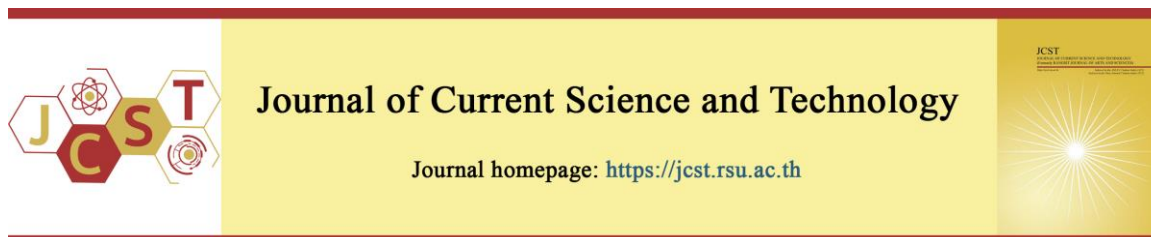


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Oral Health-promoting Potential of Standardized *Cyanthillium cinereum* (Ya Dok Kao) Extract: Anti-inflammatory and Cytoprotective Activities

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Abstract

Cigarette smoking causes oral inflammation, dental caries, and gingival hyperpigmentation, thereby impairing oral health and self-confidence. This study evaluated the potential of standardized *Cyanthillium cinereum* extract (SCCE) in oral health promotion, focusing on its anti-inflammatory, cytoprotective, antibacterial, and anti-tyrosinase activities. SCCE was prepared using microwave-assisted extraction and standardized to contain high levels of apigenin and luteolin contents. Anti-inflammatory effects were assessed in murine macrophages by measuring nitric oxide scavenging activity and the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor- α (TNF- α). Cytoprotective activity was evaluated in human gingival fibroblasts exposed to H₂O₂. Antibacterial activity against *Streptococcus mutans* was tested using disc diffusion method, while tyrosinase inhibitory activity was examined using enzymatic assays. SCCE effectively scavenged nitric oxide (IC₅₀ of 189.20 μ g/mL) and significantly suppressed iNOS, COX-2, and TNF- α expression in macrophages. Its activity was comparable to apigenin and luteolin, and in some assays superior to indomethacin. SCCE also protected gingival fibroblasts from H₂O₂-induced cell death, with cell viability up to 57.29 – 73.66%, though its cytotoxicity profile varied by cell type. However, SCCE demonstrated no antibacterial activity against *S. mutans* at 1 mg/disc and showed no significant tyrosinase inhibition. SCCE exhibits strong anti-inflammatory and cytoprotective effects, supporting its potential as a natural oral healthcare product for smokers. Despite its limited antibacterial and tyrosinase inhibitory activities, its anti-inflammatory and antioxidative properties highlight its promise in mitigating smoking-related oral health problems. Further clinical studies and prototype formulation development are warranted to advance its application.

Keywords: COX-2, flavonoids, iNOS, nitric oxide, smoking, TNF- α , oxidative stress

1. Introduction

Smoking causes various oral health problems such as tooth discoloration, tooth loss, oral tissue pigmentation, altered taste perception, gingival

discoloration, palatal lesions, dental caries, periodontitis, and oral cancer (Hedin et al., 1993; Johnson & Bain, 2000; Reibel, 2003; Warnakulasuriya et al., 2010). Studies show that smokers have a higher

prevalence and severity of periodontitis and respond less effectively to periodontal treatment (Winn, 2001). Smoking also increases dental plaque accumulation and promotes the growth of *Streptococcus mutans*, leading to a higher risk of caries (Huang et al., 2012; Reibel, 2003; Wu et al., 2019; Zonuz et al., 2008). Furthermore, it stimulates melanocyte activity, causing melanin deposition in oral tissues, which may affect self-confidence due to cosmetic concerns (Hedin et al., 1993; Johnson & Bain, 2000). Although several pharmacological smoking cessation therapies exist, such as nicotine replacement, bupropion, and clonidine, they are often costly and associated with side effects, including insomnia and nausea (McRobbie et al., 2005). This has led to increased interest in herbal alternatives.

Many other plant extracts have been studied for their antioxidant, anti-inflammatory, and anti-cancer properties on oral changes caused by smoking. For instance, extracts of *Camellia sinensis* (green tea) have been shown to modulate pro-inflammatory cytokines and prevent the formation of oral lesions (Malcangi et al., 2023). *Musa paradisiaca* (banana flower) and *Curcuma longa* (turmeric) reduce gingival melanin deposition (Sowmya et al., 2022; Leo et al., 2024). Furthermore, other extracts from *Clinacanthus nutans* have shown inhibitory activity against cariogenic *S. mutans* (Suriyaprom et al., 2024), supporting the standardization of herbal extract use in addressing multiple oral health issues.

