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Original Article

Evaluating thymol vapor for biofilm removal and biocompatibility in curved root canal models *in vitro*

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Thymol vapor;
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Abstract *Background/purpose:* Conventional irrigants such as chlorhexidine and sodium hypochlorite have strong antimicrobial properties but high cytotoxicity, limiting their use in regenerative endodontics. We hypothesized that thymol vapor could provide effective antibacterial activity with lower cytotoxicity.
Materials and methods: The antimicrobial activity of thymol in both liquid and vapor phases was tested against *Enterococcus faecalis*, *Streptococcus mutans*, and *Aggregatibacter actinomycetemcomitans* using a resin block model simulating curved root canals. The effect of thymol vapor, alone or with mechanical instrumentation, was tested on early-stage biofilms removal. Cytotoxicity was assessed using MTT assays in L-929 fibroblasts and MG-63

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osteoblast-like cells, and pro-inflammatory cytokine gene expression (*IL-1β*, *TNF-α*, *IL-6*) was measured via qRT-PCR.
Results: Thymol exhibited minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.8—1.0 mg/mL in planktonic cultures. In early-stage biofilms models, 10—100 mg/mL liquid thymol and 5.0 mg/mL thymol vapor significantly reduced bacterial viability. Combining 1.0 mg/mL thymol vapor with mechanical instrumentation enhanced early-stage biofilms removal, particularly against *E. faecalis*. Direct exposure to thymol and chlorhexidine caused significant cytotoxicity, while 1.0 mg/mL vapor showed lower cytotoxic and did not significantly induce pro-inflammatory cytokine genes in L-929 cells. At higher concentrations, MG-63 cells exhibited increased cytokine expression.
Conclusion: This study is the first to propose thymol vapor for biofilm removal in curved root canal models and to demonstrate its antibacterial activity with lower cytotoxicity than conventional irrigants. Its potential as an adjunct in regenerative endodontics merits further investigation, particularly in relation to immunomodulatory effects.

Introduction

Effective root canal treatment relies heavily on the thorough elimination of bacteria from the root canal system. However, the presence of biofilms—encased in a protective matrix of extracellular polymeric substances (EPS)—poses a significant challenge to complete disinfection. Common bacterial species isolated from infected root canals include *Streptococcus*, *Porphyromonas*, and *Enterococcus faecalis*, with *E. faecalis* being most frequently associated with persistent infections and failed root canal treatments.[1]

These biofilms protect bacteria from conventional treatment modalities, including mechanical instrumentation and chemical irrigation. Although mechanical instrumentation can reduce the overall bacterial load, it is insufficient for completely eliminating bacteria from anatomically complex regions of the root canal system, such as lateral and accessory canals.[2] As a result, chemical irrigation is essential to complement mechanical preparation and enhance disinfection efficacy.

Sodium hypochlorite (2.25 %) and chlorhexidine (2 %) are the most commonly used chemical irrigants in root canal treatment. Both agents demonstrate broad-spectrum antimicrobial efficacy, with higher concentrations yielding stronger bactericidal effects.[3] The antimicrobial mechanism of sodium hypochlorite primarily involves the disruption of the phospholipid structure of bacterial cell membranes, the formation of chloramines that interfere with cellular metabolism, the oxidative inactivation of bacterial enzymes, and the degradation of lipids and fatty acids.[4] However, its clinical application is limited by well-documented drawbacks, including high cytotoxicity,[5] potential damage to dentin, and the formation of a smear layer that can compromise sealing ability.[6]

Chlorhexidine (CHX), another widely used disinfectant, is also effective against a broad range of microorganisms. Its antimicrobial mechanism involves positively charged CHX molecules binding to negatively charged phosphate-containing components of the bacterial cell wall, leading to disruption of the cell wall, cytoplasmic leakage, and inhibition of enzymatic activity. Furthermore, CHX can form complexes with adenosine triphosphate (ATP) and nucleic acids, resulting in cytoplasmic coagulation and precipitation, ultimately causing bacterial cell death.[7] Nevertheless, CHX has several limitations, including the risk of microbial resistance,[8] tooth staining,[7] and cytotoxic effects on host tissues.[9]

In cases of apical resorption—particularly in immature teeth with an open apex and thin dentinal walls—mechanical instrumentation should be minimized or avoided to reduce the risk of root fracture.[10] In such scenarios, effective root canal disinfection depends primarily on the antimicrobial properties of irrigating solutions. However, commonly used irrigants not only exhibit significant cytotoxicity but also adversely affect the viability and function of stem cells residing in the apical region.[10]

Thymol (THY), a natural monoterpenoid phenol derived from *Thymus vulgaris* (thyme), exhibits a broad range of pharmacological activities, including antibacterial, antifungal, antitumor, and anti-inflammatory effects.[11] Its antimicrobial efficacy is primarily attributed to its ability to disrupt the cytoplasmic membrane, leading to increased membrane permeability and depolarization, which in turn causes cytoplasmic leakage.[12] This membrane disruption is also associated with elevated production of reactive oxygen species (ROS).[13] Thymol has demonstrated antimicrobial activity against a variety of oral pathogens, including *Streptococcus mutans*, *Enterococcus faecalis*, *Aggregatibacter actinomycetemcomitans*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli*.[13,14] Notably, thymol vapor has also been applied in the preservation of fruits, vegetables,[15] and seafood,[16] supporting its combined antimicrobial efficacy and safety.

