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Activated carbon attenuates nicotine and *Candida albicans*-induced oral leukoplakia via modulation of IL-17R signaling and oxidative stress: an in vivo study

Utmi Arma[1*], Abu Bakar[1], Fitria Mailiza[1], Fauzia Nilam Orienty[2] and Basri A. Gani[3]

Abstract

Objectives This study evaluated the therapeutic potential of coconut-shell-derived activated carbon (AC) in attenuating oral leukoplakia induced by cigarette smoke condensate (CSC) nicotine and *Candida albicans*, focusing on nicotine burden, fungal growth, interleukin-17 receptor (IL-17R) expression, oxidative stress (malondialdehyde, MDA), and mucosal cytotoxicity.

Materials and methods An in vivo experimental study was conducted using *Rattus norvegicus* ($n=25$), divided into five groups: negative control (CSC-nicotine + *C. albicans*), positive control (triamcinolone acetonide 1%), and AC-treated groups (1:10, 1:20, 1:30). Oral mucosal changes were assessed macroscopically and histologically. Free nicotine was measured by FTIR, IL-17R, and MDA by ELISA, and cell viability by MTT assay. Statistical analyses included ANOVA, the Kruskal–Wallis test, and correlation tests ($p<0.05$).

Results CSC-nicotine and *C. albicans* induced progressive leukoplakia-like lesions with high dysplasia scores, elevated IL-17R (182.4 ± 11.2 pg/mL), MDA (3.1 ± 0.2 nmol/mg), and low cell viability (46.2 ± 4.8%). AC at 1:10 significantly reduced fungal growth (− 53.3%), free nicotine (39 ± 7 µg/mL), IL-17R (101.5 ± 7.9 pg/mL), and MDA (1.7 ± 0.3 nmol/mg), while restoring cell viability (94.6 ± 6.1%), comparable to the positive control. Strong correlations were observed between nicotine, IL-17R, and MDA ($r>0.97$).

Conclusions Activated carbon at 1:10 effectively reduces nicotine burden, inflammation, oxidative stress, and mucosal cytotoxicity, limiting oral leukoplakia progression in vivo.

Keywords Oral leukoplakia, Nicotine, *Candida albicans*, Activated carbon, IL-17R, Malondialdehyde, MTT assay

*Correspondence:
Utmi Arma
utmiarma@fkg.unbrah.ac.id
[1]Department of Oral Medicine, Dentistry Faculty, Universitas Baiturrahmah, Padang, Sumatera Barat, Indonesia
[2]Department of Oral Biology, Dentistry Faculty, Universitas Baiturrahmah, Padang, Sumatera Barat, Indonesia
[3]Department of Oral Biology, Dentistry Faculty, Universitas Syiah Kuala, Darussalam, Banda Aceh, Indonesia

Introduction

Oral leukoplakia represents the most common premalignant lesion of the oral cavity and carries a significant risk of progression to oral squamous cell carcinoma [1]. Among its etiologic factors, tobacco exposure, particularly the nicotine fraction of cigarette smoke condensate (CSC), plays a pivotal role by inducing epithelial irritation, promoting hyperkeratosis, and driving dysplastic remodeling [2]. Concurrently, *Candida albicans* is often identified within leukoplakia lesions and further exacerbates epithelial injury through mechanisms such as hyphal invasion, secretion of aspartyl proteinases, and activation of pro-inflammatory pathways, thereby accelerating disease progression [3]. Within this pathological environment, oxidative stress and immune activation emerge as central features. The MDA serves as a key biomarker of lipid peroxidation and membrane injury [4]. At the same time, interleukin-17 receptor (IL-17R) signaling reflects the crosstalk between epithelial cells and the immune system, particularly in mediating neutrophil-driven inflammation [5]. Collectively, these interconnected processes, including nicotine burden, IL-17R-mediated inflammation, and oxidative stress, form a mechanistic triad that perpetuates the progression and persistence of oral leukoplakia [6].

The selection of interleukin-17 receptor (IL-17R) as the primary biomarker in this study is supported by strong evidence indicating that the IL-17/IL-17R axis plays a central role in the progression of oral premalignant lesions. Activation of IL-17R in the oral mucosal epithelium has been reported to enhance neutrophil recruitment, increase the production of pro-inflammatory cytokines, and stimulate reactive oxygen species (ROS) generation, all of which contribute to tissue damage, epithelial hyperproliferation, and dysplastic transformation [7]. Several studies have demonstrated that IL-17R expression is significantly elevated in leukoplakia with higher dysplasia grades and correlates with the risk of malignant transformation, making it a more specific and biologically relevant marker than broader inflammatory mediators such as TNF-α or IL-6 [8]. Therefore, IL-17R provides a stronger mechanistic basis for assessing the protective effects of activated carbon against inflammation and oxidative stress in the leukoplakia model [9].

Current management emphasizes risk-factor modification and topical/systemic anti-inflammatory agents (e.g., corticosteroids). Yet, these approaches can be variably practical and carry concerns regarding recurrence or adverse effects [10] This motivates exploration of adjunctive, locally acting strategies that are safe, inexpensive, and capable of simultaneously lowering chemical and microbial insults at the mucosal surface, as suggested by previous reports showing the benefit of topical agents in reducing lesion progression and microbial burden in oral potentially malignant disorders [11].

