Title: Anti-inflammatory, antioxidant, and antimicrobial evaluation of morin.

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Abstract

Objectives: This study aimed to evaluate the anti-inflammatory and antioxidant activity of morin in an epithelial cell culture inflammatory model, as well as its antimicrobial activity against periodontal pathogens. Effects of morin alone or slow-release from a polymer-based formulation were compared. **Methods:** An oral epithelial cell line (H400) was pre-conditioned with 0.125 mg/mL morin (free morin or morin in the formulation) for 8 h. The cells were challenged with heat-killed *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (MOI 100:1) for 24 h. The production of GM-CSF was measured by enzyme-linked immunosorbent assay (ELISA) and the gene expression levels of IL-8, GM-CSF, IL1B, NF-kB and NLRP3 were evaluated by RT – PCR. Neutrophils were pre-conditioned with 0.01 mg/mL morin and stimulated immediately with heat-killed *F. nucleatum* or *P. gingivalis* (MOI 100:1) for 2.5 h and then total reactive-oxygen-species (ROS) levels were determined using enhanced chemiluminescence. *F. nucleatum* and *P. gingivalis* as well as a multi-species biofilm (*Streptococcus oralis*, *F. nucleatum* and *P. gingivalis*) were exposed to 1 mg/mL morin for 24 h, planktonic culture and for 7 days, multi-species biofilm. After exposure, the microbial viability (CFU/mL) and biofilm biomass (crystal violet assay) were determined. Live/dead staining and confocal laser scanning microscopy were also performed. Data were submitted to one way ANOVA followed by Tukey's post-hoc test (α=0.05). **Results:** Morin and the polymer-based formulation containing morin significantly attenuated secretion of GM-CSF, significantly reduced ROS generation and the gene expression levels of the cytokines tested ($p<0.05$). Microbial viability and the biofilm biomass significantly reduced ($p<0.05$) after treatment with morin and the polymer-based formulation containing morin. **Conclusions:** Morin showed an anti-inflammatory and antioxidant effect as well as antimicrobial properties against periodontal pathogens. In addition, polymer-based formulation enhanced these effects.

Keywords: Periodontitis, Biofilm, Morin, Neutrophil, Epithelium.

1. Introduction

Periodontitis is a chronic inflammatory disease, driven and propagated by a dysbiotic shift in the subgingival pathogenic plaque biofilm and characterized by a dysregulated and excessive inflammatory host response (Chapple, 2014; Chen et al., 2018; Hajishengallis, 2015; Song et al., 2017). Periodontitis is the most prevalent disease in the world, affecting 50% of the adult population with different severity levels and is the leading cause of tooth loss in adults (Blanc et al., 2014).

Classically nonsurgical periodontal therapy is aimed at disruption of the pathogenic biofilm and dental calculus removal from the tooth / root surfaces due to its function as a plaque retentive factor. Soft plaque deposits removed daily alongside professional debridement has been the management strategy of choice, producing successful clinical outcomes. However, in certain situations where access to the biofilm and calcified deposits are challenging e.g. deep periodontal pockets and furcation defects, treatment outcomes can be compromised (Iorio-Siciliano et al., 2021; Lang et al., 2019; Suvan et al., 2020). In such situations where isolated periodontal pockets remain, after recurrent non-surgical debridement has taken place and in the absence of any other pathology, the use of locally (topical) antibiotics alongside instrumentation may be considered to enhance subgingival biofilm removal and to improve clinical outcomes (Lang et al., 2019; Tan et al., 2020). However, concerns with antibiotic use exist due to the challenges of rising antimicrobial resistance (Ansiliero et al., 2021; Ferri et al., 2017; Guentsch, 2021). Therefore, an unmet clinical need exists to develop new non-antibiotic based adjunctive antimicrobial therapies to help improve treatment outcomes in patients with periodontitis. A non-antibiotic local administration system that acts on the biofilm, “periochip”, is currently commercially available, but there are no claims for direct anti-inflammatory and antioxidant action. In this way, interest has grown in the utilization of natural derived substances that do not promote antimicrobial resistance and have antimicrobial, anti-inflammatory and antioxidant properties as these may have efficacy in chronic inflammatory diseases including periodontitis.