Given these properties, the present study aims to evaluate the potential application of thymol vapor in endodontic treatment. We propose that its antimicrobial action offers an alternative to conventional irrigants, overcoming limitations of liquid-based disinfection in complex

anatomies. Moreover, thymol vapor is expected to exhibit minimal cytotoxicity, supporting its potential as a safe adjunct in endodontic treatment.

Materials and methods

Microorganisms culture

Enterococcus faecalis (BCRC 10789), *Streptococcus mutans* (ATCC 25175), and *Aggregatibacter actinomycetemcomitans* (ATCC 33384) were used in this study. *E. faecalis* and *S. mutans* were cultured in tryptic soy broth (TSB), whereas *A. actinomycetemcomitans* was cultured in brain heart infusion (BHI) broth. All bacterial strains were incubated at 37 °C for 24 h under constant agitation at 200 rpm. Detailed culture procedures and conditions were performed as previously described.[17]

Single curved root canal resin block preparation and sterilization

Resin blocks were shaped using HERO Shaper rotary files (MICRO-MEGA, Besançon, France) at 400 rpm and sterilized with 5 % sodium hypochlorite, followed by two cycles of sonication to ensure thorough cleaning. The preparation of resin block canals involved measuring with #10 NiTi file (MICRO-MEGA, Besancon, France) and shaping them with sterile HERO Shaper rotary files (MICRO-MEGA) at 400 rpm, utilizing a TEONIKA Dentsply endomotor (Dentsply Sirona, NC, USA).

Thymol and chlorhexidine preparation

Thymol (Sigma—Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1000 mg/mL stock solution. DMSO served as the vehicle control. Chlorhexidine (CHX) (200 mg/mL; Sigma—Aldrich) was used as a positive control and diluted to working concentrations of 2 mg/mL and 20 mg/mL for experimental use.

Determination of thymol minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the medicaments were determined using the broth dilution method.[18] Bacterial suspensions 1×10^6 colony-forming units (1×10^6 CFUs) were inoculated into wells and treated with varying concentrations of thymol for 24 h. Optical density at 600 nm (O.D. 600 nm) was measured using a Varioskan Lux spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). CHX at 2 mg/mL was used as a positive control. MBC values were determined by spot-plating the treated samples onto agar plates, followed by incubation at 37 °C for an additional 24 h.

Direct treatment of thymol and CHX in single curved root canal resin block with early-stage biofilms

Bacterial suspensions (1.5×10^4 CFUs) were inoculated into resin blocks and incubated for 48 h to allow early-stage biofilms formation. For the direct treatment, thymol solutions at concentrations of 1, 10, and 100 mg/mL, and CHX at 2 and 20 mg/mL, were used to fill the root canals of the resin blocks. Following a 24-h incubation period, tenfold serial dilutions of the bacterial suspensions were prepared, and CFUs were counted to assess bacterial reduction.

Vapor treatment of thymol and CHX in single curved root canal resin block with early-stage biofilms

Early-stage biofilms in the resin blocks were prepared using the same method as in the direct treatment model. Thymol solutions at concentrations of 1, 5, and 10 mg/mL (corresponding to 0.015 mg, 0.075 mg, and 0.15 mg, respectively), along with a 2 mg/mL CHX solution (0.03 mg), were applied to cotton pellets and placed inside the resin blocks. The blocks were immediately inverted to prevent the solutions from flowing into the canals. After a 24-h incubation period, the bacterial suspensions were sonicated, and CFUs were quantified.

Mechanical instrumentation with thymol vapor treatment in single curved root canal resin block with early-stage biofilms

Early-stage biofilms in the resin blocks were prepared using the same procedure as in the direct treatment model. After the application of thymol or CHX for 1 min, a one-minute circumferential filing was performed using two HERO Shaper files. The remaining solution was aspirated with a pre-curved 27-gauge needle syringe. Subsequently, thymol or CHX solutions were applied to a cotton pellet, which was placed into the resin block. The blocks were immediately inverted to prevent the solution from flowing into the canals. After a 24-h incubation, the bacterial suspension was sonicated, and CFUs were quantified.

Cell culture

Mouse fibroblast (L-929) and human osteogenic sarcoma (MG-63) cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), 1 % antibiotic-antimycotic solution (penicillin/streptomycin/amphotericin, 100X; Gibco, Carlsbad, CA, USA), and 1 % L-glutamine (Gibco BRL).