In Thailand, *Cyanthium cinereum* (L.) H.Rob, or “Ya Dok Kao,” is traditionally used to reduce cigarette cravings and is listed in the National List of Essential Medicines B.E. 2556 (Kaji & Puttarak, 2022). Evidence from traditional use, preclinical studies, and clinical studies supports its effectiveness in reducing nicotine dependence (Puttarak et al., 2018). Its proposed mechanisms include inhibition of CYP2A6-mediated nicotine metabolism and monoamine oxidase activity, which help reduce withdrawal symptoms (Prasopthum et al., 2015). Active compounds in Ya Dok Khao, particularly apigenin and luteolin, exhibit anti-inflammatory, antioxidant, and wound-healing properties (Aziz et al., 2018; Chen et al., 2021; Shukla & Gupta, 2010).

Despite the availability of commercial *C. cinereum* products such as lozenges and capsules, many use unstandardized extracts, leading to inconsistent efficacy (Kaji & Puttarak, 2022). In a previous study, we developed a standardized extract rich in apigenin and luteolin, which demonstrated strong anti-inflammatory and antioxidant activities.

These results suggest potential applications of standardized *C. cinereum* extract in developing oral health products for smokers.

However, further investigation is required to confirm its efficacy in more specific biological contexts. These include anti-inflammatory activity at the gene expression level, protection of gingival cells from oxidative stress, antibacterial action against cariogenic bacteria, and inhibition of melanin production. Findings from these studies will help support the development of standardized *C. cinereum*-based health products for oral care in smokers.

2. Objectives

This research aimed to investigate anti-inflammatory activity at the gene expression level, and the protective effects against oxidative stress in gingival cells of standardized *C. cinereum* extract. In addition, its antibacterial action against cariogenic bacteria and inhibition of melanin production through the tyrosinase enzyme were evaluated.

3. Materials and Methods

3.1 Chemicals and Reagents

Cell lines, including murine macrophage (RAW 264.7) cells and human gingival fibroblasts (HGF-1), were purchased from the American Type Culture Collection (ATCC). Cell culture medium and all supplements, including fetal bovine serum (FBS), and penicillin/streptomycin, were supplied by Gibco. Apigenin was obtained from Axio Chemicals, while luteolin and indomethacin were obtained from Loba Chemie. Lipopolysaccharide (LPS), mushroom tyrosinase, kojic acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. The FavoPrep™ Blood/Cultured cell Total RNA Purification Mini Kit and Viva cDNA Synthesis Kit were brought from FAVORGEN Biotech Crop. and Vivantis Technologies, respectively. 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) was purchased from Solis Biodyne. Sodium nitroprusside was from HIMEDIA. Sulfanilamide was obtained from DC Fine Chemicals. The stock solutions of tested samples were prepared in dimethyl sulfoxide (DMSO).

3.2 Standardized *C. cinereum* Extract Preparation

The aerial part of *C. cinereum* was harvested from January to June 2024 from Prince of Songkla University (PSU), Songkhla Province, Thailand. The voucher specimen (SKP219220301) was identified by a botanist and kept at the herbarium of Department of

Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, PSU. The harvested plant was prepared as a dry powder and subjected to plant extraction. Standardized-*C. cinereum* extract (SCCE) was prepared following a previous report (Kaji & Puttarak, 2022). The plant powder was extracted with 75% ethanol using microwave-assisted extraction (MAE) at 900 W for 180 s at room temperature, and the extraction was performed in triplicate. The obtained crude extract was separated using a Diaion® HP-20 column using distilled water: ethanol at ratios ranging from 100:0 to 0:100 (%v/v) as the elution solvent. Subsequently, fractions of 50:50 and 25:75 of distilled water: ethanol were combined and dried to get SCCE. The apigenin and luteolin contents in SCCE were measured using high performance liquid chromatography (HPLC) following previously reported conditions (Kaji & Puttarak, 2022).

3.3 Cell Culture

RAW 264.7 and HGF-1 cells were used to investigate the anti-inflammation and cytoprotection properties of the samples, respectively. RAW 264.7 murine macrophages were used as a standard in vitro model for evaluating inflammatory responses due to their high sensitivity to LPS stimulation and their ability to produce key pro-inflammatory mediators. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Chaiwong et al., 2019). HGF-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% antibiotic (Zhu et al., 2020). Both cells were incubated under 5% CO₂ at 37°C in CO₂ incubator. The culture medium was typically changed at 3-day intervals until the cells reached 80% confluence. Then, confluent cells were sub-cultured by trypsinization.

3.4 Cell Viability

The viability of the tested cells was assessed using the MTT assay (Chaiwong et al., 2024). RAW 264.7 (4×10^4 cells/well) and HGF-1 (8×10^3 cells/well) cells were cultured in 96-well plates and incubated at 37°C in a 5% CO₂ atmosphere for 24 h to reach approximately 70% confluency. Cells were treated with SCCE, apigenin, or luteolin (1 – 100 µg/mL) in complete medium for 24 h, while untreated cells served as the control. After treatment, fresh medium containing MTT solution was added to the cells. After incubation for 3h, the medium was discarded, and DMSO was added to dissolve formazan. The optical density (OD) of mixtures

was detected at 570 nm using microplate reader. The test was performed in triplicate.

