The impact of nicotine and cigarette smoke condensate on metabolic activity and

biofilm formation of Candida albicans on acrylic denture material

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Running title: Inhibition of *C. albicans* biofilm formation and adhesion

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Abstract

Purpose: Smokers have increased denture stomatitis caused primarily by *Candida albicans*. The primary aim of this study was to demonstrate the impact of a wide range of nicotine and cigarette smoke condensate (CSC) concentrations on biofilm formation and metabolic activity of *C. albicans* on acrylic denture material.

Materials and Methods: *C. albicans* (ATCC strain 10231) was used. Standardized denture acrylic (PMMA) samples (total of 135 samples) were incubated with *C. albicans* and exposed to nicotine and CSC at different concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml) and (0, 0.25, 0.5, 1, 2 and 4 mg/ml), respectively. For each experiment, 3 samples per nicotine and CSC concentration and a total of 45 specimens (27 specimens for the nicotine- and 18 specimens for the CSC-treated samples) were used and they were selected randomly for each group. The control group consisted of 0 mg/ml of nicotine or CSC. The viability of *C. albicans* was measured using spiral plating on blood agar plates. The effect of nicotine and CSC concentrations on planktonic cells was were measured using a microplate reader. Metabolic activity of 24 h old established *C. albicans* biofilm exposed to nicotine and CSC for 24 h in microtiter plates was determined using a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide (XTT) reduction assay.

Results: The viability of *C. albicans* increased concomitant with increasing concentrations of CSC and nicotine, particularly at 0.5 and 2 mg/ml, respectively. Concentrations of CSC and nicotine above this resulted in an inhibitory effect on *C. albicans* viability. CSC and nicotine at 4 and 16 mg/ml, respectively, increased *C. albicans* biofilm metabolic activity.

Conclusion: Nicotine and CSC up to certain concentrations caused increases in biofilm formation, metabolic activity, viability and planktonic cell absorbance of *C. albicans*. This in-vitro study demonstrates the effectiveness of tobacco on promoting the growth of *C. albicans* and suggests their potential contributing factor in *C. albicans* biofilm related infections in smokers.

Keywords: Candidiasis; denture stomatitis; biofilm; XTT.

1. Introduction:

Fungi are eukaryotic organisms and can exist in two forms: yeast, or filamentous fungi.[1] In addition, they can be a combination of these two, which are called dimorphic fungi such as *Candida albicans.* The oral environment contains many microorganisms including bacteria and fungi; *C. albicans* is found in 30-50% of the population.[1] In general, as a person ages, the rate of *C. albicans* carriage increases. *C. albicans* is an opportunistic microorganism, which means that it is harmless in a normal oral environment, but if an imbalance in the oral flora occurs for whatever reason, *C. albicans* may colonize and cause infection.[1] In order for *C. albicans* to initiate infection, it needs to adhere to host surfaces facilitated by fungal surface components such as the C3d receptor, or saccharine and mannoprotein in the *C. albicans* cell wall.[1] Some additional reported *C. albicans* adhesins include Hwp1, Als5p, and Als1p. These are members of a group of proteins called glycosylphatidylinositol-dependent cell wall proteins (GPI-CWP).[1] These proteins have two termini; the N- terminal signal peptide peptide and the C- terminal that facilitates the attachment to cell wall glucan. The agglutinin-like sequence (ALS) family of *C. albicans* includes eight genes that encode several large cell-surface glycoproteins.[2] The large family of ALS genes in *C. albicans* was found to consist of putative adhesins that adhere to endothelial, epithelial and extracellular matrix proteins of the host surface.[3-5] *C. albicans* uses these adhesins to adhere to saliva-coated surfaces, such as prostheses (i.e., dentures). In addition, it was concluded by Hazen and co-workers that surface proteins on *C. albicans* attached to hydrophobic surfaces like dental materials.[6]