Cytotoxicity test: direct and vapor treatments

L-929 and MG-63 cells (5×10^3 cells per well) were seeded into 96-well cell culture plates and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 24 h. The cells were then treated with vehicle control or various concentrations of thymol (1—10 mg/mL) and CHX (2 mg/

mL) in culture media containing 10 % FBS for 1, 2, 4 min, and 24 h. For the vapor-phase cytotoxicity assay, thymol vapor or air—liquid interface CHX group was applied to filter paper placed at the center of the plate lid, and cells were exposed to the vapor for 24 h at 37 °C. At the end of each treatment period, cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[19]

Pro-inflammatory cytokine gene expression

The effects of vapor-phase thymol on pro-inflammatory cytokine gene expression in L-929 and MG-63 cells were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells (1.0×10^5) were seeded into 6-well tissue culture plates and incubated at 37 °C in 5 % CO_2 for 24 h. They were then treated with vehicle control, thymol vapor at 1 and 10 mg/mL (equivalent to 0.080 mg/well and 0.80 mg/well, respectively), or air—liquid interface CHX at 2 mg/mL (equivalent to 0.16 mg/well) in 10 % FBS-containing medium for an additional 24 h. Following treatment, cells were harvested for RT-qPCR analysis. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and reverse transcribed using Oligo$(dT)_{20}$ primers. The resulting cDNA was used as the template for PCR. To analyze *interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6)* expression, quantitative PCR (qPCR) was performed by a StepOnePlus Real-Time PCR System and PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA). The gene expression levels of pro-inflammatory cytokines were normalized to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. Primer sequences for each gene are listed in Table 1. All experiments were performed in triplicate, and data were analyzed according to previously described protocols.[20]

Statistical analysis

Each experimental condition was independently repeated in at least three separate experiments. Data are expressed as the mean ± standard deviation (SD). Bacterial growth, early-stage biofilms removal efficacy, cell viability, and pro-inflammatory cytokine gene expression were analyzed using unpaired t-tests and Ordinary one-way ANOVA and Dunnett's test with Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Antibacterial activity of thymol

E. faecalis, *S. mutans*, and *A. actinomycetemcomitans* were significantly eradicated after 24-h treatment with thymol at concentrations of 1.0, 0.9, and 0.8 mg/mL, respectively, which correspond to the MIC for each bacterium (Fig. 1A—C). To confirm the MBC, aliquots from each MIC treatment were spot-plated on agar and incubated overnight. No bacterial growth was observed at the MIC levels for all three species (Fig. 1D—F), indicating that the MBC is equal to the MIC. For CHX, both the MIC and MBC were below 2 mg/mL for all three bacteria. The slightly higher turbidity observed in CHX-treated samples was attributed to increased background absorbance caused by the drug's dissolution.

Early-stage biofilms removal ability of direct thymol treatment in the single curved root canal resin block model

Once planktonic bacteria form biofilms, their resistance to antimicrobial agents increases significantly. Therefore, the MBC of thymol was increased by 10-fold and 100-fold to evaluate its effectiveness in early-stage biofilms removal. In the resin block model, the early-stage biofilms bacterial counts of *E. faecalis*, *S. mutans*, and *A. actinomycetemcomitans* ranged from 10^6 to 10^7 CFU/mL. Direct treatment with 100 mg/mL thymol significantly reduced *E. faecalis* compared to the untreated group (Fig. 2A), while *S. mutans* was significantly reduced at 1 mg/mL thymol (Fig. 2B), and *A. actinomycetemcomitans* was significantly reduced at 10 mg/mL thymol, reaching bacterial counts of 10^4—10^5 CFU/mL. Additionally, direct treatment with 2 mg/mL CHX (CHX) significantly reduced *E. faecalis* (10^1—10^2 CFU/mL) and *S. mutans* compared to untreated groups. However, the *A. actinomycetemcomitans* group treated with CHX still showed numerous colonies, ranging from 10^4 to 10^5 CFU/mL (Fig. 2C).

Table 1 List of pro-inflammatory cytokine genes and primer sequences.

Gene (species)	Forward primer	Reverse primer
GAPDH (mouse)	*TATGTCGTGGAGTCTACTGGT*	*GAGTTGTCATATTTCTCGTGG*
IL-1β (mouse)	*TGGACCTTCCAGGATGAGGACA*	*GTTCATCTCGGAGCCTGTAGTG*
TNF-α (mouse)	*GGTGCCTATGTCTCAGCCTCTT*	*GCCATAGAACTGATGAGAGGGAG*
IL-6 (mouse)	*TGTACTCCAGGTAGCTATGG*	*GTTCTCTGGGAAATCGTGGA*
GAPDH (human)	*TGGTATCGTGGAAGGACTCATGAC*	*ATGCCAGTGAGCTTCCCGTTCAGC*
IL-1β (human)	*CCACAGACCTTCCAGGAGAATG*	*GTGCAGTTCAGTGATCGTACAGG*
TNF-α (human)	*CTCTTCTGCCTGCTGCACTTTG*	*ATGGGCTACAGGCTTGTCACTC*
IL-6 (human)	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCACGTTG

Abbreviations: *GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1β, interleukin-1β; (TNF-α), tumor necrosis factor-α; IL-6, interleukin-6.*

