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In-vitro antifungal efficacy of tissue conditioner-chitosan composites as potential treatment therapy for denture stomatitis

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ABSTRACT

Tissue conditioners are commonly used to improve the adaptation of ill-fitting dentures. These materials are easily colonized by *Candida albicans (C. albicans)*, resulting in "denture stomatitis". Chitosan and its derivative possess inherent antifungal activity. This study aims to formulate a chitosan-based tissue conditioner and assess its anti-fungal efficacy against *C. albicans* over time.

A chitosan oligosaccharide (COS) was synthesized from commercially purchased chitosan and was characterized by Fourier Transform Infrared spectroscopy (FTIR) and X-ray diffraction analysis (XRD). The minimum inhibitory concentration (MIC) of COS and commercial chitosan against *C. abicans* was evaluated using broth micro-dilution assay ($n = 3$). Next, it was incorporated into tissue conditioners and two experimental groups i.e. tissue conditioner modified by chitosan (TC-CH) and tissue conditioner modified by COS (TC-COS) were prepared. The in-vitro antifungal activity of experimental group was compared with a control group (chitosan-free tissue conditioner) by counting numbers of colony forming units (CFUs) ($n = 3$). The antifungal potential of experimental formulations over time in a simulated oral environment was also evaluated using a spectrophotometry ($n = 3$). A potent in-vitro antifungal activity against *C. albicans* was observed. The minimum inhibitory concentration (MIC) of pure commercial chitosan and COS was 0.625 mg/ml and 0.3125 mg/ml respectively. Compared to the control group, experimental groups showed a reduction in number of CFUs of *C. albicans* density. Once immersed in saliva, the TC-CH retained its inhibitory effect for 24 h, while TC-COS exhibited improved inhibition until the third day, beyond which a gradual reduction in the inhibitory effect was observed. Experimental formulations showed a reduction in *C. albicans* density compared to the control group. Thus, a novel combination of chitosan and tissue conditioners is a promising alternative for prevention and treatment of denture stomatitis.

1. Introduction

Since 1960, tissue conditioners have been used for the conditioning of traumatised oral tissues. It acts as a cushion under prosthesis and helps in redistribution of occlusal forces [1,2]. These materials are supplied in the form of powder and liquid and therefore on mixing suffer from porosities. These porosities act as potential sites for microbial growth, subsequently causing irritation of underlying mucosa, a condition known as 'denture stomatitis'. Although, several microbes accumulate and colonizes tissue conditioners, colonization of *C. albicans* plays a fundamental role [3].

It has been reported that the incorporation of antimicrobial agents into tissue conditioner will prevent contamination of these materials. In addition, it will allow treatment of denture stomatitis by allowing precise and prolonged administration of the antimicrobial agent to the affected site [4,5]. To date various antifungal drugs, herbal agents and inorganic compounds have been incorporated into tissue conditioners. It has been reported that antifungal drugs have better efficacy but, their use has been associated with re-infection and re-colonization of *C. albicans*. Moreover, emergence of resistant strain and concern of drug interaction have been reported [6]. In contrast, inorganic compounds such as silver particles, potentially have long term side effects and natural herbal agents shows time-dependent effect [7,8]. Also, scarce data is available on the impact of the addition of these agents on the properties of tissue conditioner. Therefore, a continuous search for a compound with intrinsic antimicrobial properties with a low risk of adverse effect on mammalian cells and properties of tissue conditioners should be continued [9].

Chitosan, a de-acetylated derivative of chitin, is a biocompatible and biodegradable polymer. It is approved by Food and Drug Administration

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(FDA) for biomedical and pharmaceutical purposes and possesses natural antimicrobial activity [10]. It has several advantages over other natural antimicrobial agents including broader antimicrobial spectrum, higher killing rate and lower toxicity towards mammalian cells [11,12]. However, its limited solubility in a neutral and alkaline environment limits its use. Therefore, the present study aims to synthesize water-soluble chitosan oligosaccharide and to evaluate antifungal potential of chitosan-modified tissue conditioners against *C. albicans*, with and without immersion in artificial saliva for treatment and prevention of denture stomatitis.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (448869, 50,000-190,000) and acetic acid were purchased from Sigma Aldrich, USA while ethanol and acetone were purchased from BDH, AnalaR, England. All chemicals and reagents were of analytical grade and were used without further purification. For antifungal analysis, *C. albicans* (ATCC, 90029) was obtained from the Molecular Medicine Laboratory, Department of Microbiology, Quaid-i-Azam University.