3.5 Anti-inflammation Mechanism Study

3.5.1 Nitric Oxide (NO) Scavenging Assay

NO scavenging activity of SCCE was evaluated based on the previous report (Chaiwong et al., 2019) with slight modifications. Briefly, SCCE, apigenin, and luteolin solutions (1 – 100 µg/mL) were mixed with 2 mM sodium nitroprusside (1:1 ratio) and left under light at ambient temperature for 180 min. The mixtures were reacted with Griess reagent (1:1 ratio), and then optical density of reactions were read at 546 nm using microplate reader. Quercetin was applied as positive control (Ebrahimzadeh et al., 2010). The test was done in triplicate, and 50% inhibitory concentration (IC₅₀) was calculated graphically.

3.5.2 Gene Expression Analysis

Anti-inflammation molecular mechanism of SCCE was evaluated by monitoring mRNA level of inflammation-related genes including, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and tumor necrosis factor- α (TNF- α), by real-time PCR. RAW 264.7 (2×10^5 cells) were cultured in 6-well plates under 5% CO₂ atmosphere at 37°C in CO₂ incubator for 24 h to reach approximately 70% confluency. The cells were incubated with SCCE (1 – 10 µg/mL), apigenin, luteolin, and the positive control drug, indomethacin (5 µg/mL) medium containing 1 µg/mL LPS for 24 h. The treated cells were subjected to total RNA extraction using FavoPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit. The obtained RNA was used as a template for cDNA synthesis using the Viva cDNA Synthesis Kit. Briefly, RNA (8 µg) was mixed with 40µM oligo d(T)₁₈ primer (1 µL) and 10mM dNTPs (1 µL), then incubated at 65°C for 5 min. After incubation, 5000u M-MuLv Reverse Transcriptase (1 µL), 10X buffer M-MuLV (2 µL) and nuclease-free water (up to final volume 20 µL) were added to mixture then further incubated at 42°C for 60 min followed by 85°C for 5 min. The levels of inflammation-related genes were detected by real-time polymerase chain reaction using ready-to-use PCR master-mix 5× HOT FIREPol® EvaGreen® qPCR Mix Plus. The PCR mixture consisted of cDNA (300 ng), 0.5 µL of forward and reverse primers (10 ng/µL), and 5 µL of PCR master-mix in a final volume of 25 µL. The amplification condition consisted of polymerase activation (95°C, 15 min) followed by 40 cycles of denaturation (95°C,

15 sec), annealing (60°C, 30s), and extension (72°C, 45 sec). β -actin was used as the endogenous control gene, and the untreated control served as the calibrator. Primer sequences are listed in Table 1. Relative gene expression levels were determined using the cycle threshold (C_T) method. All experiments were performed in triplicate.

3.6 Cytoprotective Study of SCCE on H_2O_2 -Induced HGF-1 Cell Death

The efficacy of SCCE in preventing H_2O_2 -induced HGF-1 cell death from oxidative stress was investigated by the MTT assay (Zhu et al. 2020). HGF-1 (8×10^3 cells/well) were cultured in 96-well plates under a 5% CO_2 atmosphere at 37°C in a CO_2 incubator for 24 h. HGF-1 cells were pre-treated with SCCE, apigenin, or luteolin at concentrations ranging from 1 – 100 μ g/mL for 24 h, followed by exposure to H_2O_2 for 18 h to induce oxidative stress and cell death. Cells cultured in complete medium without treatment served as the untreated control. Cell viability was assessed using the MTT assay. All experiments were performed in triplicate.

3.7 Anti-carries Activity Study

The anti-carries activity of SCCE was evaluated using the disc diffusion method against *S. mutans* based on previous reports (Techaoei, 2022; Macambira et al., 2024). Briefly, bacteria were cultured in nutrient broth at 37°C for 24 h and the turbidity was adjusted to McFarland No. 0.5 prior to use. The SCCE, apigenin, and luteolin solutions were dropped onto sterile paper discs to obtain 1 mg/disc and placed over agar swabbed with bacteria. The plates were incubated at 37°C for 24 h and the clear zones that appeared were measured. Chlorhexidine (10 μ g/disc) was used as standard antibiotic. All samples were assayed in triplicate.

3.8 Anti-pigment Accumulation Activity Study

The anti-pigment accumulation activity of SCCE was examined using tyrosinase inhibition assay following previous method (Dej-Adisai et al., 2021). Briefly, SCCE, apigenin, and luteolin stock solutions were diluted in phosphate buffer pH 6.8 to obtain desired concentrations (25 – 800 μ g/mL). In the reaction, 140 μ L of buffer, 20 μ L of sample solution, and 2 μ L of tyrosinase enzyme solution were mixed in a 96-well plate and incubated at 25°C for 10 min. Then, 20 μ L of L-Dopa was added, and the OD of the mixtures was measured at 492 nm. After further incubation for 20 min, the OD of reactions was detected again. Kojic acid was served as positive control. The test was done in triplicate and IC_{50} was calculated graphically.