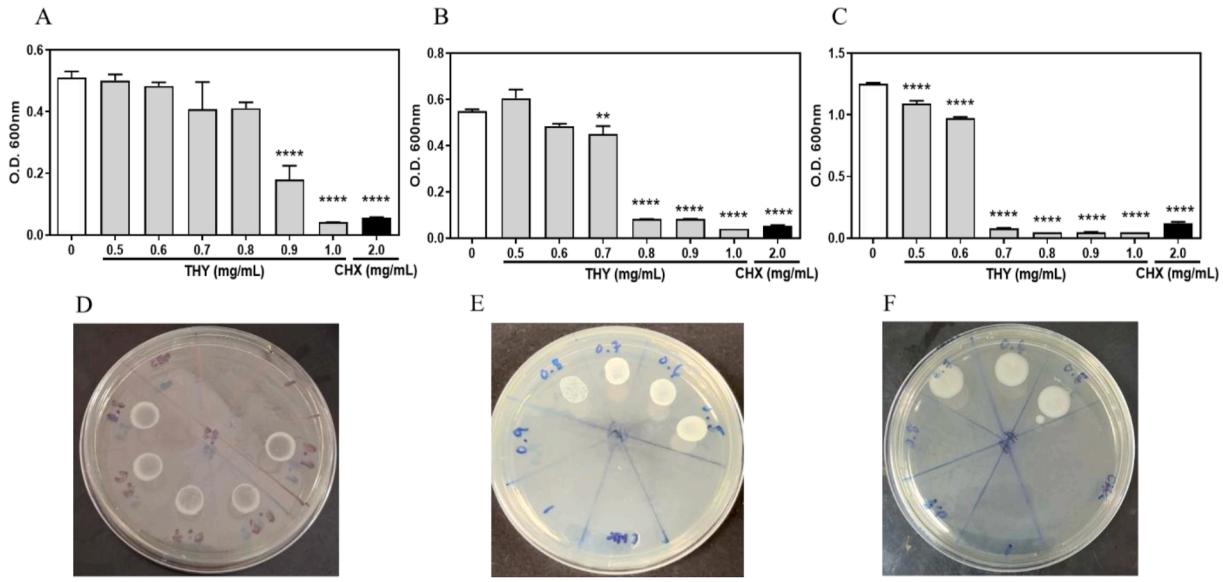

Figure 1 Antibacterial activity of thymol. MIC and MBC of thymol and CHX against oral pathogens. O.D. 600 nm measurements of *E. faecalis* (A), *S. mutans* (B), and *A. actinomycetemcomitans* (C) were recorded after treatment with various concentrations of thymol and 2 mg/mL CHX. Colony formation assays confirmed complete eradication of *E. faecalis* (D), *S. mutans* (E), and *A. actinomycetemcomitans* (F) at 1.0, 0.9, and 0.8 mg/mL thymol, respectively. Histograms represent mean ± standard deviation (SD). The thymol and CHX treatment groups were compared with the untreated control (0 group). **($P < 0.01$), and **** ($P < 0.0001$), one-way ANOVA. Abbreviations: O.D., optical density; THY, thymol; CHX, chlorhexidine; MI, mechanical instrumentation.

Early-stage biofilms removal ability of mechanical instrumentation and thymol vapor combination treatment in the single curved root canal resin block model.

Mechanical instrumentation (MI) alone slight reduced the bacterial load of *E. faecalis* (10^5–10^6 CFU/mL) and *S. mutans* (10^5–10^6 CFU/mL), but did not *A. actinomycetemcomitans* compared to the untreated groups (10^6–10^7 CFU/mL). Thymol vapor at 1 mg/mL effectively reduced *E. faecalis* (10^3–10^4 CFU/mL), while 5 mg/mL thymol vapor was effective against *S. mutans* (10^4 CFU/mL) and *A. actinomycetemcomitans* (10^4–10^5 CFU/mL). The combination of MI with thymol vapor further decreased bacterial loads. However, *A. actinomycetemcomitans* was less sensitive to this combined treatment compared to *E. faecalis* and *S. mutans* (Fig. 2D–F). The air–liquid interface CHX group significantly reduced all three bacteria ($P < 0.0001$) (Fig. 2D–F), although small colonies were still observed for *E. faecalis* and *S. mutans*, indicating that early-stage biofilms in the resin block model exhibited a significant bactericidal response after only one minute of exposure to 2 mg/mL CHX. It was shown that the early-stage biofilms of *A. actinomycetemcomitans* and *E. faecalis*, the resistance to thymol increased by about 100-fold compared to planktonic condition MIC/MBC (Fig. 2A and C).

Thymol vapor treatments reduced the cytotoxicity of direct thymol treatment

Cell viability of both L-929 and MG-63 significantly decreased following direct treatment with 2 mg/mL CHX and thymol at concentrations of 1, 5, and 10 mg/mL for 1, 2, 4 min, and 24 h (Fig. 3A and B). In contrast, vapor treatment with 1 mg/mL thymol or CHX caused only a slight, and non-significant reduction in cell viability compared to the untreated control in L-929, and MG-63, respectively. However, vapor treatments with 5 and 10 mg/mL thymol induced significant cytotoxicity in both cell lines (Fig. 3C and D).