2.2. Synthesis of chitosan oligosaccharide

Water soluble COS was synthesized via hydrolysis under reflux condition according to the procedure described in the literature [13]. The percentage recovery and percentage solubility was calculated by using Eq. (1) and Eq. (2) respectively [14].

$$\text{Percentage Recovery} = \frac{\text{weight of water soluble chitosan}}{\text{weight of crude chitosan}} \times 100 \quad (1)$$

$$\text{Percentage Solubility} = \frac{(W1-W2)}{W1} \times 100 \quad (2)$$

where *W1* is the initial weight of chitosan oligosaccharide (g) and *W2* is the weight of un-dissolved section (g).

2.3. Characterization of chitosan oligosaccharide

2.3.1. Infrared spectroscopy

FTIR spectra of synthesized COS and commercial chitosan were recorded in the range of 400–4000 cm^{-1} on FTIR spectrometer (Bruker, Tensor-II, Germany).

2.3.2. Powder X-ray diffraction analysis

The diffraction pattern of commercial chitosan and COS was recorded on Bruker D8 Advance (Germany) X-ray diffractometer between a diffraction angle (2θ) of 10° to 70° at 4 kV, ambient temperature and at the scanning rate of 1°/min.

2.4. Antifungal susceptibility testing

It includes three sets of experiments. Initially, the minimum inhibitory concentration of commercial chitosan and COS against *C. albicans* was evaluated. Then, experimental tissue conditioner formulation containing chitosan or COS were prepared and their effect on *C. albicans* attachment was compared. Lastly, the antifungal efficacy of experimental formulations in a simulated oral environment over time was evaluated.

2.4.1. Determination of MIC

MIC was determined by broth micro-dilution assay adapted from the National Committee for Clinical Laboratory Standards (NCCLS) [15,16]. Briefly, stock solutions of chitosan and COS (2.5% wt./v) were prepared and the pH of solutions were adjusted to 5.6–5.8 [12]. This was done to ensure that the acidic state would not interfere with the determination of antifungal activity of chitosan. Next, 50 μl of stock solution was added to a sterile 96-well micro-titration plate and two-fold serial dilutions were made. Then, 50 μl of prepared inoculum of 1×10^5 cells/ml of *C. albicans* was added to each well to obtain a final concentration of chitosan and COS ranging from 25 to 0.048 mg/ml. The last two wells were used as positive and negative control. For positive control, 50 μl *C albicans* with 50 μl of sabouraud broth were used, while 50 μl of sabouraud broth and 50 μl of stock solution were used as a negative control [17]. Micro-titration plate was then sealed with aluminium foil and incubated in an aerobic condition at 37 ± 2 °C for 24 h. The entire experiment was carried out in triplicate. The MIC was recorded by visual inspection for turbidity in comparison to the positive control (drug free).

2.4.2. Antifungal assay of chitosan modified tissue conditioner on C. albicans

2.4.2.1. Preparation of tissue conditioner discs. In this study, three types of tissue conditioner discs were prepared. GC tissue conditioner without chitosan was used as a control. For experimental specimens, part of GC tissue conditioner powder was replaced either by chitosan or COS at concentrations, equivalent to 2 and 4 times MIC and the powder was ball milled for 4 h (Table 1). Next, the powder was mixed with monomer according to manufacturer's recommendations and material was poured in custom made prefabricated Teflon molds (40 mm × 10 mm × 1 mm) sandwiched between the two glass slabs lined with acetate paper.

After gelation, the material was removed from the mould and 5 mm discs were cut out with a sterile cork borer ($n = 3$). The entire procedure was done in a laminar air flow chamber (aseptic condition) and all prepared discs were sterlized by UV for 15 min [4].