3.9 Statical Analysis

The data are presented as the mean $\pm$ standard deviation (SD). The statistical significance of the data was analyzed using GraphPad Prism Software 5, using one-way ANOVA followed by Tukey's test. Microsoft Excel was employed to calculate IC_{50} values. The statistically significant was considered at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

4. Results

4.1 Percent Yield and Active Compound Contents of SCCE

The preparation of SCCE using MAE and Diaion® HP-20 column chromatography provide 11.95 % yield of extract. For the quantitative analysis of apigenin and luteolin by HPLC, the standard curves of apigenin (62.5 – 1,000 μ g/mL) and luteolin (1.56 – 25 μ g/mL) were $y = 40.536x - 200.69$ ($R^2 = 0.9999$) and $y = 7936x - 2957.9$ ($R^2 = 0.9998$), respectively. The amount of apigenin was 91.20 ± 1.23 mg/g extract while luteolin was 167.00 ± 0.49 mg/g extract.

Table 1 Sequences of primers used in gene amplification

Primer	Sequence	Size in bp (Reference)
iNOS_Foward	5'-ATCTGGATCAGGAACCTGAA-3'	580
iNOS_Reverse	5'-CCTTTTTTGCCCCATAGGAA-3'	(Sae-wong et al., 2009)
COX-2_Foward	5'-GGAGAGACTATCAAGATAGTGATC-3'	860
COX-2_Reverse	5'-GGAGAGACTATCAAGATAGTGATC-3'	(Sae-wong et al., 2009)
TNF- α _Foward	5'-ATGAGCACAGAAAGCATGATCCGC-3'	502
TNF- α _Reverse	5'-CTCAGGCCCGTCCAGATGAAACC-3'	(Chen et al., 2007)
β -actin_Foward	5'-TGTGATGGTGGGAATGGGTCAG-3'	514
β -actin_Reverse	5'-TTTGATGTCACGCACGATTTCC-3'	(Sae-wong et al., 2009)

4.2 Effect of SCCE on Cell Viability

To investigate the efficacy of SCCE, apigenin, and luteolin on anti-inflammation and cytoprotective properties, the cell viability of RAW 264.7 and HGF-1 cells affected by tested compounds was first assessed using MTT assay. The cells were not treated with tested agents basically counted as 100% of cell viability. As shown in Figure 1, all tested concentrations of SCCE, apigenin, and luteolin did not exert cytotoxic effects against HGF-1 cell which providing 97.57 - 117.28% cell viability. Additionally, SCCE at all tested dosages presented no toxicity towards RAW 264.7 cell with recorded cell viability of 104.99-110.65%. On the other hand, apigenin and luteolin at high concentrations of 15 and 20 $\mu\text{g/mL}$ suppressed RAW 264.7 cell viability to 19.90 - 51.27%. Additionally,

indomethacin (15 – 20 $\mu\text{g/mL}$) was used as a standard anti-inflammatory drug and was found to be non-toxic to RAW 264.7 cells, exhibiting 100.78 – 106.75% cell viability.

4.3 NO Scavenging Efficacy of SCCE

SCCE, apigenin, and luteolin were investigated for the capacity of NO radical scavenging by monitoring nitrite formation from the reaction of NO and oxygen. The NO scavenging activity of tested compounds is in Table 2. The results show that SCCE exhibited good NO quenching activity with IC_{50} of 189.20 $\mu\text{g/mL}$ (contains 17.26 $\mu\text{g/mL}$ of apigenin and 31.60 $\mu\text{g/mL}$ of luteolin). The pure active flavonoid compounds, apigenin and luteolin also exhibited strong NO scavenging ability comparable to standard agent quercetin.