Thymol vapor induced pro-inflammatory cytokine gene expression in L-929 cells and MG-63 cells

After exposure to thymol vapor at 1 and 10 mg/mL for 24 h, L-929 cells showed a decreasing trend in *IL-1β* expression (Fig. 4A), while *TNF-α* and *IL-6* expression showed an increasing trend (Fig. 4B and C). In the group treated with 2 mg/mL CHX at the air–liquid interface for 24 h, *IL-1β* expression showed a decreasing trend (Fig. 4A), *TNF-α* expression remained unchanged (Fig. 4B), and *IL-6* expression showed an increasing trend (Fig. 4C). In MG-63 cells treated under the same conditions, thymol increased the expression of *IL-1β*, *TNF-α*, and *IL-6*, whereas CHX increased *IL-1β* and *IL-6* expression but had no effect on *TNF-α* expression (Fig. 4D–F). These results indicate that both thymol vapor and air–interphase CHX treatments upregulate *TNF-α* and *IL-6* expression in L-929 and MG-63 cells, but their regulation of *IL-1β* expression differs.

Discussion

Root canal infections may arise from reinfection, periodontitis, or deep carious lesions that invade the pulp. Successful treatment requires both mechanical and

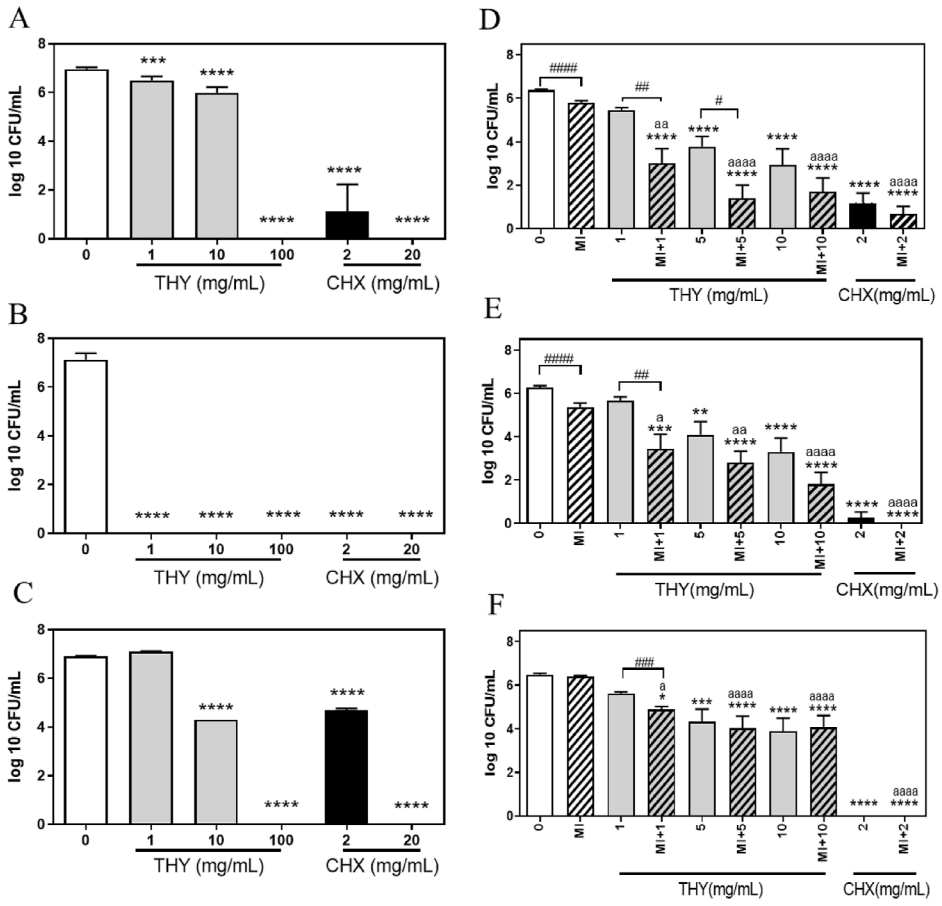

Figure 2 Early-stage biofilms removal ability of direct thymol treatment in the resin block model. A 48-h bacterial early-stage biofilms model in resin canals was used to assess the antibacterial effects of thymol and CHX through direct contact, vapor treatment, and combination with mechanical instrumentation. Log reductions in CFUs/mL of *E. faecalis* (A), *S. mutans* (B), and *A. actinomycetemcomitans* (C) were determined after direct contact treatment. Log reductions in CFUs/mL of *E. faecalis* (D), *S. mutans* (E), and *A. actinomycetemcomitans* (F) were assessed after vapor-only treatment and vapor combined with mechanical instrumentation. Histograms represent the mean ± standard deviation (SD). The thymol and CHX treatment groups were compared with the untreated control (0 group). * ($P < 0.05$), **($P < 0.01$), *** ($P < 0.001$), and **** ($P < 0.0001$), The MI and vapor-thymol cotreatment groups were compare with MI only treatment. a ($P < 0.05$), aa ($P < 0.01$), and aaaa ($P < 0.0001$). One-way ANOVA. The MI and vapor-thymol cotreatment groups were compare with each vapor-thymol dose treatment. # ($P < 0.05$), ## ($P < 0.01$), ### ($P < 0.001$) and #### ($P < 0.0001$). Unpair t-test. Abbreviations: THY, thymol; CHX, chlorhexidine; MI, mechanical instrumentation.