Fungal infections are common in patients with various systemic conditions.[7,8] Patients who are at high risk include those with cancer who are undergoing immunosuppressive chemotherapy, especially if this treatment is causing neutropenia. In these patients, mucosal colonization with *C. albicans* has been found to be associated with increased incidence of systemic candidiasis.[9] Many studies indicate that the prevalence of *C. albicans* varies widely, and a variety of factors seem to affect the prevalence. One factor is denture wearing; 56% of elderly denture wearers are prone to *C. albicans* infection[1], which is the leading microorganism in denture stomatitis. Denture stomatitis is a pathological condition of the soft tissue of the maxilla, typically covered by a denture, and is caused by trauma from ill-fitting dentures, xerostomia, psychological factor, allergy, denture cleanliness resulting in *C.*

albicans colonization of denture acrylic material.[10,11] The most widely used classification of denture stomatitis is the Newton classification that is based on the severity of the condition. Based on the Newton classification there are three types of denture stomatitis: (1) pinpoint hyperaemic foci; (2) diffuse hyperaemia of the denture supporting tissue; and (3) papillary hyperplasia.[12]

Another major factor that is related to the prevalence of *C. albicans* is smoking, however the exact effect of smoking on the oral microflora including *C. albicans* is not yet clear. Many studies found no difference in the prevalence of *C. albicans* between smokers and non-smokers and concluded that smoking did not increase or decrease the oral mycotic flora.[13,14,17,18] Beasley (1969) reported that smoking was not the causative factor of oral candidiasis.[15] However, Arendorf and Walker in 1980 found that smoking significantly increased the carriage rate of *C. albicans* by 70%, while the rate in non-smokers was increased by 30%.[16] Moreover, Tapper-Jones et al.[19] examined diabetics and healthy subjects of the same age, sex, dental status and smoking habits, and observed that smoking increased the candidal carrier rate in both diabetic and healthy patients. In a recent in vitro study by Semlali et al.[20] on the effect of cigarette smoke condensate (CSC) on *C. albicans*, generated by complete burning of commercial cigarettes in an in house smoking machine where 1 cigarette provides up to 1 mg of nicotine and 4 mg of CSC, it was concluded that *C. albicans* adhesion, growth and biofilm formation increases with exposure to CSC. The study, however, had limitations in that they used glass slides to measure *C. albicans* adhesion and did not use acrylic material specimens. Furthermore, Baboni et al. [21] focused only on the attachment of *C. albicans* to the acrylic surface and did not measure biofilm formation. Both are important differences as the yeast may attach in a different manner depending on the surface used and biofilm formation is much more complicated than simple attachment.

The combination of the two factors, smoking and denture wearing, was found to increase the candidal carriage in the oral cavity, which is the leading microorganism in denture stomatitis.[22] The mechanism by which smoking may affect *C. albicans* has not yet been established. However, several researchers have provided hypotheses. One of the hypotheses is that smoking may cause localized epithelial alterations, which may expedite candidal

colonization. A second hypothesis is that smoking may provide some type of nutrition for *C. albicans*.[22]

The hypothesis of this study was that CSC and nicotine will increase *C. albicans* metabolic activity and biofilm formation of this opportunistic microorganism on denture acrylic material. According to previous studies from this laboratory using *Streptococcus mutans,* increased biofilm formation and metabolic activity at 1 mg/ml of nicotine and 0.5 mg/ml of CSC was observed.[23,24] The primary aim of this study was to demonstrate the impact of a wide range of nicotine and CSC concentrations on biofilm formation and metabolic activity of *C. albicans* on acrylic denture material.

2. Materials and Methods:

2.1 Yeast and media:

C. albicans was obtained from the American Type Culture Collection (ATCC 10231). *C. albicans* cells were inoculated into 5 ml of yeast-peptone-dextrose (YPD) broth medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose; Fisher Scientific, Newark, DE, USA). Nicotine was purchased from Sigma Chemical Co., St. Louis, MO. CSC was purchased from Murty Pharmaceuticals, Lexington, KY and was made by burning five cigarettes in an in house smoking device and concentrated in 100 ml of water at the end of this device. Approximately 2.4% of the CSC concentration is nicotine and the remainder of the CSC consists of the other 4000 components of cigarette smoke.

2.2 PMMA specimen preparation:

Acylic specimens were made using a thermoplastic sheet of 2 mm thickness. This sheet was flasked with dental stone (Microstone, Whip Mix), packed with Polymethacrylate Lucitone 199 and cured by heating water for 8 h following the manufactor's instructions. Once cured, it was removed from the flask and placed in an ultrasonic to remove stone remnants. Specimens were cut to a size of 8 mm x 8 mm with an automatic saw machine. The total number of specimens fabricated was 135. For each experiment, 3 specimens per concentration and a total of 45 specimens (27 specimens for nicotine- and 18 specimens for the CSC-treated samples) were used and they were selected randomly for each group .