Activated carbon (AC) is a highly porous and biocompatible adsorbent material widely used for the removal of low–molecular-weight, hydrophobic compounds, including alkaloids, aromatic hydrocarbons, and microbial metabolites [12]. Owing to its large specific surface area and tunable micro- and mesoporosity, AC has been shown to effectively bind nicotine, polycyclic aromatic hydrocarbons, and other cigarette smoke–derived toxicants, thereby reducing their bioavailability and tissue exposure [13]. Coconut-shell–derived AC is characterized by a predominantly microporous architecture, high chemical stability, and a favorable safety profile for oral contact, and it is abundantly available in tropical regions at low cost [14]. In addition to its strong adsorptive capacity, AC has been reported to sequester minor hydrophobic alkaloids, including nicotine, capture microbial toxins and quorum-sensing metabolites, reduce biofilm biomass and surface colonization, and indirectly attenuate oxidative stress by limiting the accumulation of redox-active species and downstream inflammatory responses [15]. These combined properties have supported the use of AC in medical detoxification, toxin adsorption, and emerging biomedical applications aimed at mitigating chemically and microbially induced tissue injury. (R2).

This study is the first to explore coconut shell-derived AC as an adjuvant therapy in a CSC-nicotine and *Candida albicans*-induced oral leukoplakia model. Its novelty lies in combining macroscopic, histopathological, and biochemical evaluations, covering free nicotine, IL-17R expression, and MDA levels, alongside correlation analysis, to clarify the interplay of nicotine burden, inflammation, and oxidative stress. The research assessed whether topical AC could lower nicotine and fungal loads, suppress IL-17R–mediated inflammation and oxidative damage, and improve mucosal histology. It was hypothesized that higher sorbent density would provide the most effective protection by attenuating dysplasia progression.

Materials and methods

Study design

This study was granted ethical approval. It was conducted using an in vivo experimental model of oral leukoplakia induced by nicotine exposure and *Candida albicans* infection. A total of 25 healthy Wistar rats (*Rattus norvegicus*), aged 8–10 weeks and weighing 200–250 g, were randomized into five groups ($n = 5$ each): negative control (nicotine + *C. albicans* without therapy), positive control (Triamcinolone acetonide 0.1% for orabase), and three treatment groups receiving AC at ratios of 1:10, 1:20, and 1:30. Only healthy rats were included. At the same time, those who showed signs of illness or stress were excluded.

All animals were acclimated for seven days on a standard diet and water ad libitum before the intervention.

Preparation of activated carbon

Activated carbon was prepared from coconut shell (*Cocos nucifera* endocarp) through sequential pyrolysis and chemical activation. Briefly, the shells were cleaned, sun-dried, and carbonized at 500 °C for 2 h under limited oxygen. The resulting charcoal was ground and sieved to a particle size of 100–200 mesh, chemically activated by immersion in 1 M KOH for 24 h, rinsed to neutral pH, oven-dried, and subjected to a second pyrolysis at 700 °C for 1 h to enhance pore development. The activated material was subsequently washed with 0.1 M HCl and deionized water, dried, and stored in airtight containers until use [16]. Following completion of the induction phase, activated carbon was administered as a localized topical intervention to evaluate its therapeutic effects on established leukoplakia-like lesions [17]. The application was performed directly onto the buccal mucosa using a sterile cotton applicator, allowing close contact with the epithelial surface. Adhesion of the material was facilitated by its fine particle size, high surface area, and the naturally moist oral environment, ensuring adequate epithelial contact. Importantly, activated carbon treatment was initiated only after lesion establishment and was not administered concurrently with the induction phase. (R2).

Preparation of cigarette smoke condensate

For the preparation of cigarette smoke condensate (CSC), cigarettes were placed at one end of a silicone tube connected to an Erlenmeyer flask containing 200 mL of 0.9% sodium chloride solution (Merck KGaA, Darmstadt, Germany). The other end of the silicone tube was connected to a vacuum pump. Cigarettes were extracted under vacuum, and the smoke was drawn directly into the sodium chloride solution. This procedure was repeated until a total of 10 cigarettes (each containing 2.3 mg of nicotine) had been processed. The resulting CSC solution containing nicotine was filtered through a 0.22 µm filter paper, sterilized, and stored at 4 °C until further use [18].

Oral leukoplakia animal model

A 14-day induction period was selected based on evidence demonstrating that exposure to nicotine and *C. albicans* can induce early leukoplakia-like alterations within this timeframe. (R2) Nicotine has been reported to induce epithelial hyperkeratosis, oxidative stress, and early dysplastic remodeling within 7–14 days of exposure [19]. Likewise, *C. albicans* has been shown to establish stable mucosal colonization, form adherent white plaques, and promote epithelial hyperplasia and parakeratosis within 7–10 days, particularly in the presence of chemical irritants such as cigarette smoke or nicotine [20]. Moreover, combined exposure to nicotine and *C. albicans* exerts a synergistic effect, accelerating keratinization, inflammatory responses, and the onset of early dysplastic changes within two weeks [21]. Based on these findings, a 14-day induction period was considered sufficient and biologically appropriate for reproducing early histopathological features of oral leukoplakia rather than advanced lesions [22]. (R2) The oral leukoplakia model was established according to previously reported experimental protocols in which topical nicotine exposure, particularly when combined with *C. albicans* infection, induces leukoplakia-like epithelial alterations. The nicotine concentration (20 mg/mL) and induction duration were selected based on earlier studies demonstrating consistent epithelial hyperkeratosis, inflammation, and early dysplastic changes within this timeframe. *Candida albicans* (ATCC 10231; 1×10^7 CFU/mL) was applied every 48 h to ensure stable mucosal colonization and to exacerbate nicotine-induced epithelial injury. Accordingly, this model was intentionally designed to represent early-stage mucosal alterations, characterized by adherent white plaques, epithelial thickening, inflammatory infiltration, and early dysplastic remodeling, which are hallmarks of early leukoplakia-like lesions. (R2).