Morin (2′,3,4′,5,7-pentahydroxyflavone), a flavonoid obtained from plants, fruits and herbs, has emerged as a promising non-antibiotic adjunctive therapy in periodontal treatment due to is strong antioxidant, anti-inflammatory and antibacterial activities (Caselli et al., 2016; Ola et al., 2014; Venu Gopal, 2013; Wang et al., 2016). Morin demonstrates inhibitory activity against Gram-positive bacteria (Kang et al.,

2006) and suppresses pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), NLRP3 inflammasome expression and the NF-κB signaling pathway (Leu et al., 2019; Tianzhu et al., 2014; Yu et al., 2020) whilst demonstrating low toxicity (Suntres et al., 2015). Furthermore, morin neutralizes reactive oxygen species (ROS), preventing oxidative stress - a central component in the progression of periodontitis (Ola et al., 2014). It also inhibits osteoclast-mediated bone resorption and stimulates bone formation, which are critical processes in periodontal tissue preservation (Pereira et al., 2025; Rajput et al., 2021). In addition, morin does not induce antimicrobial resistance (Gupta & Birdi, 2017). Despite these promising properties, the effects on Gram-negative bacteria, such as *F. nucleatum* and *P. gingivalis*, and its anti-inflammatory activvity in oral cells stimulated with periodontal pathogens are still largely unexplored.

However, when in its free form, morin - like other natural compounds- shows low solubility, limited stability in the oral environment and low bioavailability, which affects its distribution at the desired site. In order to overcome these limitations, recent studies (de Farias et al., 2020, 2022; Sales et al., 2023) have developed formulations based on sodium alginate and gellan gum. These biocompatible polymers protect morin from degradation and enable its sustained, controlled release directly at the periodontal site, thereby overcoming rapid clearance and enhancing local bioavailability. Moreover, these natural polymers themselves contribute to wound healing and tissue regeneration, further enhancing the therapeutic outcome (Yuan et al., 2023; Zhang et al., 2023). Another advantage of these formulations, especially when compared to non-sustained release products such as mouthwashes, is the release of the active compound over a period of time at a therapeutic dose to allow best efficacy, prolonged effect and potentiate its effect. Unlike conventional agents such as chlorhexidine or antibiotic-loaded chips, which can disrupt the oral microbiome and promote resistance, morin's multifaceted mechanism—including anti-biofilm, anti-inflammatory, and antioxidant actions— may offer a broader spectrum of benefits with minimal side effects and improved patient compliance due to less frequent dosing and non-invasive application.

Thus, natural compounds have the key characteristics (antioxidant, antimicrobial and anti-inflammatory) that may provide important clinical benefits when used alongside conventional non-surgical periodontal treatments and might be an alternative to antibiotics for controlling microorganisms associated with periodontal

disease. Therefore, the aim of this study was to evaluate the potential of morin, specifically: (i) anti-inflammatory activity, by investigating proinflammatory gene expression changes and downstream cytokine production, (ii) antioxidant capacity, by evaluating the modulation of extracellular and total reactive oxygen species production, and (iii) antimicrobial activity against key periodontal pathogens. Additionally, these effects were compared with those of a previously developed polymer-based formulation containing morin (de Farias et al., 2020, 2022; Sales et al., 2023).

2. Materials and methods

2.1 Materials

The flavonoid morin hydrate (2′,3,4′,5,7-Pentahydroxyflavone, purity >85 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The same batch (M4008) was used throughout all tests. Dimethyl sulfoxide (DMSO) was used to dissolve morin. When morin was tested as its free form, a fresh stock solution (60 mg/mL) was prepared in DMSO. As a vehicle control, DMSO, without morin, at an equivalent concentration was used.

The polymer-based formulation used was based on natural polymers containing morin and prepared as reported in the literature (Sales et al., 2023). Briefly, a suspension containing sodium alginate (ALG) and gellan gum (GG) in a 25:75 ratio (ALG:GG) and 500 mg of morin (equivalent to 20% of the final polymer mass) was prepared and dried in a spray dryer (MSD 0.5 spray dryer, LabMaq do Brasil, Ribeirão Preto, Brazil). The final concentration of morin in the formulation was 15.44 mg morin / 100 mg powder (Sales et al., 2023). A formulation without morin, was also prepared and used as blank polymer-based formulation. The polymer-based formulations were previously decontaminated in ultraviolet light for 20 min before each experiment was carried out.

2.2 Experimental groups

The experimental groups are described in Table 1.