2.3 Biofilm formation on the acrylic samples:

The acrylic samples were sterilized in 70% ethanol for 5 min and rinsed with sterile water and then placed in sterile 6 well tissue culture plates. Three specimens were placed in each well with 50 μl from an overnight yeast culture. The wells were exposed to nicotine and CSC in 3 ml of YPD at different concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml) and (0, 0.25, 0.5, 1, 2 and 4 mg/ml), respectively. The 0 mg/ml nicotine or CSC group served as control groups. The plates were incubated for 48 h at 37°C in 5% CO_2. The acrylic samples were removed from the wells and placed in 1 ml of sterile saline in a 15 ml centrifuge tube. The attached biofilm were dislodged by sonication for 10 sec followed by vortexing for 10 sec. The

viability of *C. albicans* biofilm was measured using spiral plating on blood agar plates at 2 dilutions (1:10 and 1:1000). Plates were incubated for 48 h at 37°C in 5% CO_2. The yeast colonies were counted by an automated colony counting instrument (Synbiosis, Inc., Frederick, MD, USA) using specialized Windows – based software which calculated the numbers of colony forming units (CFU)/ml.

In order to determine the relative amount of planktonic growth the planktonic cells which were not attached to acrylic specimens were measured at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The effect of nicotine and CSC concentrations on planktonic cells was determined by pipetting 120 µl of the planktonic cell suspension from each well into a fresh 96 well microtiter plate and measuring the absorbance at 595 nm.

2.4 Biofilm metabolic activity:

C. albicans biofilm was established by incubating 10 µl of a *C. albicans* overnight culture in 190 µl of YPD without CSC or nicotine to allow biofilm to grow in sterile 96-well microtiter plates for 24 h at 37°C. The biofilm was washed with sterile saline. These wells were exposed to nicotine and CSC in YPD at different concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml) and (0, 0.25, 0.5, 1, 2 and 4 mg/ml), respectively, in quadruplicate for 24 h at 37°C. The metabolic activity of *C. albicans* was calculated using a method described by Pierce et al. [25,26], by reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide (XTT) by biofilm cells into a water soluble orange compound. XTT was prepared as a saturated solution, filtered, aliquoted and stored at -70°C. Before assay this solution was thawed and menadione was added to the final concentration. The XTT/menadione solution was added to the prewashed biofilm, the plate was incubated in the dark for 2 h at 37°C and the color change of these plates was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

2.5 Statistical analysis:

The effect of each concentration of nicotine and CSC was measured in triplicate and each experiment was repeated three times. One-way ANOVAs (with a random effect for experiment) were performed to compare the concentrations of CSC or nicotine for differences in absorbance, CFU and metabolic activity. Due to non-normality, a natural log transformation

was used prior to analysis. Pair-wise comparisons between groups were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. Distribution of the data was examined and a transformation of the data was used. The study had 80% power to detect a 3.5x difference in CFU/mL between any groups. The calculations assumed two-sided tests each conducted at a 5% significance level. All data were expressed as mean values with the corresponding standard errors of the mean.

3. Results:

The viability of *C. albicans* was significantly increased ($p \leq 0.05$) concomitant with increasing concentrations of nicotine up to 2 mg/ml (mean value 5×10^7 and SD 2×10^7) and CSC up to 0.5 mg/ml (mean value 4×10^7 and SD 2×10^7) (Fig. 1) from the mean of ?? and SD of ?? at 0 mg/ml of nicotine. However, increasing the concentrations beyond these levels resulted in a significant inhibitory effect on *C. albicans* viability. Using higher concentrations of nicotine (4, 8, 16 and 32 mg/ml) (mean value 2×10^6 , 3×10^5 , 10^2, 0) and CSC (1, 2 and 4 mg/ml) (mean value 1×10^7, 9×10^5, 6×10^5) resulted in significant inhibition of *C. albicans* viability.