ELISA assay

IL-17R and MDA levels were quantified from buccal mucosal tissue homogenates prepared in cold PBS and centrifuged at 3000 rpm for 10 min at 4 °C. Supernatants were stored at – 80 °C until analysis. IL-17R concentrations were measured using a Rat IL-17R ELISA Kit (Cusabio, Wuhan, China) and expressed in pg/mL. At the same time, MDA levels were determined with a Rat MDA ELISA Kit (Cusabio, Wuhan, China) and expressed as nmol/mL (plasma) or nmol/mg (tissue), based on respective standard curves. Absorbance for both assays was read at 450 nm, and all measurements were performed in triplicate to ensure reproducibility, serving as quantitative indicators of inflammation and oxidative stress in the experimental model [23].

Hematoxylin and Eosin (H&E) staining

Buccal mucosal tissues from leukoplakia lesion sites were fixed in 10% buffered formalin for 24 h, dehydrated in graded ethanol, cleared with xylene, and embedded in paraffin. Sections of 4–5 µm thickness were cut, mounted on slides, and stained with hematoxylin and eosin (H&E). The procedure included deparaffinization, rehydration, nuclear staining with hematoxylin, bluing, cytoplasmic staining with eosin, dehydration, clearing, and coverslipping. Histopathological evaluation was performed under a light microscope (40×–400×) to assess the morphology of epithelial and connective tissues. Dysplasia was scored

based on hyperplasia, keratinization, pleomorphism, loss of polarity, altered nucleus-to-cytoplasm ratio, and mitotic activity, and categorized as mild, moderate, or severe [24].

Candida albicans Growth in oral mucosa

Swab samples from the buccal mucosa were cultured in Sabouraud Dextrose Broth (SDB) at 37 °C for 24 h, then *Candida albicans* was cultured on selective media (CHROMagar™, Paris, France) and incubated at 37 °C for 48 h. Colony counts were expressed as CFU/mL, with morphology and color confirming *C. albicans*. Fungal growth in broth was also assessed by optical density at 600 nm. All procedures were performed in triplicate to ensure accuracy and reproducibility [25].

Free nicotine level assessment

Free nicotine levels in buccal mucosa were determined using Fourier Transform Infrared Spectroscopy (FTIR). Tissue samples were collected, homogenized in PBS, and then centrifuged. The resulting supernatant was analyzed. Infrared spectra (4000–400 cm^{-1}) were recorded with an FTIR spectrophotometer (PerkinElmer Spectrum Two™) at 4 cm^{-1} resolution and 32 scans. Nicotine-specific absorption peaks were identified and compared with pure standards. Nicotine concentrations (µg/mL) were calculated using a calibration curve and the linear equation C = a·A + b (Lambert–Beer law), where C = nicotine concentration, A = absorbance, a = slope, and b = intercept [26].

MTT assay for mucosal cell viability

Cell viability of buccal mucosal tissue was evaluated using the MTT assay. Tissue samples obtained after treatment were homogenized in cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged to obtain the supernatant. Aliquots of the homogenate were incubated with MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a final concentration of 0.5 mg/mL and maintained at 37 °C for 4 h in the dark. Following incubation, the resulting formazan crystals were dissolved using dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the negative control, with higher absorbance values indicating greater mitochondrial activity and cell viability. All measurements were performed in triplicate [27].

Statistical analyses

All quantitative variables (IL-17R expression, *C. albicans* colony counts, free nicotine levels, and MDA concentrations) were analyzed using one-way ANOVA after verifying assumptions of normality and homogeneity. Normality of continuous variables (free nicotine, IL-17R, and MDA) was assessed using the Shapiro–Wilk test, which confirmed that all parameters were normally distributed ($p > 0.05$). Therefore, Pearson's correlation coefficient was used to examine associations among these variables. Histological dysplasia scores, which did not meet parametric assumptions, were analyzed using the Kruskal–Wallis test. Correlation analyses used Pearson's or Spearman's coefficients as appropriate. A significance level of $p < 0.05$ was applied for all statistical tests. Statistical analyses were performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, New York, USA).

Results

Table 1 shows the progressive macroscopic changes in the oral mucosa of *Rattus novergicus* subjected to CSC-nicotine exposure and *Candida albicans* infection. By day 7, the mucosa displayed mild erythema, slight surface roughness, and thin white plaques in scattered areas, with no ulceration and only mild edema. By day 14, these features had advanced to intense discoloration, surface keratinization, and dryness, accompanied by thick, widespread white plaques resembling leukoplakia, ulcerations in certain areas, and moderate to severe edema, indicating a marked progression of inflammatory and pathological changes.

Table 2 shows that AC reduced Candida albicans colony counts in a concentration-dependent manner. The negative control had the highest fungal load ($12.0 \pm 0.8 \times 10^4$ CFU/mL). AC at a 1:10 ratio achieved the greatest antifungal effect, lowering counts to $5.6 \pm 0.6 \times 10^4$ CFU/mL (a 53.3% decrease, $p < 0.05$), which was comparable to the positive control (a 57.5% decrease). By contrast, the 1:20 and 1:30 ratios showed only moderate, non-significant reductions (34.2% and 24.2%, respectively). These results suggest that the antifungal efficacy of AC is most

Table 1 Macroscopic profile of the oral mucosa of *Rattus novergicus* infected by *Candida albicans*

Time and Treatment	Mucosal Color	Surface	Moisture	Lesion/Plaque	Ulcer/Erosion	Inflammation
Day 7 (CSC-nicotine + *C. albicans*)	Diffuse redness (*erythema*)	Slightly rough	Slightly dry	Thin white plaque in several mucosal areas	None	Mild edema
Day 14 (CSC-nicotine + *C. albicans*)	Intense red to whitish-yellow	Rough and keratinized	Dry	Thick, widespread white plaque resembling leukoplakia	Present in some areas	Moderate–severe edema