chemical debridement. However, complex anatomical structures—such as lateral and accessory canals—pose challenges for complete bacterial eradication, necessitating the use of chemical disinfectants.[2] Relying solely on mechanical instrumentation can lead to dentin debris accumulation, which may obstruct the apical constriction and accessory canals, thereby compromising disinfection efficacy.[21] Additionally, commonly used chemical irrigants are often cytotoxic to human cells.[22] Therefore, this study explores the vapor-phase application of thymol, evaluating its antibacterial efficacy and cytotoxicity on L-929 fibroblasts and MG-63 osteoblast-like cells.

CHX is a widely used dental disinfectant, commonly applied at 0.2 % for periodontal therapy and 2 % for root canal treatments. Previous studies have shown that 0.2 % CHX can eliminate *E. faecalis* within 30 s, with the liquid formulation demonstrating superior antibacterial efficacy compared to the gel form, likely due to enhanced bacterial contact and diffusion.[23] Moreover, applying 0.2 % CHX to dentin surfaces for 15 min does not significantly alter dentin roughness or microhardness, in contrast to higher concentrations of sodium hypochlorite and EDTA.[24] In this study, 0.2 % CHX (equivalent to 2 mg/mL) was employed as a positive control to evaluate and compare the antibacterial efficacy and cytotoxicity of thymol. All three tested bacterial species exhibited significantly reduced viability following 2 mg/mL CHX treatment, supporting its clinical use in managing periodontitis and persistent endodontic infections.[25] However, complete bacterial eradication was not achieved, as residual colonies of *A. actinomycetemcomitans* and *E. faecalis* remained after 24 h of thymol and CHX treatment. Nonetheless, even brief exposure—one minute—to 2 mg/mL CHX resulted in marked cytotoxicity in both L-929 fibroblasts and MG-63

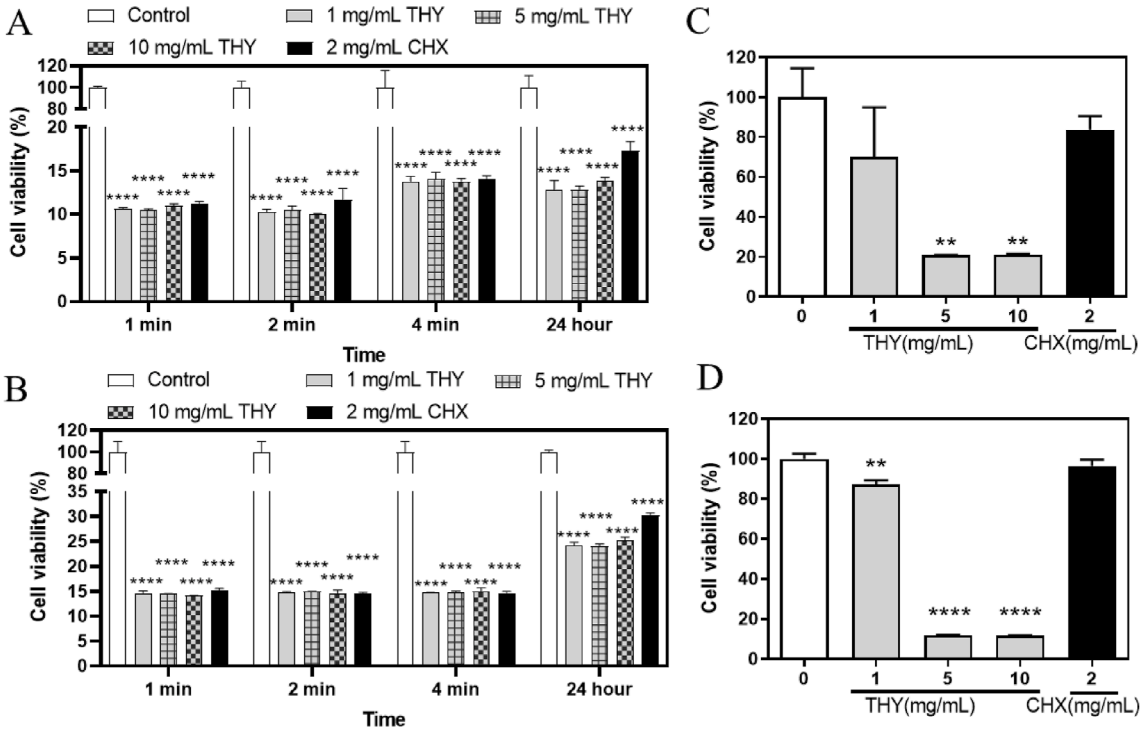

Figure 3 Thymol vapor treatments reduced the cytotoxicity of direct thymol treatment. Cytotoxic effects were evaluated using the MTT assay. Cell viability of L-929 fibroblasts (A) and MG-63 osteoblast-like cells (B) was measured after direct treatment with thymol and CHX for 1, 2, 4 min, and 24 h. Cell viability of L-929 (C) and MG-63 (D) cells was also assessed after 24-h vapor exposure to thymol and CHX. Data are presented as mean ± standard deviation (SD). The thymol and CHX treatment groups were compared with the untreated control (0 group). **($P < 0.01$), and **** ($P < 0.0001$), one-way ANOVA. Abbreviations: THY, thymol; CHX, chlorhexidine.

osteoblast-like cells (Fig. 3A and B), raising concerns about its biocompatibility.