To provide a more quantitative assessment of cellular activity of established *C. albicans* biofilm in the presence or absence of nicotine and CSC, the ability of yeast to reduce XTT was measured (Fig. 2). The results demonstrated that nicotine at 16 mg/ml (mean value 3.4 and SD 0.7) and CSC at 4 mg/ml (mean value 1.1 and SD 0.1), significantly increased *C. albicans* biofilm metabolic activity. However, increasing the concentrations above these levels resulted in a decline of *C. albicans* metabolic activity. Higher concentrations of nicotine at 32 mg/ml (mean value 3 and SD 0.7) and CSC at 8 mg/ml (mean value 1 and SD 0.1) resulted in a significant inhibitory effect on *C. albicans* metabolic activity.

Planktonic cell absorbance results with different nicotine concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml) were consistent with *C. albicans* viability at similar concentrations of nicotine (Fig. 3). Increasing planktonic cell absorbance was observed with increasing concentrations of nicotine. As the concentration of nicotine increased from 0 mg/ml (mean

value 0.3 and SD 0.05) up to 2 mg/ml (mean value 0.4 and SD 0.03), the measurement of planktonic cell absorbance increased as well and then with higher concentrations of nicotine from 2 mg/ml (mean value 0.4 and SD 0.03) up to 32 mg/ml (mean value 0.02 and SD 0.01), the planktonic cell absorbance started to decline. However, planktonic cell absorbance results with different concentrations of CSC (0, 0.25, 0.5, 1, 2, 4 and 8 mg/ml), was significantly different than the viability of *C. albicans* with the various CSC concentrations. Planktonic cell absorbance continued to increase with increasing concentrations of CSC up to 8 mg/ml (mean value 0.9 and SD 0.07).

Overall, there were significant differences between the various CSC concentrations for *C. albicans* absorbance and CFU. Furthermore, there were significant differences between the concentrations of nicotine for *C. albicans* absorbance, CFU, and metabolic activity.

4. Discussion:

1. Fungal infections are common in patients with various systemic conditions.[7,8] Many studies demonstrate that the prevalence of *C. albicans* varies widely, and a variety of factors seem to affect the prevalence. One factor related to the prevalence of *C. albicans* is smoking, however the exact effect of smoking on the oral microflora including *C. albicans* is not yet clear. The present study demonstrated that nicotine and CSC at certain concentrations had an impact by increasing *C. albicans* viability, planktonic cell absorbance and cellular metabolic activity. Then at specific higher nicotine and CSC concentrations the impact starts to be inhibitory by reducing *C. albicans* viability, planktonic cell absorbance and cellular metabolic activity. These results are in agreement with earlier data that indicated the impact of smoke and CSC on *C. albicans*.[19,20,21] However, these results were in contrast to several studies which did not find any relationship between smoke and the prevalence of *C. albicans*.[13,14,15,17,18] Nicotine has been demonstrated to stimulate microbial biofilm formation, which increases the risk of oral health problems. Nicotine enhances biofilm

formation of different microorganisms in the oral cavity including *L. casei, A. viscosus, A. naeslundii, E. faecalis* and *C. albicans*. In addition, many studies indicated that nicotine and CSC increase the adhesion of *C. albican, S. mutans,* and *S. pneumonaiae* to hard and soft oral surfaces.[24] The present study demonstrated that an increase in nicotine or CSC concentrations resulted in a decrease of *C. albicans* viability and increasing nicotine concentrations resulted in a decrease in planktonic cell absorbance while the opposite was reported for CSC. These results are possibly due to other components of CSC than nicotine. All of these data support the effect of nicotine on oral biofilm and enhancement of bacterial and fungal growth.

Approximately 1 mg of nicotine is absorbed with each smoked cigarette.[27] The nicotine concentrations used in the present study were (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml). These concentrations are physiologically relevant to the smoker. After smoking, nicotine accumulates in oral biofilm but there is no evidence indicating the total nicotine accumulation in the dental plaque of smokers.

Although there is no direct association that can be ascribed clinically to changes in the absorbances observed in the biofilm, planktonic or metabolic activity measurements, it is assumed that reduced measurements may be correlated with reduced *C. albicans* attached to the denture material and subsequently there should be less inflammation and therefore less denture stomatitis disease.