Source: own research

Table 2 Colony count of *Candida albicans* on CHROMagar™ Candida medium

Treatment Group	*N*	Mean ± SD (×10^4 CFU/mL)	Reduction vs. Negative Control (%)	*p*-value*
Negative Control	5	12.0 ± 0.8	—	—
AC 1:10	5	5.6 ± 0.6	53.3	0.003
AC 1:20	5	7.9 ± 0.7	34.2	0.081
AC 1:30	5	9.1 ± 0.5	24.2	0.114
Positive Control	5	5.1 ± 0.4	57.5	0.002

**p*-values were obtained from one-way ANOVA followed by post hoc LSD test, compared with the negative control. *AC* Activated carbon, *CFU* Colony-forming units, *SD* Standard deviation, *ns* Not significant

Table 3 Histology dysplasia scores in oral mucosa of *Rattus norvegicus*

Treatment Group	*N*	Dysplasia Score (Mean ± SD)	Histological Grade	*p*-value*
Negative Control	5	3.5 ± 0.3	Moderate–Severe	—
AC 1:10	5	1.2 ± 0.2	Mild	0.004
AC 1:20	5	2.1 ± 0.4	Mild–Moderate	0.018
AC 1:30	5	2.8 ± 0.3	Moderate	0.091
Positive Control	5	1.1 ± 0.2	Mild	0.003

**p*-values were obtained using the Kruskal–Wallis test followed by post hoc pairwise comparisons, with the Negative Control as the reference group. *AC* Activated carbon, *SD* Standard deviation

pronounced at the 1:10 concentration, while higher dilutions diminish its effectiveness.

Table 3 shows that exposure to CSC-nicotine and *Candida albicans* resulted in the highest dysplasia scores in the negative control group (3.5 ± 0.3; moderate to severe), characterized by hyperplasia, abnormal keratinization, pleomorphism, and inflammatory infiltration. AC at a 1:10 ratio significantly reduced dysplasia (1.2 ± 0.2; mild), with results comparable to those of the positive control (1.1 ± 0.2). The 1:20 concentration produced moderate improvement (2.1 ± 0.4; mild–moderate), while 1:30

Table 4 FTIR analysis of buccal mucosa nicotine concentration at 2925 cm^{-1}

Treatment Group	*N*	Absorbance (AU) Mean ± SD	Nicotine (µg/mL) Mean ± SD	Reduction vs. Negative Control (%)	*p*-value*
Negative Control	5	0.275 ± 0.015	100 ± 5	—	—
AC 1:10	5	0.124 ± 0.017	39 ± 7	61.0	0.002
AC 1:20	5	0.155 ± 0.018	51 ± 6	49.0	0.014
AC 1:30	5	0.180 ± 0.013	60 ± 5	40.0	0.031
Positive Control	5	0.131 ± 0.017	42 ± 6	58.0	0.004

**p*-values were obtained using one-way ANOVA followed by LSD post hoc test, with the Negative Control as the reference group. *AU* Absorbance units, *AC* Activated carbon, *FTIR* Fourier transform infrared spectroscopy, *SD* Standard deviation

showed only a slight, non-significant reduction (2.8 ± 0.3). These findings highlight that AC 1:10 provides the strongest histological protection against dysplasia, equivalent to corticosteroid treatment, whereas higher dilutions were less effective.

Figure 1 shows that exposure to nicotine and *Candida albicans* induced marked epithelial dysplasia, characterized by loss of polarity, elongated rete ridges, abnormal keratinization, pleomorphic nuclei, and frequent mitotic changes, consistent with a precancerous state. In contrast, after 14 days of treatment with AC 1:10, the oral mucosa exhibited notable repair, characterized by a restored basal layer, an intact basement membrane, reduced pleomorphism, and orderly epithelial organization. These improvements indicate that AC 1:10 effectively mitigates mucosal injury and promotes epithelial normalization, suppressing dysplastic progression and supporting tissue recovery.

Table 4 shows that the negative control group had the highest nicotine concentration (100 ± 5 µg/mL; 0.275 ± 0.015 AU). Treatment with AC significantly reduced nicotine levels, with the 1:10 concentration

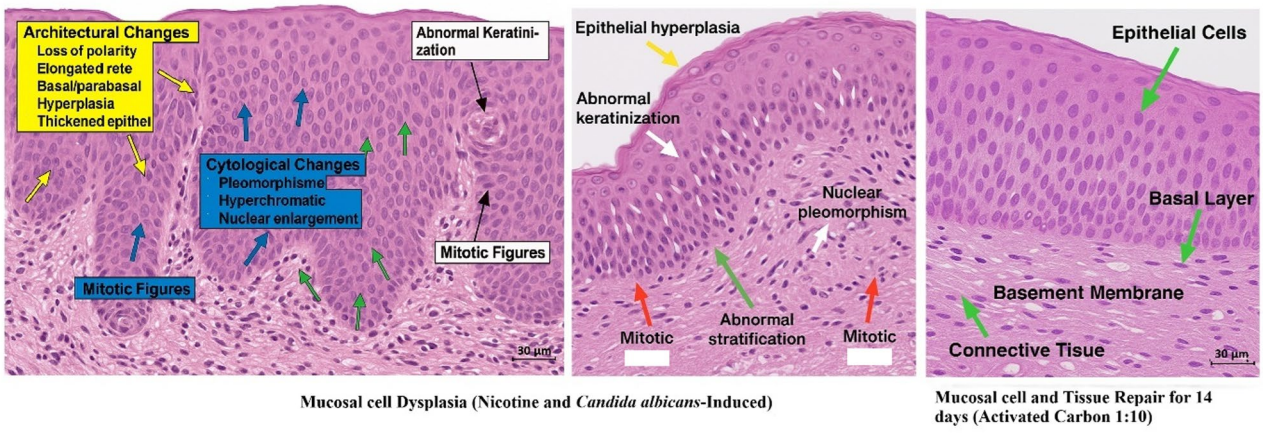

Fig. 1 Histopathology of the oral mucosal epithelial tissue stained with Hematoxylin-Eosin (H&E, 30 µm)