Table 1. Experimental groups of the study

Experimental groups	Description
Control	Growth control (cells or microorganisms with culture medium)
Free morin	Flavonoid morin hydrate dissolved in DMSO
DMSO	Only DMSO (vehicle control)
Polymer formulation	Blank polymer-based formulation (without morin)
Polymer formulation + morin	Polymer-based formulation with encapsulated morin
Polymer formulation + free morin	Blank Polymer-based formulation (without morin) + flavonoid morin hydrate dissolved in DMSO

2.3 Evaluation of anti-inflammatory activity

2.3.1 Cell Culture

Oral epithelial cells (H400), passage numbers 20-35, were cultured in Dulbeco's Modified Eagle's Media (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and 5% glutamine at 37 °C in 5% CO_2. The cell line was purchased from the Europe culture collection (ECACC 06092006) and was chosen for presenting close similarities to gingival epithelium, besides being one of the most well-characterised oral epithelial cell lines available (Prime et al., 1990). In addition, a validated methodology using this cell line stimulated with heat-killed bacteria has already been established (Milward et al., 2013).

2.3.2 Heat-killed bacteria

Fusobacterium nucleatum (ATCC 23726) and *Porphyromonas gingivalis* (ATCC 33277) were grown anaerobically (80% N_2, 10% CO_2, and 10% H_2; Don Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK) at 37 °C on Schaedler anaerobe agar plates (SAA; Sigma-Aldrich/Merck, Darmstadt, Germany) and blood agar plates (Oxoid, Basingstoke, UK), respectively. Schaedler anaerobe broth (SAB; Oxoid, Basingstoke, UK) was used to grow the microorganisms overnight in liquid cultures. After growth, the bacteria were washed and centrifuged two times in sterile phosphate buffered saline (PBS) and heat-killed for 1 hour at 80 ºC. The optical density at 600 nm was determined and used to calculate the bacterial concentration for ensuing multiplicity of infection (MOI 100:1) calculations. Heat inactivation was confirmed by plating. The presence of the desired species, *F.*

nucleatum or *P. gingivalis,* in the heat-killed suspension was confirmed by PCR using 16S ribosomal RNA (rRNA) universal primers (16s - Forward: 5-'AGAGTTTGATCATGGCTCAG'-3 and 16s - Reverse: 5-'CGGTTACCTTGTTACGACTT'-3).

2.3.3 Anti-inflammatory activity

Prior to assessing the anti-inflammatory properties, preliminary experiments were carried out. These included epithelial cell cytotoxicity tests and confirmation that bacterial stimulation (heat-killed *F. nucleatum* and *P. gingivalis*) induced cytokine production (IL-8 and GM-CSF) in H400 cells. Additionally, morin stability with and without the formulation was also assessed. A time- and dose-response study was also conducted to determine the optimal dose and treatment duration for evaluating IL-8 and GM-CSF expression. Further details of these experiments are provided in the Supplementary Material.

To evaluate the anti-inflammatory effect of morin, H400 cells were seeded as previously described (2.3.1). The experimental groups were: i) control - cells without stimulation; ii) *F. nucleatum* or *P. gingivalis* stimulation (MOI: 100:1) - cells stimulated with heat-killed periodontal bacteria; iii) polymer formulation (0.82 mg/mL) - formulation without morin; iv) DMSO (2 µl/mL); v) morin (0.125 mg/mL); vi) polymer-based formulation (0.82 mg/mL) + free morin (0.125 mg/mL); vii) polymer formulation with encapsulated morin (final dose of morin was 0.125 mg/mL). The dose of morin chosen was based on the dose-response study (Supplementary Material). After treatment for 8 h, the cells were immediately challenged with heat-killed *F. nucleatum* and *P. gingivalis* (MOI 100:1) for 24 h.

Cytokine production in cell culture medium was measured by enzyme-linked immunosorbent assay kit (ELISA; Diaclone; IDS Ltd.) following the manufacturer's protocol. Genes expression levels of IL-8, GM-CSF, IL1B, NF-kB and NLRP3 were evaluated by RT – PCR. Total RNA was extracted by using a commercial kit (RNeasy Plus Mini Kit) according to the manufacturer's instructions and relative quantification of genes were performed using reverse transcription polymerase chain reaction (RT-PCR) by using a LightCycler® 480 system (Roche, Switzerland) with a SYBR Green I Master mix (Roche). The experiments were carried out in triplicate. Cytokine expression results were presented as pg/mL, by using a calibration curve and the gene

expression levels were presented as a calibrated normalised relative quantity obtained by the QBase+ method according to (Hellemans et al., 2007).

