphala	65.0 mg/mL
	Mimusops elengi extract	-

*Three individual tests were performed in triplicates

Discussion

Plant based natural products are popular alternatives to antibiotics due to less toxicity. Further, scientific community pay more attention on these natural products as a control measure to emergence of resistant microbial strains and low cost[19-21]. This study evaluated the anti-biofilm efficacy of two aqueous plant extracts, namely Triphala and *M. elengi* bark against common pathogenic *Candida* species using a series of *in-vitro* experiments.

Antifungal activity of Triphala against *C. albicans* and some dermatophytes have been previously reported using the well diffusion assay[7]. The effect of triphala on biofilm forming sessile cells was found to be different than that of planktonic cells of *C. albicans*. It is known that biofilm forming sessile cells are different phenotypically and functionally to their plaktonic counterparts[1]. Sessile *C. tropicalis* and mixed species biofilms were susceptible for Triphala, but not susceptible to *M. elengi* bark extract.

Interestingly a biofilm growth enhancement was observed with increasing concentrations of Triphala, Triphala decoction contains 47 types of tannins mainly and around 35 other phytoconstituents including chebulinic acid, gallic acid, chebulagic acid and ellagic acid[22-25]. These tannins are toxic for some fungi, bacteria or yeast while on the other hand some organisms can detoxify tannins by synthesis of tannin-complexing polymers, oxidation or biodegradation of tannins[26]. According to Aoki *et al.*[27], some *Candida* species produce an enzyme "Tannin acyl hydrolase/Tannase". Tannase acts on the hydrolysable tannins of the extract converting tannins into gallic acid, glucose and galloyl esters. Gallic acid is reported to exhibit poor cytotoxic effect against *C. albicans* biofilms[28]. Hence with increasing concentrations of Triphala growth enhancement of *C. albicans* biofilms could result in as observed in this study.

Table 2 — Reduction of biofilm viability with the exposure to 0.2 mg/mL CHL, 65.0 mg/mL Triphala and 12.0 mg/mL *Minusops elengi**

Organism	Chlorhexidine gluconate (0.2 mg/mL)				Triphala (65.0 mg/mL)				*Mimusops elengi* (12.0 mg/mL)			
	30s	60s	90s	120s	30s	60s	90s	120s	30s	60s	90s	120s
C. albicans	97%	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%
C. tropicalis	98%	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%
Mixed sp.	98%	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%

**Three individual tests were performed in triplicates

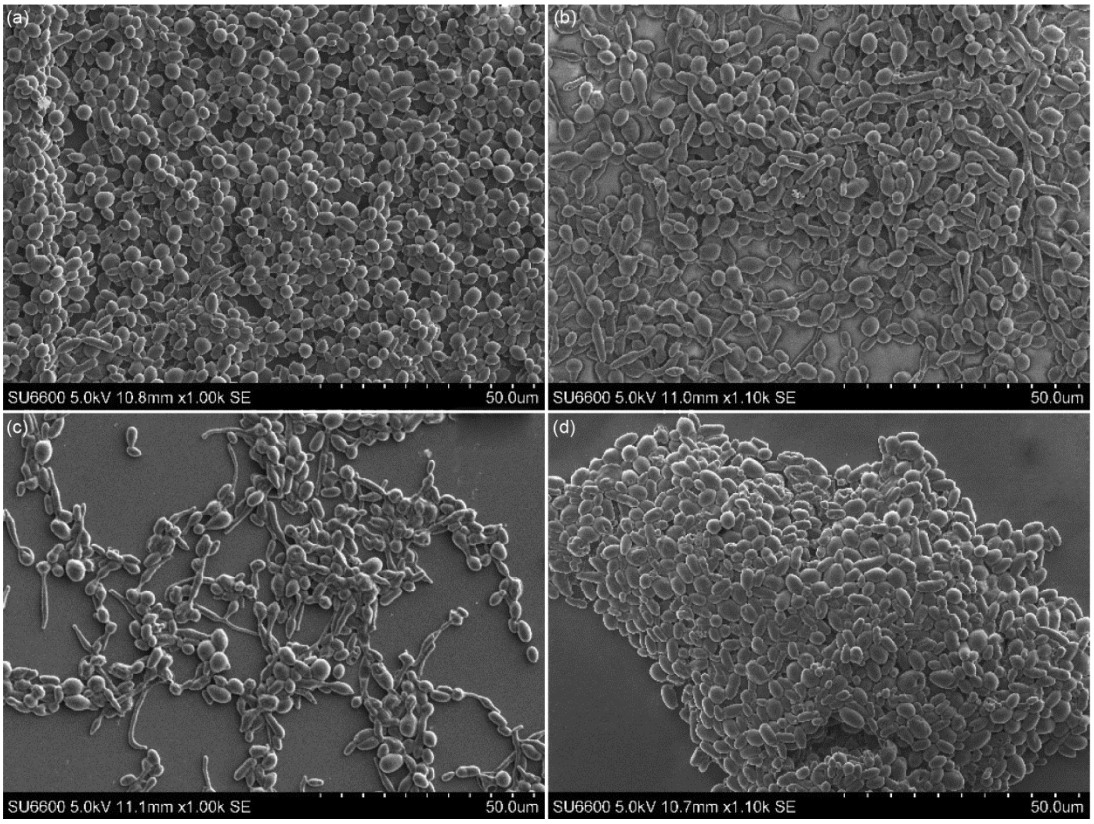

Fig. 2 — (a) Biofilm of *C. albicans* after 48 h treatment with triphala. Dense, abundant hyphal formation of *Candida* cells can be seen after Triphala treatment. (b) Biofilm of *C. albicans* negative control (without treatment). (c) Biofilm of *C. tropicalis* after 48 h treatment with Triphala. Significant reduction of *Candida* cell density were observed after 48 h exposure of Triphala. (D) Biofilm of *C. tropicalis* negative control.

As opposed to triphala, *M. elengi* had no effect on the two candidal species tested. The aqueous extract of the bark of *M. elengi* contains wax, starch and ash forming inorganic salts and quercitol as major phytochemicals. Its bark extract is also known to have activity against number of oral and non-oral microorganisms including *Streptococcus mutans*, *S. sanguis*, *S. Salivarius*, *Staphylococcus aureus* and the fungus *C. albicans*[9,29]. In contrast to previous reports, this study failed to demonstrate any anti-*Candida* effect of aqueous *M. elengi* bark extract against sessile cells of *Candida* spp.

Conclusion

The findings of the current study suggest that Triphala has potential antibiofilm activity against *Candida tropicalis*. However, further studies are needed to determine the antifungal effect of triphala against different species of *Candida* biofilms.

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Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

GKW & TAJ: Conceptualization; Formal analysis; Funding acquisition; Resources; Software; original draft; writing; review & editing. KAAD, MMW & LPS: Supervision; Conceptualization; Funding acquisition; Resources; Software; original draft; writing; review & editing. CG; NK, NF: original draft; writing; review &

editing. All authors read and approved the final manuscript.

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