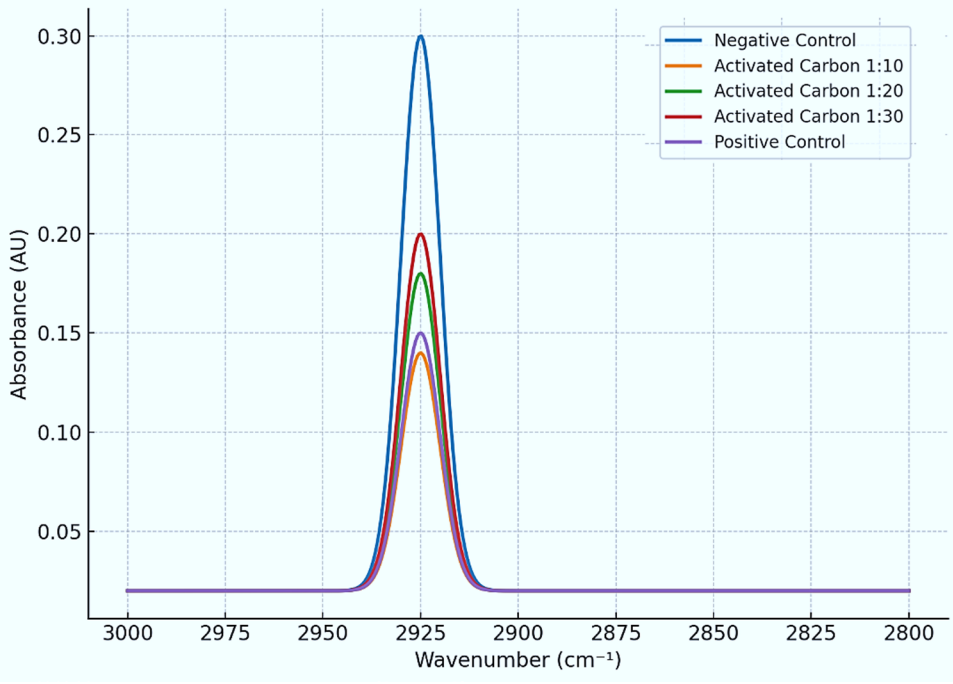

Fig. 2 FTIR spectra of tissue nicotine in Rattus norvegicus at the characteristic wavenumber 2925 cm^{-1} following activated carbon treatment. The graph shows absorbance peaks corresponding to the C–H asymmetric stretching vibration of methyl ($–CH_3$) and methylene ($–CH_2–$) groups within nicotine's pyrrolidine ring and aliphatic chain. The Negative Control group exhibits the highest absorbance (~0.28 AU), while treatment with AC at a 1:10 ratio markedly reduces the peak intensity (~0.12 AU), closely resembling the Positive Control (~0.13 AU). AC at 1:20 (~0.16 AU) and 1:30 (~0.18 AU) displays moderate peak reductions

Table 5 IL-17R expression in oral mucosa of *Rattus norvegicus*

Treatment Group	*N*	IL-17R (pg/mL) Mean ± SD	Reduction vs. Negative Control (%)	*p*-value*
Negative Control	5	182.4 ± 11.2	—	—
AC 1:10	5	101.5 ± 7.9	44.4	0.002
AC 1:20	5	124.7 ± 9.5	31.6	0.017
AC 1:30	5	139.2 ± 10.1	23.7	0.041
Positive Control	5	94.8 ± 8.6	48.0	0.001

*p-values were obtained using one-way ANOVA followed by LSD post hoc test, with the Negative Control as the reference group. *AC* Activated carbon, *IL-17R* Interleukin-17 receptor, *SD* Standard deviation.

being most effective (39 ± 7 µg/mL; 0.124 ± 0.017 AU, $p < 0.01$), comparable to the positive control (42 ± 6 µg/mL, $p < 0.01$). AC at 1:20 (51 ± 6 µg/mL) and 1:30 (60 ± 5 µg/mL) also reduced nicotine, but less effectively. These findings confirm that the 2925 cm^{-1} FTIR peak is a reliable marker of nicotine.

Figure 2 presents the spectral profiles of nicotine in buccal mucosa across different treatment groups, with a focus on the absorption band at 2925 cm^{-1}. This peak reflects the C–H asymmetric stretching vibration, a reliable marker for nicotine quantification. The Negative Control showed the most prominent peak, indicating the highest nicotine concentration. Treatment with AC, especially at a 1:10 ratio, significantly attenuated the absorbance peak, confirming its strong capacity to adsorb free nicotine, which is comparable to that of the Positive Control.

Table 5 shows that IL-17R expression was highest in the negative control group (182.4 ± 11.2 pg/mL), reflecting strong inflammation. The AC significantly reduced IL-17R in a dose-dependent manner, with the 1:10 concentration producing the most significant decrease (101.5 ± 7.9 pg/mL; 44.4%, $p < 0.01$), which was comparable to the positive control (94.8 ± 8.6 pg/mL; 48.0%, $p < 0.01$). Lower reductions were observed at 1:20 (124.7 ± 9.5 pg/mL; 31.6%, $p < 0.05$) and 1:30 (139.2 ± 10.1 pg/mL; 23.7%, $p < 0.05$).