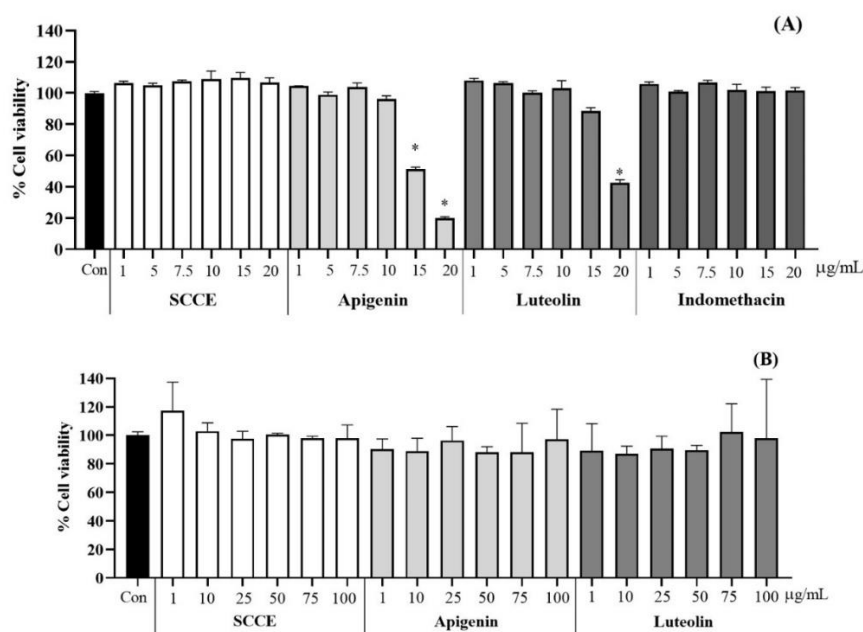


Figure 1 Effects of SCCE, apigenin, and luteolin on the viability of RAW 264.7 and HGF-1 cells. Cell viability of RAW 264.7 (A) and HGF-1 (B) cells was evaluated 24 h after treatment with the test compounds. * indicates cytotoxic concentrations that resulted in cell viability below 80%

Table 2 NO scavenging, anti-*S. mutans*, and tyrosinase inhibitory activities of SCCE, apigenin, and luteolin

Compound	NO scavenging activity (IC_{50} , $\mu\text{g/mL}$)	Anti- <i>S. mutans</i> (Clear zone, mm)	Tyrosinase inhibitory activity (IC_{50} , $\mu\text{g/mL}$)
SCCE	189.20	ND	> 1,000
Apigenin	70.50	ND	660.02
Luteolin	56.95	ND	117.37
Quercetin	91.97	-	-
Chlorhexidine	-	37.60 $\pm$ 1.85	-
Kojic acid	-	-	21.57

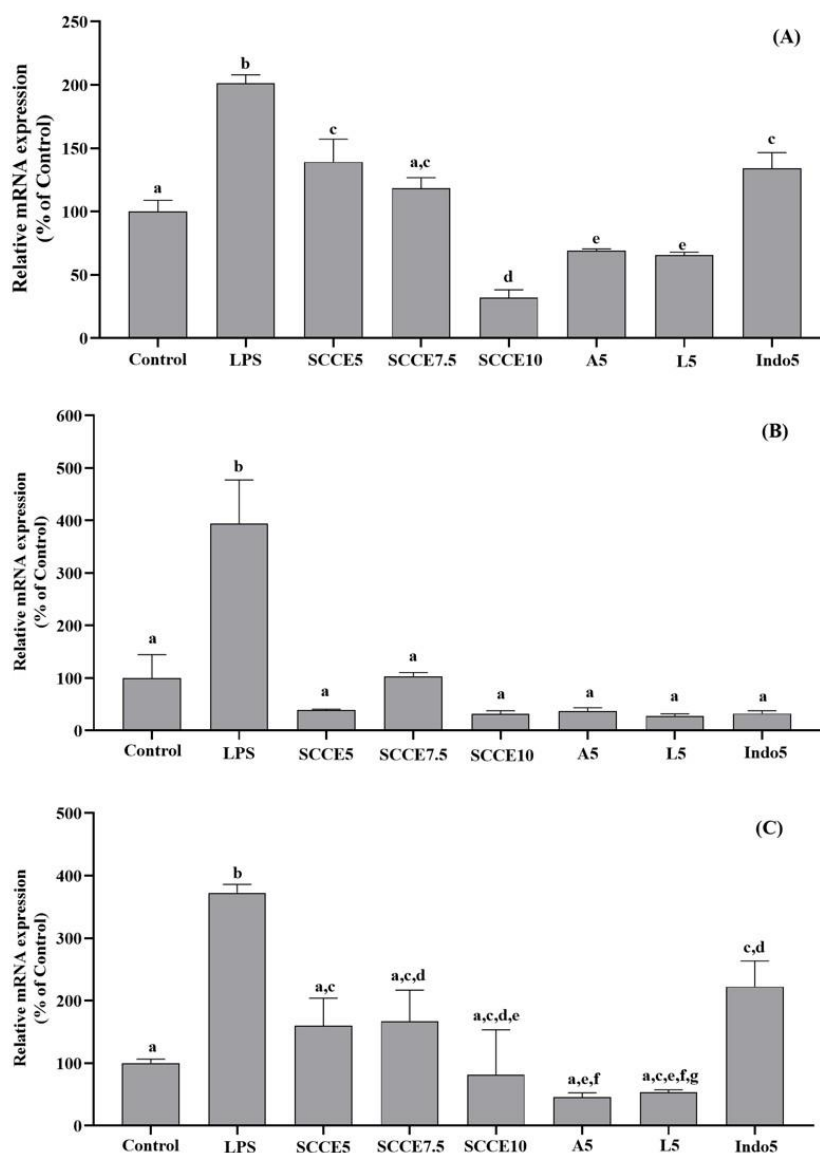


Figure 2 Relative mRNA expression levels of inflammatory related genes in RAW 264.7 cell after treated with SCCE for 24 h determined by real-time PCR. iNOS (A), COX-2 (B), TNF- α (C). Alphabets over the bar indicated statistic significant at $*p < 0.05$. The bars having same letters are not statistically significant. SCCE, Standardized *C. cinereum* extract; A, apigenin; L, luteolin; Indo, indomethacin