2.4.2.2. Susceptibility testing. C. albicans was cultured onto Sabouraud dextrose agar (SDA). Twenty-four hours before placing tissue conditioner disc, 1 ml of sterile nutrient broth was inoculated with standard colony of *C. albicans* [18]. After 24 h, the inoculums size was adjusted equivalent to 0.5 McFarland Standard and tissue conditioner discs (control and experimental) were immersed in triplicate in eppendorf containing 900 μl Sabouraud broths. All discs were seeded with 100 μl of prepared *C. albicans* cell suspension and incubated at 37 ± 2 °C for 24 h. After incubation, the broth was removed with a sterile pipette and discs were rinsed 5 times with sterile water to remove the loosely adherent *C. albicans*.

Then discs were placed in sterile eppendorf containing 1 ml of sterile saline and vortex for 30 s to remove surface organism. Ten-fold serial dilutions were made and100 μl of elute from each eppendorf were placed

Table 1
Materials used for preparation of experimental and control disc.

Sr.No.	Material	Group name	Purpose
1.	GC Tissue Conditioner powder and liquid	Control	Control
2.	GC-Tissue Conditioner powder + 2 times MIC of commercial chitosan	TC-2CH	Experimental
3.	GC-Tissue Conditioner powder + 4 times MIC of commercial chitosan	TC-4CH	Experimental
4.	GC-Tissue Conditioner powder + 2 times MIC of COS	TC-2COS	Experimental
5.	GC-Tissue Conditioner + 4 times MIC of COS	TC-4COS	Experimental

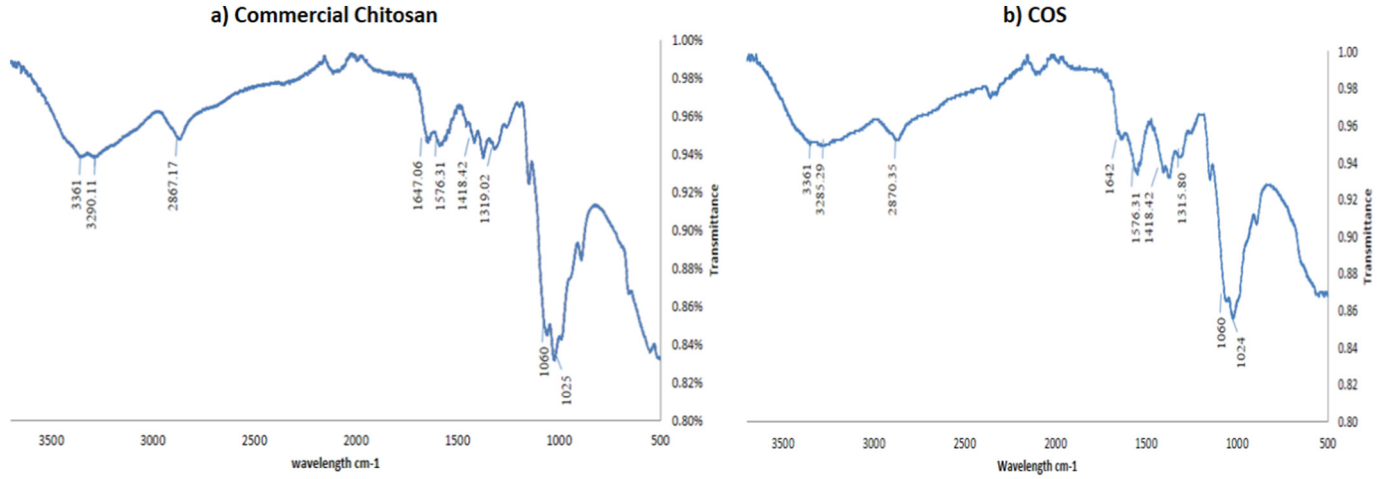

Fig. 1. FTIR spectra of a) commercial Chitosan and b) COS.

in duplicate on the SDA plates and incubated at 37 °C for 24 h [19]. After incubation, the numbers of colony forming units (CFU) were counted.

2.4.3. Antifungal efficacy of chitosan modified tissue conditioner on C. albicans over time

Experimental and control discs ($n = 3$) were immersed seperately in a glass bottles containing 2 ml of sterile artificial saliva and stored in incubator at 37 °C for 1, 3, 5 and 7 days. After immersion period, discs were removed from the artificial saliva and washed with sterile water to remove any traces of storage liquid [20]. Each disc was immersed in triplicate in eppendorf containing 900 μl Sabouraud broths and 100 μl of *C. albicans* cell suspension. These were incubated at 37 ± 2 °C for 24 h.