Table 6 shows that the negative control group had the highest MDA concentration (3.1 ± 0.2 nmol/mg), indicating severe oxidative stress induced by nicotine and *Candida albicans*. AC 1:10 significantly reduced MDA to 1.7 ± 0.3 nmol/mg, comparable to the positive control (1.8 ± 0.3 nmol/mg), indicating the strongest protective effect. The 1:20 and 1:30 groups also lowered MDA (2.1 ± 0.4 and 2.5 ± 0.5 nmol/mg), but less effectively than the 1:10 group.

Table 7 shown the MTT assay results demonstrated that nicotine and *Candida albicans* exposure markedly

Table 6 Malondialdehyde (MDA) levels in the oral mucosa of *Rattus norvegicus*

Treatment Group	*N*	MDA (nmol/mg) Mean ± SD	Reduction vs. Negative Control (%)	*p*-value*
Negative Control	5	3.1 ± 0.2	—	—
AC 1:10	5	1.7 ± 0.3	45.2	0.009
AC 1:20	5	2.1 ± 0.4	32.3	0.021
AC 1:30	5	2.5 ± 0.5	19.4	0.114
Positive Control	5	1.8 ± 0.3	41.9	0.012

**p*-values were obtained using one-way ANOVA followed by LSD post hoc test, with the Negative Control as the reference group. *AC* Activated carbon, *MDA* Malondialdehyde, *SD* Standard deviation

Table 7 Cell viability of oral mucosal tissue of *Rattus norvegicus*

Treatment Group	*N*	Absorbance (OD 570 nm) Mean ± SD	Cell Viability (%)	Comparison vs. Negative Control	**p*-value
Negative Control	5	0.38 ± 0.04	46.2 ± 4.8	—	—
AC 1:10	5	0.78 ± 0.05	94.6 ± 6.1 *	104.7%	0.001
AC 1:20	5	0.63 ± 0.06	76.4 ± 7.3 *	65.4%	0.018
AC 1:30	5	0.55 ± 0.05	66.7 ± 6.2 *	44.4%	0.031
Positive Control	5	0.80 ± 0.04	96.9 ± 5.4 *	109.8%	0.005

Data are presented as mean ± SD. * significantly different compared with the negative control ($p < 0.05$). *AC* Activated carbon, Statistical analysis: One-Way ANOVA followed by post hoc LSD test

reduced oral mucosal cell viability in the negative control group (46.2 ± 4.8%). Treatment with activated carbon significantly improved cell viability in a concentration-dependent manner. The 1:10 concentration showed the highest protective effect, restoring viability to 94.6 ± 6.1%, comparable to the positive control (96.9 ± 5.4%; $p < 0.01$). Lower concentrations (1:20 and 1:30) also significantly increased cell viability compared with the negative control ($p < 0.05$), although to a lesser extent. These findings indicate that activated carbon, particularly at a 1:10 ratio, exhibits minimal cytotoxicity and provides optimal protection to oral mucosal cells.

All variables met the assumption of normality as confirmed by the Shapiro–Wilk test ($p > 0.05$). Accordingly, Pearson's correlation analysis was performed to evaluate relationships among free nicotine levels, IL-17R expression, malondialdehyde (MDA) concentrations, and buccal mucosal cell viability, as assessed by the MTT assay. The analysis revealed strong and significant associations among these parameters. Free nicotine levels showed a very strong positive correlation with IL-17R expression ($r = 0.974$, $p = 0.0049$) and MDA concentrations ($r = 0.972$, $p = 0.0056$), indicating that increasing nicotine burden was closely associated with enhanced inflammatory signaling and oxidative stress. In parallel, IL-17R expression demonstrated a robust positive correlation with MDA levels ($r = 0.986$, $p = 0.0019$), suggesting that activation of IL-17–mediated immune responses occurred concomitantly with increased lipid peroxidation.

Conversely, MTT assay results showed a strong negative correlation with free nicotine levels ($r \approx -0.95$, $p < 0.01$), IL-17R expression ($r \approx -0.96$, $p < 0.01$), and MDA concentrations ($r \approx -0.94$, $p < 0.01$), indicating that higher nicotine burden, inflammatory activity, and oxidative stress were consistently associated with reduced mucosal cell viability. These inverse relationships confirm that cellular toxicity and loss of mitochondrial metabolic activity occur as downstream consequences of nicotine-induced inflammation and oxidative damage. Collectively, the correlation analysis supports a mechanistic continuum in which nicotine acts as the primary upstream insult, driving IL-17R–mediated inflammation and oxidative stress, which, in turn, culminate in impaired mucosal cell viability and the progression of epithelial dysplasia.

Discussion

This study confirms that combined exposure to CSC-nicotine and *Candida albicans* accelerates oral mucosal damage, progressing from mild erythema and thin plaques on day 7 to thick, leukoplakia-like lesions with ulceration and edema by day 14. Nicotine disrupts epithelial integrity by inducing keratinization and dysplastic remodeling, while *C. albicans* exacerbates these effects through virulence factors and chronic inflammation [19]. This high efficacy is linked to the dense availability of active adsorption sites, which capture nicotine, fungal toxins, and virulence factors. In contrast, the 1:20 and 1:30 dilutions only moderately suppressed fungal growth, with no significant effect, suggesting that reduced adsorbent density limits microbial inhibition. Previous research has confirmed that AC can adsorb microbial metabolites, disrupt quorum sensing, and reduce biofilm activity, thereby supporting its therapeutic potential in oral health [28].