4.4 Effect of SCCE on Inflammation-related Gene Expression

To elucidate the molecular mechanism of SCCE, apigenin, and luteolin on anti-inflammation, the response at transcriptional level of iNOS, COX-2, and TNF- α was monitored using real-time PCR. The transcriptional responses were demonstrated in terms of relative mRNA expression as shown in Figure 2.

From the results, cells solely triggered with LPS obviously up-regulated inflammatory genes

including iNOS, COX-2, and TNF- α by approximately 210 – 394% when compared to control. As shown in Figure 2A, SCCE dose-dependently suppressed iNOS mRNA expression. Interestingly, at highest tested concentration, SCCE could down-regulate this gene comparable to pure active compounds apigenin and luteolin. Moreover, iNOS mRNA suppression activity of SCCE was superior to indomethacin about 4.19 and 1.96 times, respectively. For COX-2 gene expression (Figure 2B), SCCE significantly inhibited LPS-

induced COX-2 gene expression by approximately 3.83 – 12.31 folds, which was comparable to apigenin, luteolin, and indomethacin. In addition, although SCCE did not significantly suppress TNF- α gene expression, its suppression manner was similar to its action on iNOS and obviously superior to standard drug approximately 2.74 times (Figure 2C).

4.5 Cytoprotective Efficacy of SCCE on H₂O₂-Induced HGF-1 Cell Death

Since smoking can induce cellular oxidative stress, the cytoprotective efficacy of SCCE against H₂O₂-induced oxidative damage was evaluated in HGF-1 cells. As shown in Figure 3, H₂O₂ treatment markedly reduced cell viability to 40.43%, confirming the induction of oxidative damage. SCCE at concentrations of 25 – 75 μ g/mL significantly protected against oxidative stress-induced cell death, increasing cell viability to 57.29 – 73.66%. Similarly, the pure compounds apigenin and luteolin also exhibited cytoprotective effects against H₂O₂-induced cell damage. Apigenin demonstrated moderate

protective activity, with cell viability ranging from approximately 50.23% to 58.48% at concentrations of 10 – 50 μ g/mL, although its effect was less pronounced at higher concentrations. In contrast, luteolin showed superior cytoprotective efficacy, increasing cell viability to 59.76 – 84.89% at 25 – 75 μ g/mL. Interestingly, the cytoprotective effect of SCCE was comparable to that of luteolin at similar concentrations, suggesting that the combined phytoconstituents in SCCE may contribute to its overall protective activity.

4.6 Anti-*S. mutans* and Anti-tyrosinase Activities

To evaluate the protective properties of SCCE on dental caries and pigment accumulation that predominant in smoking, anti-*S. mutans* and anti-tyrosinase assays were conducted, respectively. The results are presented in Table 2 and show that all tested substances showed no activity against *S. mutans* when tested by disc diffusion method. Additionally, SCCE also showed mild efficacy on anti-tyrosinase activity.