CSC contains several carcinogenic materials other than nicotine such as heterocyclic amine, and 2-amlno-1-methyl-6-phenylimidazo[4,5-b] (PhIP) compounds. The PhIP compounds including benzene, cadmium, arsenic, nickel, chromium, 2-naphthylamine, vinyl chloride and beryllium, which might explain the differences in biofilm formation between nicotine and CSC observed in this study. All of these components in CSC may have an impact on *C. albicans* growth or inhibition.[28,29] The impact of CSC on *C. albicans* was reported earlier by Baboni et al. which indicated increasing *C. albicans* cell adhesion was associated with increasing CSC concentrations. But as the CSC concentration increased up to 50% it started to have an inhibitory effect on cell adhesion.[20] In addition, CSC increased the adhesion of *C. albicans* on

all non-metal materials including acrylic resin.[30] CSC can be a contributing factor to oral candidiasis due to increasing the virulence of *C. albicans* with increasing the secretion of histolytic enzymes.[30] Appropriate further studies will be necessary to investigate the mechanism that led to the results reported here between *C. albicans* and CSC.

Some of the limitations of this study were that this was a controlled in-vitro study and in the oral environment we have many variables that it is difficult to duplicate in the lab. It would be more beneficial if clinical isolates of *C. albicans* in saliva samples were taken directly from the smokers. Another limitation was that at specific nicotine and CSC concentrations the impact was inhibitory by reducing *C. albicans* viability, planktonic cell absorbance and cellular metabolic activity but the exact mechanism is still unknown and further investigations are needed to clarify the inhibitory effect.

As a result of increased *C. albicans* metabolic activity and biofilm formation of this opportunistic microorganism on denture acrylic material correlated with increased concentrations of CSC and nicotine up to a certain dose, the hypothesis of this study was accepted.

5. Conclusion

Within the limitations of this study the following conclusions can be drawn:

1. Increased growth of *C. albicans* in the presence of CSC and nicotine suggests that they may be potential contributing factors to *C. albicans* biofilm related infections in smokers.

2. Nicotine and CSC caused a dose–dependent increase in the biofilm formation, metabolic activity, the viability and planktonic cell absorbance of *C. albicans.*

3. High concentrations of CSC and nicotine resulted in inhibition of the viability of *C. albicans.*

6. Clinical Significance

From a clinical perspective, the results from the current study emphasized the importance of communicating with the patient regarding the effect of smoking on the oral environment. In addition, dentists must be aware that some strategies must be applied to reduce the risk of microbial colonization, such as the importance of maintaining good oral hygiene particularly if the patient is wearing a dental appliance that is made of acrylic resin and the importance of regular dental visits.