The activated carbon markedly reduced dysplasia, Histological analysis confirmed epithelial repair, with reduced pleomorphism and restoration of tissue architecture. These findings suggest AC protects against precancerous progression by mitigating oxidative stress and inflammation, consistent with earlier reports linking nicotine and *C. albicans* to dysplasia and carbon-based adsorbents to antioxidant and tissue-protective effects. Previous studies have highlighted that nicotine exposure can accelerate oral epithelial dysplasia by inducing DNA damage, oxidative stress, and epithelial proliferation, while *C. albicans* infection acts synergistically to worsen these effects by invading epithelial tissue and triggering chronic inflammation [29]. On the other hand, carbon-based adsorbents have been shown to scavenge reactive oxygen species and reduce epithelial injury in

experimental models, supporting the findings of this study that AC can attenuate dysplastic progression [30].

In addition to its adsorptive capacity, the biological effects of activated carbon observed in this study are comparable to those of other antioxidant-based interventions used for oral mucosal protection. Several natural and synthetic antioxidants such as resveratrol, curcumin, catechins from green tea, and nanoparticle-based antioxidants have been shown to reduce epithelial damage by lowering ROS levels, inhibiting lipid peroxidation, and modulating pro-inflammatory cytokines, including IL-17 and TNF-α [31]. Similar patterns were observed in our study, in which activated carbon significantly reduced MDA concentrations and IL-17R expression, indicating attenuation of oxidative and inflammatory stress [32]. This suggests that activated carbon may exert a dual mode of action: first, by physically adsorbing harmful chemical and microbial components (nicotine, fungal metabolites), and second, by indirectly reducing oxidative stress and downstream inflammatory signaling [33]. Such dual-action therapeutic strategies have been proposed as promising approaches for mitigating the progression of potentially malignant oral disorders [34]. (R2) The ability of activated carbon to simultaneously impact microbial burden, chemical toxicity, and inflammatory pathways places it on par with several emerging mucosal-protective agents currently being explored in preclinical models.

The results of this study showed the strongest effect in reducing nicotine levels in the oral mucosa. FTIR analysis at 2925 cm^{-1} reliably detected these changes, with spectral peaks significantly attenuated in the 1:10 group. These results reinforce FTIR as a sensitive tool for nicotine monitoring and highlight the high adsorption capacity of AC, as supported by prior evidence on its efficiency against nicotine and tobacco toxins. Studies have similarly reported that FTIR spectroscopy can provide sensitive detection of nicotine in biological matrices, owing to its ability to detect molecular vibrations of functional groups specific to nicotine [35]. Additionally, AC has been widely documented for its adsorptive properties against nicotine and other tobacco-derived toxins, with adsorption efficiency strongly dependent on pore-size distribution and surface chemistry [36].

The AC has the most substantial anti-inflammatory effect by significantly lowering IL-17R levels (101.5 ± 7.9 pg/mL). Since IL-17R overexpression is closely linked to chronic inflammation and malignant transformation in oral lesions, these findings suggest that AC not only reduces nicotine toxicity but also mimics corticosteroid-like activity in suppressing IL-17R signaling. This highlights its potential as a novel adjunctive therapy for preventing the progression of oral premalignant conditions. Previous research has shown that IL-17/IL-17R signaling contributes to epithelial dysplasia and tumor progression by enhancing chronic inflammation and oxidative stress [37]. Moreover, research has demonstrated that reducing IL-17R activity, either pharmacologically or through natural compounds, is associated with the attenuation of mucosal inflammation and a reduced risk of malignant transformation [38].

The results showed that the negative control group had the highest MDA levels (3.1 ± 0.2 nmol/mg), indicating severe oxidative stress from exposure to nicotine and Candida albicans. AC at 1:10 significantly reduced MDA (1.7 ± 0.3 nmol/mg), nearly equal to the positive control, whereas 1:20 and 1:30 produced only moderate reductions. These findings highlight the AC's antioxidant-like role in reducing lipid peroxidation and protecting the mucosa from ROS damage, supporting earlier reports that both nicotine and C. albicans contribute to oxidative stress and the progression of dysplasia [39]. Meanwhile, lowering MDA improves mucosal healing and reduces the risk of carcinogenesis [40].

The MTT assay on buccal mucosal tissue demonstrated that combined exposure to cigarette smoke condensate (CSC)–nicotine and *Candida albicans* markedly reduced cell viability, as reflected by low absorbance values and decreased viability percentages in the negative control group. This finding indicates substantial cellular toxicity driven primarily by the cytotoxic effects of nicotine, which are further exacerbated by inflammatory and oxidative insults associated with *C. albicans* infection. Nicotine has been widely reported to impair mitochondrial function, increase reactive oxygen species (ROS) production, and disrupt cellular metabolic processes, thereby reducing the ability of viable cells to convert MTT into formazan as an indicator of mitochondrial activity [41]. In line with these mechanisms, the present study's correlation analysis revealed strong positive associations among nicotine burden, IL-17R expression, and malondialdehyde (MDA) levels, confirming that elevated nicotine directly promotes inflammatory signaling and lipid peroxidation. This parallel activation of immune dysregulation and oxidative stress provides a mechanistic basis for the persistence and progression of epithelial dysplasia [42].