After incubation period, the broth was removed with a sterile pipette. The discs were rinsed 5 times with sterile distlled water and 1 ml of sterile saline was added to eppendorf. This was vortex for 30 s. Next, 100 μl of this solution was transferred to test tubes containing 9.9 ml of sabouraud dextrose broth. Test tubes were incubated at 37 ± 2 °C for 24 h. To evalute candidal growth, optical density (OD) of broth in test tubes was recorded spectophotometerically at 530 nm. Broth without inoculum, was used as refrence for monitoring OD.

3. Results and discussion

3.1. Synthesis of chitosan oligosaccharide

COS can be prepared via acid or enzymatic hydrolysis, oxidative treatment or a physical treatment of chitosan [21,22]. In current study, acid hydrolysis of commercially purchased low molecular weight chitosan was carried out. It has been reported that acid hydrolysis is less expensive compared to enzymatic hydrolysis [21,23]. The synthesized COS was brown in colour and its percentage yield and solubility were 84.4% and 27.8% respectively.

FTIR spectra of commercial chitosan and COS are shown in Fig. 1. It was observed that the commercially purchased chitosan had all the bands reported in other studies [24,25]. Two characteristic peaks around 3500 to 3290 cm^{-1} can be attributed to the O—H and N—H stretching frequencies respectively [24–26]. The presence of residual *N*-acetyl group were represented by peaks around 1647 cm^{-1} (amide I) and 1319 cm^{-1} (amide III) [24,27]. The band at around 1576 cm^{-1} was attributed to N—H bending of the primary amine whereas the absorption band at around 1418 cm^{-1} and 1374 cm^{-1} represented CH2 bending and CH3 deformation respectively. The IR spectrum of COS was similar to that of commercial chitosan, confirming no structural changes except for the reduction in molecular weight.

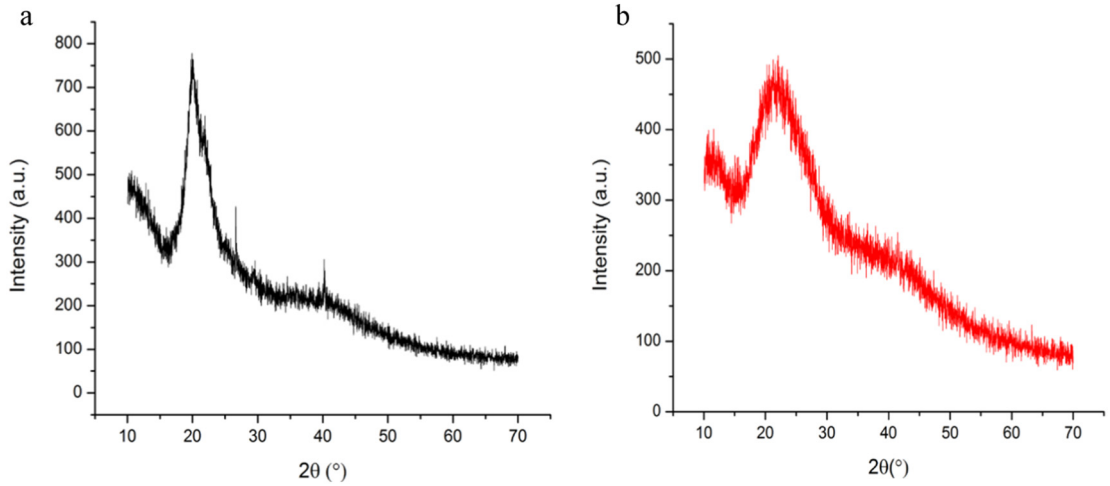

Fig. 2. X-ray diffraction pattern of a) commercial chitosan and b) COS.