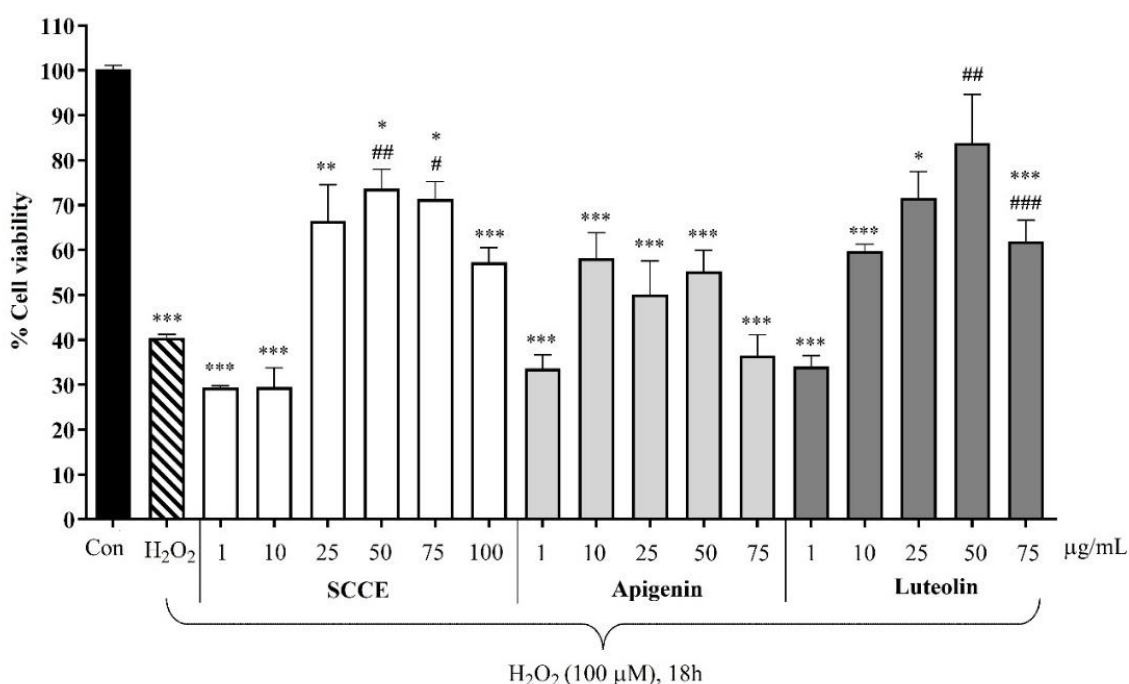


Figure 3 Cytoprotective effect of SCCE, apigenin, and luteolin on H₂O₂-induced HGF-1 cell death. The percentage of cell viability of cell was evaluated by MTT assay, after pre-treatment with SCCE, apigenin, and luteolin 24 h followed by exposed to H₂O₂ at 18 h. The data are presented as a mean $\pm$ SD (n=3). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control. # p < 0.05, ## p < 0.01, ### p < 0.001 compared to H₂O₂ treated group

5. Discussion

Cigarette smoking is a well-established risk factor contributing to oral inflammation, gingival hyperpigmentation, oxidative tissue damage, and smoking-related lesions such as smokers' stomatitis, ultimately leading to poor oral health outcomes (Bielecka-Kowalska et al., 2022; Gajendra et al. 2023). This study demonstrates that SCCE, rich in apigenin and luteolin, possesses promising biological activities that may support oral health protection in smokers.

SCCE exhibited multiple oral health-promoting effects, particularly through modulation of inflammation and oxidative stress, two key mechanisms involved in smoking-related oral pathologies. Notably, SCCE suppressed the expression of TNF- α , a key upstream pro-inflammatory cytokine that induces iNOS and COX-2, thereby reducing the production of nitric oxide, which are major mediators of inflammatory tissue damage. The yield of SCCE obtained using MAE combined with Diaion® HP-20 chromatography contained high levels of apigenin and luteolin, flavonoids known for their potent pharmacological activities and widely used as reliable chemical markers for extract standardization.

According to ISO 10993-5, compounds that provide cell viability above 80% were categorized as non-cytotoxic. Substances that provide cell viability within 80 - 60%, 60 - 40%, and lower than 40% were categorized weak, moderate, and strong cytotoxic compounds, respectively (International Organization for Standardization, 2009; López-García et al., 2014). The result of the cell viability study indicated that SCCE, apigenin, and luteolin were non-cytotoxic toward HGF-1. However, only SCCE was classified as non-cytotoxic substance toward HGF-1, whereas at high dosages of apigenin (15 - 20 $\mu\text{g/mL}$) and luteolin (20 $\mu\text{g/mL}$), these compounds demonstrated moderate to strong cytotoxic against RAW 264.7 cells.

SCCE exhibited a dose-dependent NO radical scavenging activity. According to previous data, apigenin and luteolin can directly scavenge NO radical (Crasci et al., 2018; Jin et al., 2009). The findings of the present study suggest that these flavonoids may contribute substantially to the NO scavenging activity of SCCE. According to Ebrahimzadeh et al. (2010), plant extracts with $\text{IC}_{50} < 200 \mu\text{g/mL}$ are considered to possess notable NO scavenging efficacy, supporting the biological relevance of the observed activity. The relatively lower activity of SCCE compared to the isolated flavonoids may be attributed to the complex composition of the extract, in which

active constituents are present at lower effective concentrations.

In addition, matrix effects commonly observed in phytocomplexes such as interactions affecting solubility, accessibility, or redox behavior may influence the overall activity, rather than direct antagonistic interactions (Ysrafil et al., 2023).