In contrast, administration of activated carbon (AC), particularly at a 1:10 concentration, significantly restored mucosal cell viability to levels comparable to or exceeding those of the positive control, indicating a pronounced protective effect against tissue toxicity. This effect is likely mediated by AC's high adsorptive capacity to sequester nicotine, toxic metabolites, and pro-oxidant compounds, thereby reducing epithelial exposure to harmful agents and preserving mitochondrial integrity [43]. Although AC at 1:20 and 1:30 concentrations also improved cell viability relative to the negative control, their effects were

less pronounced, supporting a dose–response relationship dependent on the availability of active adsorption sites [44]. These findings are consistent with the observed reductions in IL-17R expression and MDA concentrations, suggesting that attenuation of inflammation and oxidative stress directly contributes to the recovery of mucosal cell viability. Previous studies corroborate these observations, demonstrating that nicotine induces ROS and inflammatory mediators, *C. albicans* amplifies immune-oxidative injury, and IL-17–mediated signaling accelerates ROS accumulation and epithelial damage in oral premalignant lesions [45].

Although activated carbon has been extensively used for systemic detoxification and gastrointestinal toxin adsorption, its direct topical application to the oral mucosa has been minimally explored in previous in vivo studies [46]. To our knowledge, only limited reports have addressed mucosal or surface-based applications of carbon materials, primarily in the context of wound care, antimicrobial coatings, or dental materials, rather than as a therapeutic intervention for oral potentially malignant disorders. In the present study, topical activated carbon application was intentionally designed as an exploratory and localized therapeutic strategy aimed at maximizing adsorption of nicotine residues, microbial metabolites, and pro-inflammatory mediators at the lesion site while minimizing systemic exposure. This localized approach represents a novel adaptation of activated carbon's established adsorptive properties to the oral mucosal environment. The observed biological effects support the feasibility of this approach; however, further studies are required to optimize formulation, assess mucosal safety, and validate translational applicability. (R2).

This study has several limitations that should be considered when interpreting the findings, including the small sample size ($n = 5$), the intentional use of a relatively short 14-day induction period designed to model early-stage oral leukoplakia rather than advanced or ulcerative lesions, the lack of validation of FTIR-based nicotine quantification against reference analytical methods such as HPLC, and the absence of local or systemic toxicity assessment. At this early disease stage, clinical manifestations are typically characterized by adherent white plaques and epithelial thickening, with ulceration or edema not yet evident. The histological findings observed in untreated animals, including epithelial dysplasia and inflammatory changes, support the validity of this early-stage leukoplakia-like model. Nevertheless, the relatively short induction period remains a limitation, as longer exposure durations may be required to reproduce more advanced lesions. (R2).

In addition, the absence of validation using human clinical samples limits the immediate generalizability and translational relevance of the results. Despite these limitations, the findings provide important insights into the potential clinical applicability of activated carbon as an adjunctive intervention for oral potentially malignant disorders. By effectively reducing nicotine burden, fungal proliferation, inflammatory signaling, and oxidative stress, activated carbon targets key pathogenic mechanisms involved in oral leukoplakia. Future studies should include larger sample sizes, extended observation periods, comprehensive toxicity evaluations, analytical validation of FTIR measurements, and preliminary clinical validation using human leukoplakia specimens or saliva samples. Furthermore, the development of optimized delivery formulations, such as mucoadhesive films, topical gels, or mouthrinse preparations, along with early-phase clinical trials, will be essential to confirm the safety, efficacy, and clinical feasibility of activated carbon before translation into routine clinical practice. (R2).

Conclusions

This study demonstrates that exposure to CSC-nicotine and *Candida albicans* induces progressive leukoplakia-like lesions with severe epithelial dysplasia in untreated animals. Activated carbon (AC), particularly at a 1:10 ratio, significantly reduced fungal growth, nicotine levels, IL-17R expression, and MDA concentrations, resulting in marked histological improvement comparable to that of the positive control. The strong correlations observed among nicotine burden, inflammatory signaling, and oxidative stress highlight their interconnected roles in lesion progression. Among the tested formulations, AC 1:10 was the most effective, protecting against nicotine adsorption, demonstrating antifungal activity, and suppressing inflammation and oxidative damage. Nevertheless, further studies are required, including comprehensive toxicity evaluations, longer-term investigations, and validation using human clinical samples, before activated carbon can be considered for clinical application as an adjunctive therapy for oral potentially malignant disorders.

Abbreviations

AC	Activated Carbon
ATCC	American Type Culture Collection
AU	Absorbance Unit
CFU	Colony-Forming Unit
CSC	Cigarette Smoke Condensate
DLS	Dynamic Light Scattering
ELISA	Enzyme-Linked Immunosorbent Assay
FTIR	Fourier Transform Infrared Spectroscopy
H&E	Hematoxylin and Eosin
HCl	Hydrochloric Acid
HRP	Horseradish Peroxidase
IL-17R	Interleukin-17 Receptor
KOH	Potassium Hydroxide
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical Density
PBS	Phosphate-Buffered Saline
ROS	Reactive Oxygen Species
SDB	Sabouraud Dextrose Broth

SD	Standard Deviation
SEM	Scanning Electron Microscopy
SPSS	Statistical Package for the Social Sciences
TMB	3,3′,5,5′-Tetramethylbenzidine

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

U.A. conceived and designed the study. A.B. and F.M. performed the in vivo experiments and collected the data. F.N.O. conducted the histopathological and biochemical analyses. B.A.G. assisted with data interpretation and critical review of the manuscript. U.A. wrote the first draft of the manuscript. All authors contributed to the revision, read, and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Committee on Veterinary Ethics of the Faculty of Veterinary Medicine, Universitas Syiah Kuala (No. 428/KEPH/VIII/2025). Every effort was made to minimize animal suffering and to use the minimum number of animals required for scientific validity.

Consent for publication

Not applicable. As this study was conducted exclusively on laboratory animals, informed consent is not required. All procedures were performed in accordance with institutional and national guidelines for the care and use of laboratory animals.

Competing interests

The authors declare no competing interests.

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