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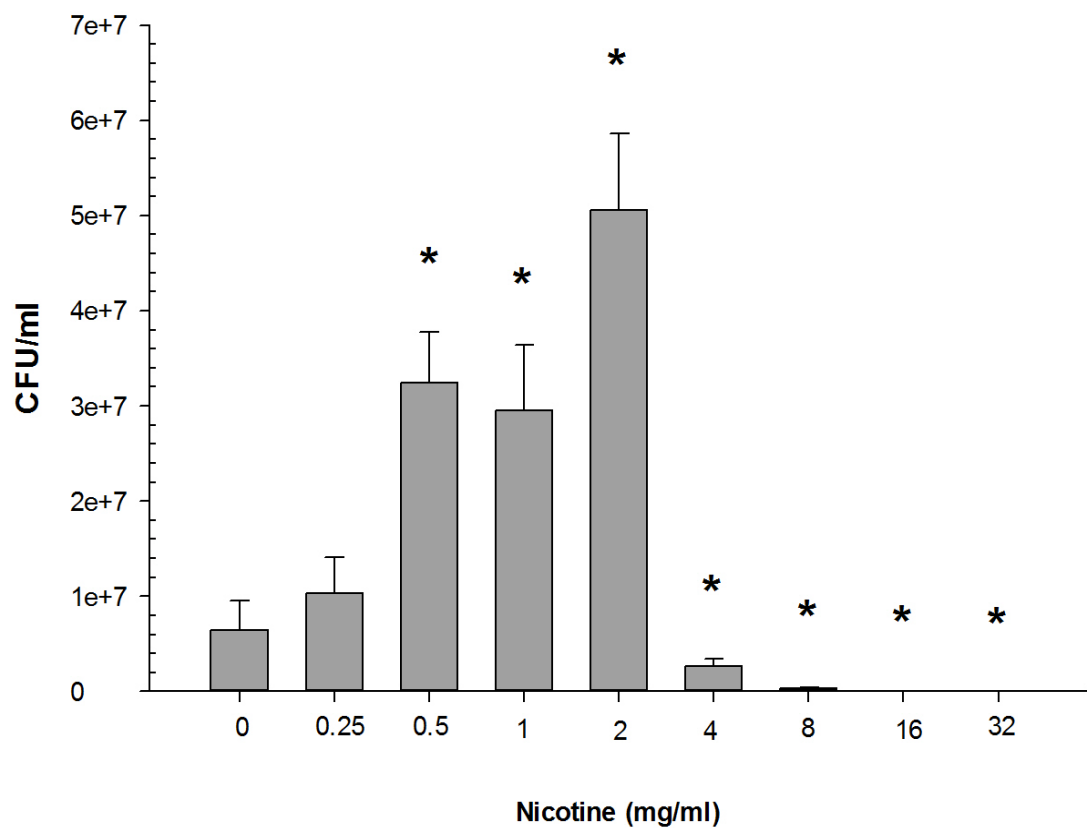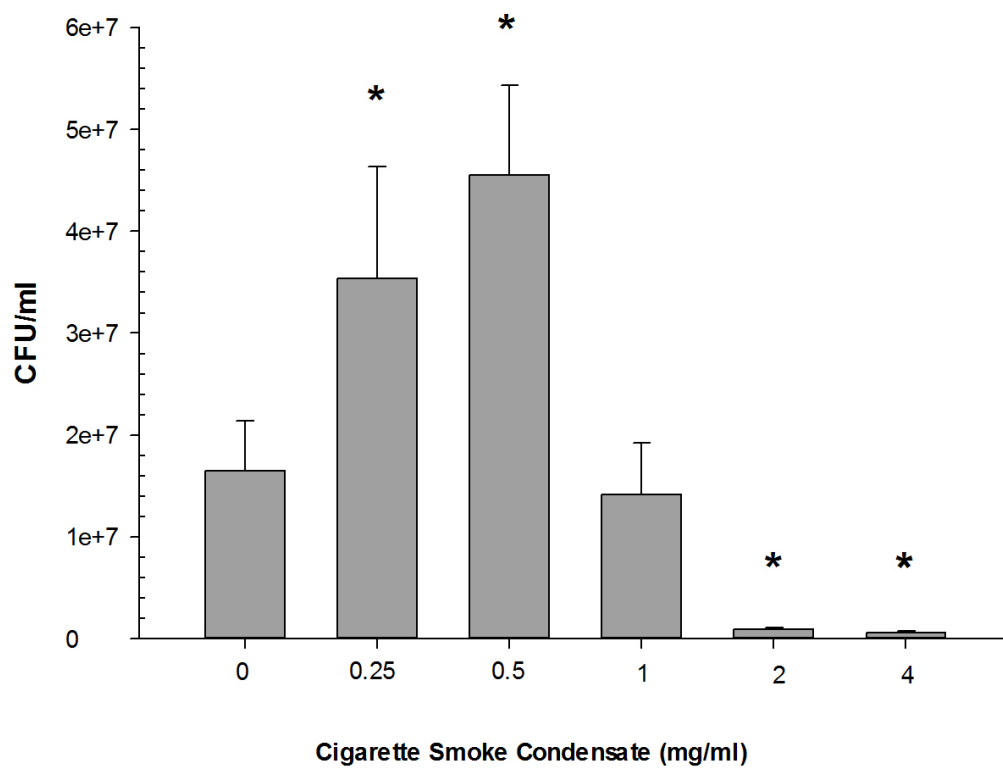
Fig. 1A