Real-time PCR analysis demonstrated that SCCE significantly suppressed the gene expression of pro-inflammatory markers, including iNOS, COX-2, and TNF- α , in LPS-stimulated RAW 264.7 cells. Inhibitory effects of SCCE were comparable with those obtained with either apigenin or luteolin alone at similar concentrations and greater than indomethacin at a similar concentration, suggesting that the combined effects of the constituent flavonoids in the SCCE extract may themselves produce additive or even synergistic enhancement of anti-inflammatory activity when compared with the actions of a single flavonoid. However, the present study did not explicitly investigate potential synergistic effects by customary methods such as combination index or isobolographic analyzes. Hence, while higher activity of SCCE in comparison to individual agents suggests the possibility of synergistic effects, definitive confirmation of such synergism is beyond the scope of this work. Confirmation of this effect would require further studies using combination design and quantitative synergy analysis. Modulation of central signaling pathways upstream of gene expression may explain the general down-regulation of inflammatory genes. TNF- α triggers iNOS and COX-2, leading to mediator production (Lee, 2014). To the best of our knowledge, this finding confirms that SCCE carries anti-inflammatory potential not only through inhibition of iNOS and COX-2 gene expression, which play important roles in the production of NO and prostaglandins, respectively, but also suppresses expression of TNF- α , an important pro-inflammatory mediator that acts as an iNOS and COX-2 stimulator. It should be noted that gene expression of pro-inflammatory mediators is involved in an important transcription factor- nuclear factor kappa-B (NF- κB) (Chen et al., 2007). Since apigenin and luteolin have been demonstrated to be NF- κB inhibitors (Funakoshi-Tago et al., 2011), the inhibitory effect of SCCE on iNOS, COX-2, and TNF- α gene expression possibly involves NF- κB inactivation. Notably, although indomethacin is primarily recognized as a cyclooxygenase inhibitor, previous studies have demonstrated that it can also suppress the expression of pro-inflammatory genes, including iNOS, COX-2,

and TNF- α , in LPS-stimulated macrophages, possibly through inhibition of NF- κ B signaling pathways. Therefore, its inclusion in this study serves as a reference anti-inflammatory drug at both enzymatic and transcriptional levels (Bradbury et al., 2001; Gupta et al., 2010). However, additional investigations are crucial to clarify the transcriptional response mechanisms underlying the downregulation of inflammation-related gene expression by SCCE.

In addition to its anti-inflammatory actions, SCCE also demonstrated cytoprotective effects against oxidative stress in HGF-1 cells exposed to H₂O₂. Luteolin exhibited superior efficacy, likely due to its known ability through suppression of the nuclear translocation of NF- κ B (Chen et al., 2020) and reduction of intracellular reactive oxygen species (ROS) (Xu et al., 2019). Although the antioxidative property of apigenin are well-documented (Kashyap et al., 2022), it showed less pronounced protection, possibly owing to its cell-specific response profile. These findings position luteolin as a primary contributor to SCCE's cytoprotective activity.

Despite these promising effects, SCCE exhibited no antibacterial activity against *S. mutans* at 1 mg/disc and failed to significantly inhibit tyrosinase at tested concentrations. This suggests that the extract's oral health benefits are more likely derived from anti-inflammatory and antioxidant actions rather than direct antimicrobial or anti-pigmentation effects.

6. Conclusion

In the present study, SCCE demonstrated several potential applications for smoking-induced oral diseases, attributable to the high concentrations of apigenin and luteolin in the extract, including strong anti-inflammatory activity through nitric oxide scavenging and inhibition of iNOS, COX-2, and TNF- α gene expression in activated macrophages. In addition, SCCE protected human gingival fibroblast cells from oxidative stress-induced damage, which may indicate that it has the potential to reduce the effects of smoking on gingival tissue of the oral cavity. The antioxidative properties may be due to the flavonoids apigenin and luteolin, which act by scavenging reactive oxygen species, modulating the NF- κ B signaling pathway, and inhibiting pro-inflammatory mediators. SCCE's activity may also be due to synergistic effects of the bioactive compounds. SCCE showed no antibacterial effects against *S. mutans*, and only weak tyrosinase inhibition occurred at the tested concentration. Taken together, these results suggest the potential use of SCCE as a natural

ingredient in oral healthcare products, particularly topical oral formulations (e.g., mouthwash, gels) where SCCE's effects on oral tissues may best support its anti-inflammatory and antioxidant properties in smokers. Future research should focus on the translational development, bioavailability, and formulation of SCCE for oral use.

7. Acknowledgements

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8. Conflicts of interest

The authors declare no conflict of interest.

9. Abbreviations

Abbreviation	Full Term
C	Celsius
COX-2	Cyclooxygenase-2
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
H ₂ O ₂	Hydrogen peroxide
HGF-1	Human gingival fibroblast
HPLC	High performance liquid chromatography
IC ₅₀	Half Maximal Inhibitory Concentration
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAE	Microwave-assisted extraction
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor kappa B
NO	Nitric oxide
OD	Optical density
PCR	Polymerase chain reaction
Sec	Second
SD	Standard deviation
TNF- α	Tumor necrosis factor- α
SCCE	Standardized <i>Cyanthillium cinereum</i> extract

10. CRediT Statement

Suriya Chaiwong: Conceptualization; Methodology; Formal analysis; Investigation; Writing - Original Draft; Writing - Review & Editing; Funding acquisition.

Panupong Puttarak: Conceptualization; Methodology; Investigation; Formal analysis; Writing - Original Draft; Funding acquisition.

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Sutthinee Wisutthathum: Methodology; Investigation; Formal analysis; Writing - Original Draft.

Watchara Damjuti: Resources; Investigation; Formal analysis; Writing - Original Draft; Writing - Review & Editing.

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