Thymol vapor, known for its high volatility, exhibits a time-dependent decline in antimicrobial activity, with significant reduction observed after 96 h. Previous studies have reported that the antibacterial activity of thymol decreases at temperatures above 80 °C, and that vapor-phase thymol is inhibited by the presence of water.[13] Importantly, the cytotoxicity of thymol also decreases as its antimicrobial activity wanes,[26] suggesting that thymol vapor may serve as a biocompatible adjunct in endodontic treatment. In this study, vapor-phase thymol at 5 mg/mL significantly reduced the viability of *E. faecalis, S. mutans* and *A. actinomycetemcomitans*. Although 1 mg/mL thymol vapor showed a reduction in bacterial load, the effect was not statistically significant compared to the untreated control (Fig. 2D–F). These results are consistent with previous findings demonstrating the mild antibacterial efficacy of thymol vapor against both Gram-positive and Gram-negative bacteria.[13] A dissertation from Rutgers University reported that vapor-phase thymol showed stronger antibacterial activity against *E. coli* DH5α than liquid or solid forms, with much lower effective concentrations. The findings indicate that the headspace concentration, despite being low, is the main contributor to antimicrobial activity, and that controlled release can achieve comparable inhibition with reduced dosage, suggesting sustained vapor availability may underlie its efficacy at the biofilm interface.

Mechanical instrumentation remains a fundamental protocol in endodontic therapy, facilitating bacterial reduction and enhancing the efficacy of chemical irrigants during root canal debridement.[21] Prior studies have demonstrated that mechanical preparation significantly decreases bacterial load, even more effectively than sodium hypochlorite irrigation alone.[2] Our findings are consistent with these reports, showing that mechanical instrumentation enhances the antibacterial effects of both CHX and thymol vapor, even at low concentrations (Fig. 2D–F). Although mechanical instrumentation alone did not significantly reduce bacterial counts compared to the untreated group, its combination with 1 mg/mL thymol vapor resulted in a significantly greater reduction in *E. faecalis, S. mutans* and *A. actinomycetemcomitans* (Fig. 2D–F). This enhanced efficacy is likely attributed to the sustained antimicrobial activity of thymol vapor.[13,27]

To minimize the risk of periapical tissue damage, root canal obturation is typically recommended to be 1 mm short of the working length.[28] However, the presence of bacteria within lateral and accessory canals complicates complete disinfection. While disinfecting up to the apical foramen may reduce the likelihood of reinfection, it carries a risk of over-instrumentation and extrusion of irrigants. Despite such precautions, cytotoxicity remains a significant concern.[22] In the present study, both L-929 fibroblasts and MG-63 osteosarcoma cells exhibited significant cytotoxic responses following direct exposure to thymol and CHX at various time intervals (1, 2, 4 min, and 24 h) (Fig. 3A and B).