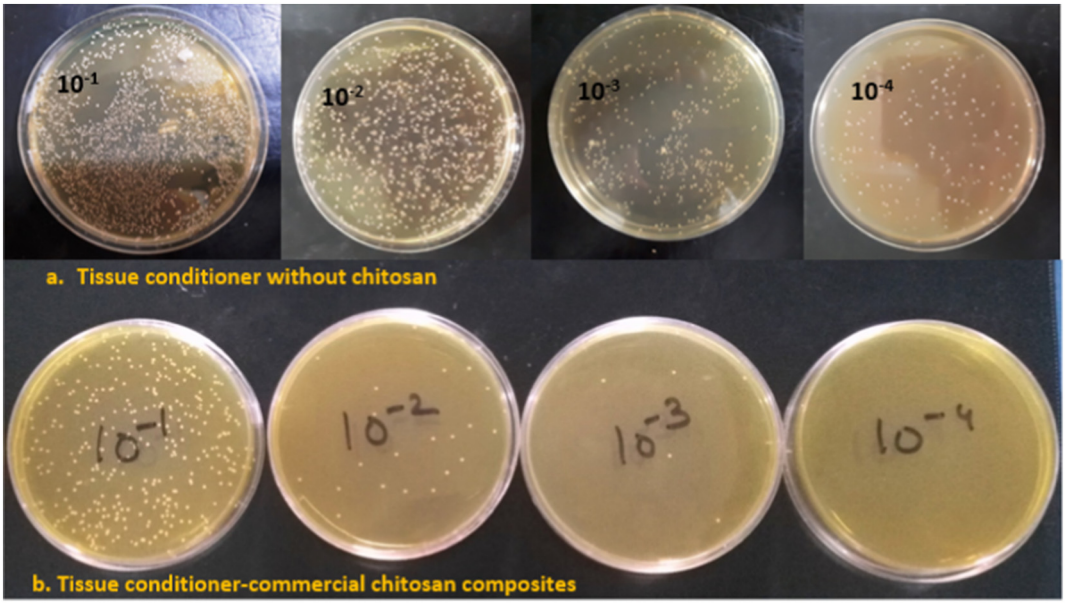

Fig. 3. CFUs of *C. albicans* at different dilutions (a) tissue conditioner without chitosan (b) tissue conditioner- commercial chitosan composite disc.

Fig. 2 shows the X-ray diffraction pattern of commercial chitosan and COS. XRD pattern of commercial chitosan showed strongest reflection at 20.1°. This was consistent with crystalline form II, as reported by Jiang et al. (2010) and Tian et al. (2003) [25,27]. On contrary, COS had a broad and less intense peak at 20.1°. A similar pattern has previously been reported for the chitosan derivatives, indicating a decrease in crystallinity and increase in the amorphous structure of chitosan [25,27–29]. It has been reported that the amorphous polymers have better solubility [30]. The findings of current study were consistent with the reported statement.

3.2. Antifungal analysis

3.2.1. Minimum inhibitory concentration (MIC)

The MIC of commercial chitosan and COS was 0.625 mg/ml and 0.3125 mg/ml respectively, thus, indicating better antifungal activity of COS compared to commercial chitosan. This further supports our results that water soluble derivatives of chitosan exhibit better anticandidal activity [9,10]. The findings can be correlated with the ability of low molecular weight chitosan to interfere with the metabolic activity of yeast cell and disrupt the cell wall [9,10].