Fig. 1B

Fig. 2A
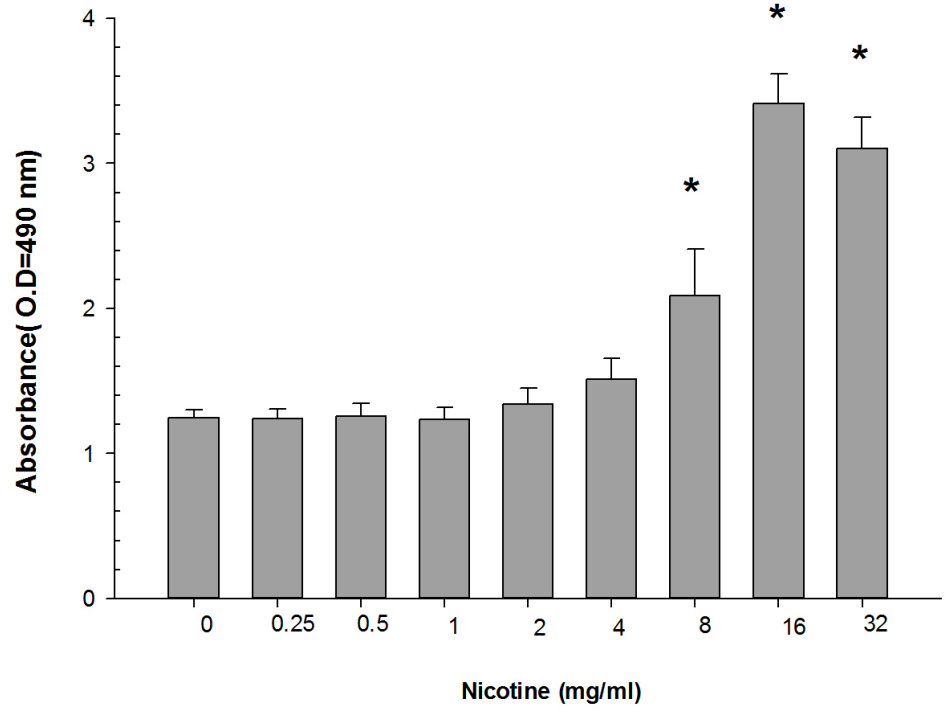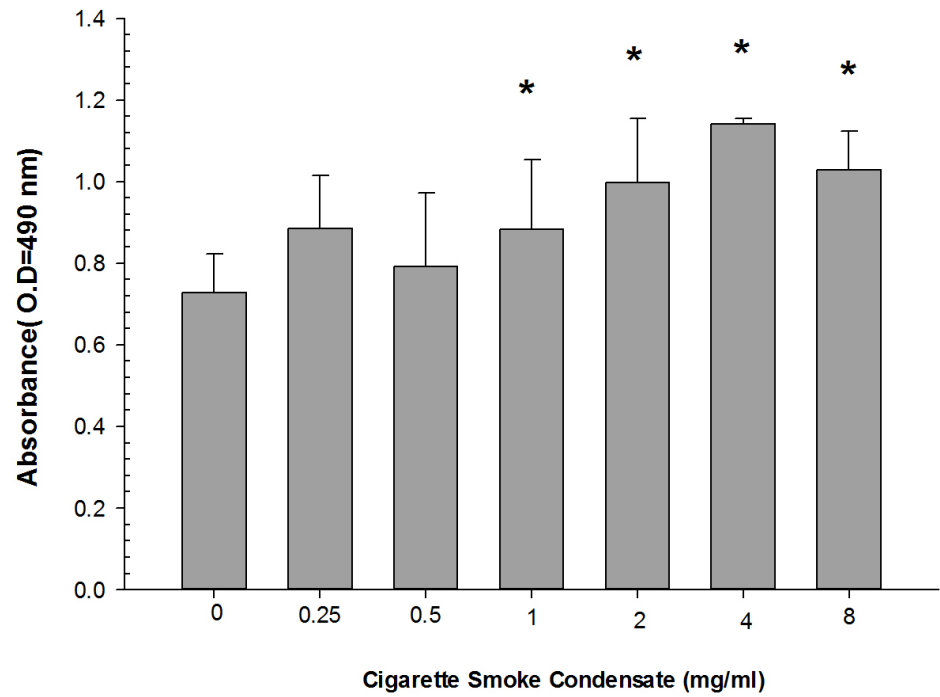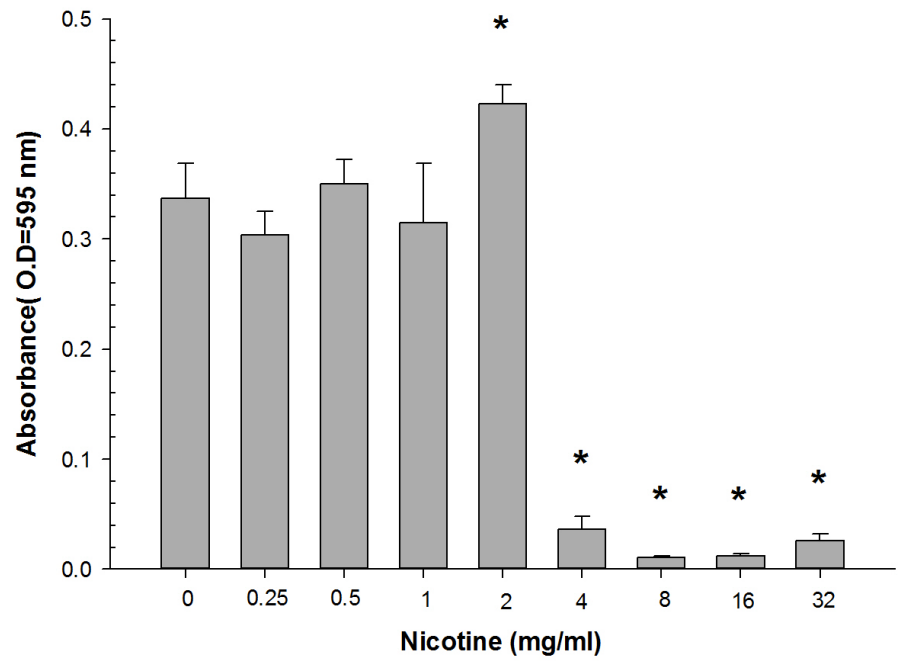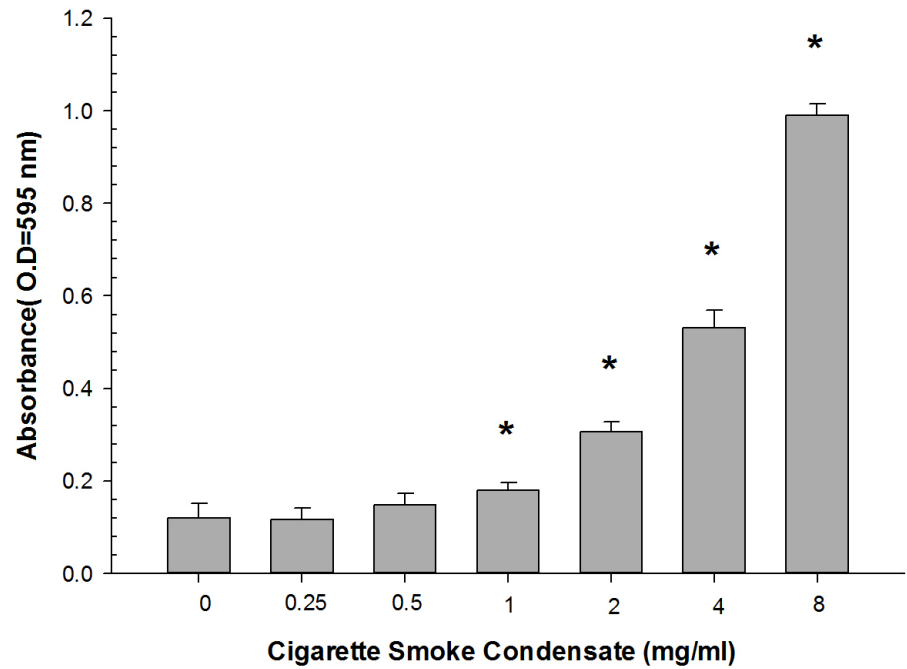

Fig. 2B

Fig. 3A

Fig. 3B

Figure legends

Figure 1. The viability of *C. albicans* incubated with different concentrations of (A) nicotine and (B) CSC after 24 h at 37°C. The data are expressed as mean colony forming units (CFU)/ml ± S.E.M. Asterisks indicate significant differences ($p \leq 0.05$) when compared with the no nicotine/CSC groups.

Figure 2. Biofilm metabolic activity of *C. albicans* incubated with different concentrations of (A) nicotine and (B) CSC after 48 h at 37°C. The data are expressed as mean absorbance (490 nm) ± S.E.M. Asterisks indicate significant differences ($p \leq 0.05$) when compared with the no nicotine/CSC groups.

Figure 3. Planktonic cell absorbance of *C. albicans* incubated with different concentrations of (A) nicotine and (B) CSC after 48 h at 37°C. The data are expressed as mean absorbance (595 nm) ± S.E.M. Asterisks indicate significant differences ($p \leq 0.05$) when compared with the no nicotine/CSC groups.