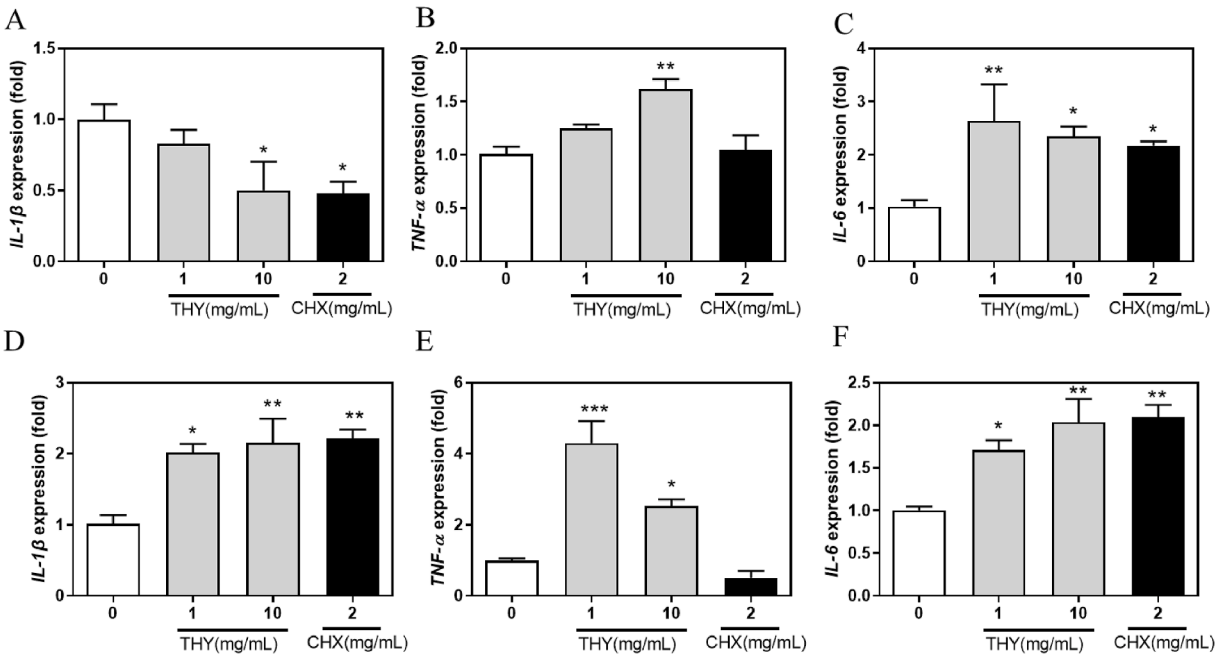

Figure 4 Thymol vapor induced pro-inflammatory cytokine gene expression in L-929 Cells and MG-63 cells. Relative mRNA expression levels of pro-inflammatory cytokines were assessed by RT-qPCR following 24-h vapor treatment with varying concentrations of thymol and CHX. Expression of *IL-1β* (A), *TNF-α* (B), and *IL-6* (C) in L-929 fibroblast cells; and *IL-1β* (D), *TNF-α* (E), and *IL-6* (F) in MG-63 osteoblast-like cells. Data are presented as mean ± standard deviation (SD). The thymol vapor and CHX treatment groups were compared with the untreated control (0 group). *($P < 0.05$), and ** ($P < 0.01$), one-way ANOVA. Abbreviations: THY, thymol; CHX, chlorhexidine; *GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1β, interleukin-1β; (TNF-α), tumor necrosis factor-α; IL-6, interleukin-6.*

These findings underscore the cytotoxic potential of many endodontic medicaments, particularly phenolic compounds.[26] The cytotoxicity appears to be dose- and time-dependent, emphasizing the importance of preventing medicament extrusion beyond the apical constriction to avoid impairing cellular viability and compromising the apical seal during obturation.[5,26] Thymol has been shown to induce reactive oxygen species (ROS) generation and trigger cell death pathways, potentially involving mitochondrial-mediated apoptosis in both L-929,[29] and MG-63 cells.[30] Thymol vapor at 1 mg/mL exhibited mild cytotoxicity toward both L-929 and MG-63 cells, whereas higher concentrations (5—10 mg/mL) showed significant cytotoxic effects (Fig. 3C and D). In this study, the combination of MI with 1 mg/mL thymol vapor provided effective bacterial early-stage biofilms removal in the root canal while minimizing cytotoxicity, suggesting its potential as a safer approach for endodontic treatment.

Inflammation plays a dual role in tissue dynamics—while a balanced inflammatory response is critical for tissue repair, dysregulated or excessive inflammation can lead to tissue damage and impaired healing. Among these mediators, IL-1β, IL-6, and TNF-α are well-established regulators of immune responses and inflammatory tissue remodeling. Pro-inflammatory cytokines are central to initiating immune responses and recruiting mesenchymal stromal cells (MSCs) to injury sites, which are essential for tissue regeneration.[31] These MSCs—originating from sources such as bone marrow, dental pulp, or gingival fibroblasts—secrete growth factors and soluble mediators that facilitate cell proliferation, migration, and differentiation, thereby promoting tissue repair.[31] However, these pro-inflammatory cytokines decrease the differentiation ability of dental pulp stem cells (DPSCs) in vitro.[32] In this study, thymol vapor induced a dose-dependent increase in *IL-1β* expression in MG-63 cells, whereas L-929 cells exhibit significant decrease in *IL-1β* levels following similar treatment (Fig. 4A and D). This suggests a cell-type-specific inflammatory response to thymol vapor. In MG-63 cells, thymol vapor-induced upregulation of pro-inflammatory cytokine gene expression may inhibit osteogenic differentiation,[33] while in L-929 cells, it could potentially delay wound healing.[34] However, in vivo, interactions among different cell types may modulate these effects. Therefore, more rigorous studies are needed to verify the impact of vapor-phase thymol on periapical tissue responses. Interestingly, the air—liquid interface CHX group also showed altered expression of pro-inflammatory cytokines, despite CHX being less volatile than thymol. Whether trace amounts of CHX may have leached into the cell culture medium or other factors contributed to the observed effects remains unclear and warrants further investigation.

This study demonstrates, for the first time, the potential of thymol vapor in root canal disinfection, showing effective antibacterial activity with reduced cytotoxicity. These findings highlight its promise as an adjunct in regenerative endodontics, warranting further validation of its immunomodulatory effects. Nonetheless, as these experiments were performed in an in vitro model that cannot fully replicate the clinical root canal environment, further

validation through animal studies or clinical trials is necessary to confirm its immunomodulatory effects.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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