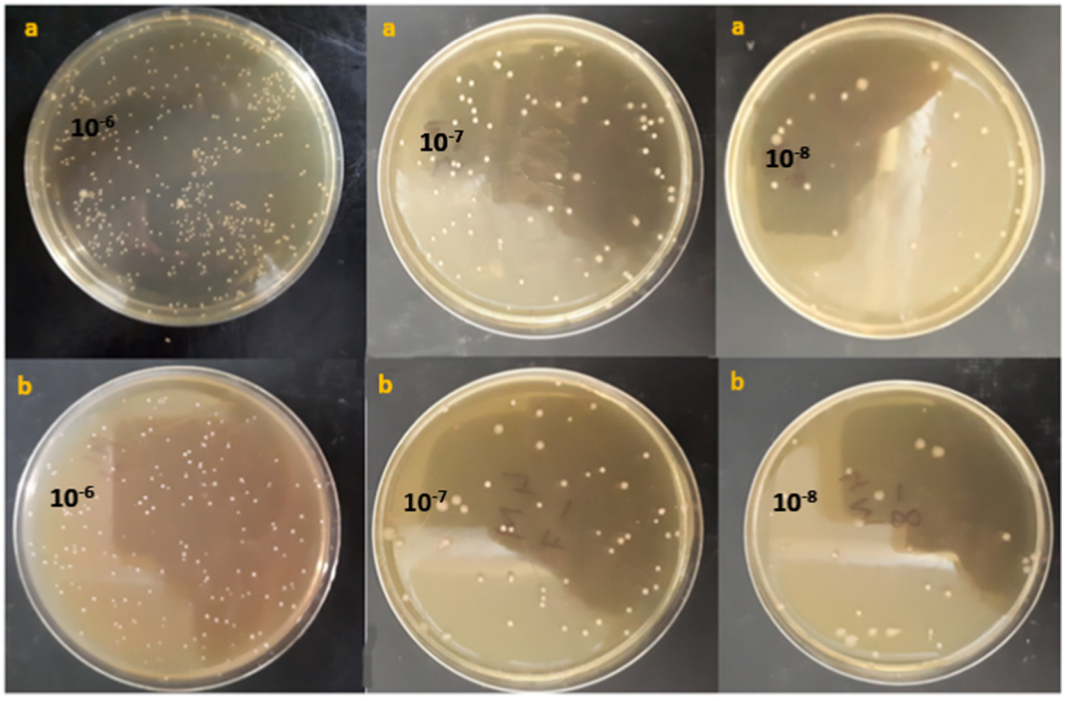

Fig. 4. CFUs of *C. albicans* at different dilutions (a) tissue conditioner without chitosan (b) tissue conditioner- COS composite disc.

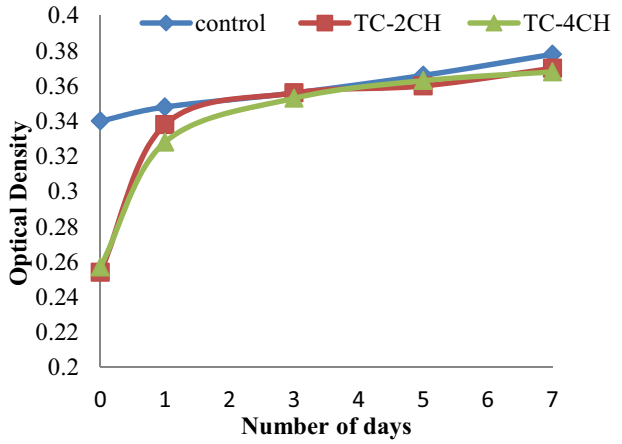

Fig. 5. The optical density (at 530 nm) growth curves of *C. albicans* attached to tissue conditioner modified by commercial chitosan at different time intervals.

3.2.2. Antifungal assay of chitosan modified tissue conditioner on C. albicans

Antifungal assay of tissue conditioner modified by chitosan were based on Clinical and Laboratory Standards Institute (CLSI) method [31]. Based on the results of the pilot study, a concentration greater than MIC was incorporated into the tissue conditioner. Similar findings were reported by Amornvit et al. It was thought that amount greater than MIC is needed to achieve a sufficient level of active compound, as once the drug is incorporated into the tissue conditioner an indirect contact between the microorganism and the antifungal agent occur [32].

In present study, tissue conditioners supplemented by chitosan or COS exhibited antifungal activity while no inhibition of *C. albicans* was observed in the control group as shown in Figs. 3 and 4. Results were in accordance with previous in-vitro studies which showed that a tissue conditioner alone lacks antifungal activity and incorporation of antifungal agents into tissue conditioners would inhibit fungal colonization [32,33].

Several studies on the use of chitosan for treatment of denture stomatitis have been published. P. Aksungur et al. evaluated in-vitro and in-vivo effect of chitosan and chitosan-nystatin formulations for the treatment of chemotherapy induced oral mucositis [34]. They observed that the survival of animal receiving treatment was higher compared to untreated group [34]. Another study evaluating the efficacy of chitosan for the treatment of resistant *Candida* species demonstrated that low molecular weight chitosan could provide a therapeutic alternative for resistant *Candida* species [35].

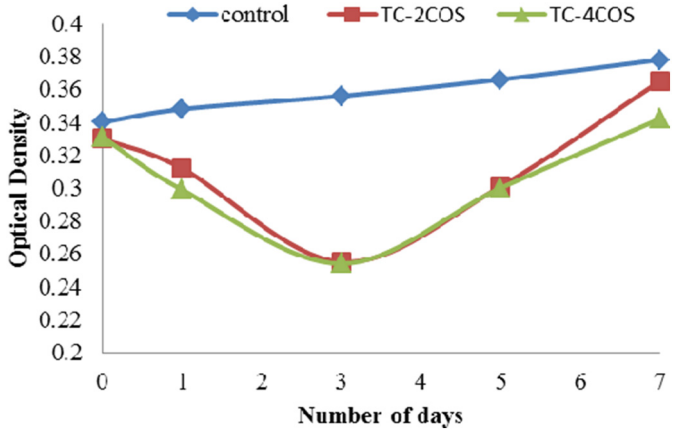

Fig. 6. The optical density (at 530 nm) growth curves of *C. albicans* attached to tissue conditioner modified by COS disc at different time intervals.

Lee et al. also demonstrated that tissue conditioner modified by chitosan or its derivative such as quaternized chitosan had significantly fewer fungal colonies than the tissue conditioner alone. In addition, chitosan and its derivatives have no effect on the viability of human gingival epithelial cells [36]. Reduction in *C. albicans* colonies in the experimental group can be attributed to the cationic nature of chitosan and its ability to disrupt the metabolic activity of yeast cells. In addition, inhibition of adhesion of *C. albicans*, can be responsible for the observed results [9,34,37,38].

3.2.3. Antifungal efficacy of chitosan modified tissue conditioner on C. albicans over time

Viable cell counting is considered a benchmark for microbial quantification. However, its biggest drawback is the laborious nature of the technique [39,40]. Several techniques have been developed to replace viable cell counts, including bioluminescence, electrical impedance, infrared spectroscopy, electrical counting and spectrophotometry [39]. The present study uses the spectrophotometric method to analyse the growth of *C. albicans* over time, as no statistically significant difference between the results obtained with the spectrophotometric method and the reference method has been reported in the literature [39].

OD was analysed statistically using a two-way analysis of variance (ANOVA). A significant difference in the optical density of the control group and the tissue conditioner modified by commercial chitosan was observed in the non-immersed samples ($p \leq 0.001$) and on day 1 of the immersed samples ($p = 0.021$). However, on continuous immersion in artificial saliva, the efficacy of *C. albicans* inhibition was reduced (Fig. 5). A pairwise comparison using Tukey post-hoc test revealed that there was no statistically significant difference between tissue conditioner containing 2 times (TC-2CH) or 4 times (TC-4CH) MIC of commercial chitosan.

On contrary, tissue conditioner modified by COS showed an improvement in the candida inhibition up to third day, beyond which a gradual reduction of inhibitory effect was observed. A significant difference ($p \leq 0.05$) in optical density between control group and experimental was observed at an interval of day 1, 3 and 5 (See Fig. 6). The trend was similar to the reported literature as the release of a drug was relatively high initially due to the difference in diffusion gradient between composite disc and the immersion medium [4,5].

4. Conclusion

The method was studied to prepare and explore the antifungal activity of chitosan oligosaccharide (COS) on the growth and attachment of *C. albicans* when incorporated into a tissue conditioner for the prevention and treatment of denture stomatitis. COS was prepared by acid hydrolysis under reflux conditions and its chemical structure was characterized by FTIR. The XRD study indicated a decrease in crystallinity. The minimum inhibitory concentration (MIC) of chitosan and COS was 0.625 mg/ml and 0.3125 mg/ml respectively. In addition, the number of *C. albicans* attached to a tissue conditioner showed a significant reduction after incorporation of chitosan and COS. The antifungal effect lasted for 24 h and 5 days in a simulated oral environment for chitosan and COS, respectively. Therefore, it can be concluded that chitosan and COS provide safe and alternative therapy to conventional synthetic antifungals for the treatment and prevention of denture stomatitis. These findings support a continued investigation of the physical properties of the chitosan modified tissue conditioner. In addition, such studies need to be continued in the future with other chitosan derivatives to allow a comprehensive comparison of *C. albicans* inhibition.

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