2.4 Evaluation of antioxidant activity

2.4.1 Neutrophil isolation and stimulation

Neutrophils were isolated from the peripheral venous blood of systemically and periodontally healthy volunteers (University of Birmingham ethics code: 19/SW/0198) using discontinuous Percoll gradient ($\delta = 1.079:1.098$) followed by erythrocyte lysis (0.83% NH_4Cl containing 1% $KHCO_3$, 0.04% EDTA and 0.25% BSA) and then re-suspended in PBS at 10^6 cells/mL. Trypan blue was used to assess cell viability, mostly >98% (Matthews et al., 2007).

Neutrophils were stimulated with heat-killed periodontal bacteria (*P. gingivalis* and *F. nucleatum)* as described in item 2.3.2.

2.4.2. Measurement of total ROS generation

ROS generation after stimulation with periodontal bacteria and respective treatments were determined using enhanced chemiluminescence. Neutrophils (1×10^5) from five different donors were added in pre-blocked (PBS BSA 1%, overnight at 4 °C) white 96-well plate with PBS supplemented with glucose (GPBS) and luminol (3 mM). After being equilibrated for a 30-min baseline period, the following treatments were added to the cells (10 µl): i) Control group - cells were kept in GPBS and were not infected with heat-killed bacteria; ii) *F. nucleatum* or *P. gingivalis* stimulation (MOI 100:1) – cells infected with heat-killed periodontal bacteria; iii) polymer formulation (0.07 mg/mL) – formulation without morin; iv) DMSO (0.165 µl/mL); v) morin (0.01 mg/mL); vi) polymer formulation without morin (0.07 mg/mL) + free morin (0.01 mg/mL)]; vii) polymer formulation with encapsulated morin [polymer formulation (0.07 mg/mL) + morin (0.01 mg/mL)]. The dose of morin chosen was based on a previous study (Matthews et al., 2007). In this step, the experimental groups polymer formulation and polymer formulation + morin could not be added directly to the wells containing the neutrophils, due to minimal dosage employed and the brief analysis period (2.5 hours), which would not allow morin release in full. Therefore, solutions containing these two groups were prepared in GPBS and vortexed until completely solubilized. After adding the treatment, cells were stimulated immediately with heat-killed *F. nucleatum* or *P. gingivalis* (MOI 100:1) or PBS as

control. The ROS generation was determined over the subsequent 2.5 hours. All readings were expressed as relative light units (RLUs) and obtained at 37 °C (Berthold Technologies microplate-reader; LB96v: Bad Wilbad, Germany).

2.5 Evaluation of antimicrobial activity

2.5.1 Microorganisms and growth conditions

Periodontal bacteria, *F. nucleatum* (ATCC 23726) and *P. gingivalis* (ATCC 33277) were cultured as described in 2.3.2. *Streptococcus oralis* (ATCC 35037) was grown at 37 °C in 5% CO_2 on Brain heart infusion agar (BHI; Oxoid, Basingstoke, UK). BHI broth was used to grow *S. oralis* overnight in liquid cultures. Prior to evaluating the antimicrobial activity of the system, the minimum inhibitory concentration (MIC) was determined (Supplementary Material).

2.5.3 Planktonic culture assay

Overnight *F. nucleatum* or *P. gingivalis* cultures were diluted in fresh SAB to adjust the optical density to OD_{600}= 0.01 (10^7 CFU/mL). The experimental groups i) polymer formulation (6.58 mg/mL) - formulation developed without morin; ii) DMSO (16 µl/mL); iii) morin (1 mg/mL); iv) polymer formulation (6.58 mg/mL) + morin (1 mg/mL) - formulation developed with encapsulated morin were added to the wells of a 24 well plate. The dose of morin chosen was based on the MIC (approximately 10 times the MIC value - Supplementary Material). Next, 1 mL of diluted culture was transferred into a 24-well plate. Untreated wells were used as a control. Plates were incubated anaerobically at 37 °C for 24 h. To determine bacterial cell viability after the treatment colony forming units (CFU) assays was performed. The results were expressed in log (1+ CFU/mL). The experiments were performed in triplicate.

2.5.4 Multi-species biofilm assay

A multi-species biofilm was formed on 13 mm diameter Thermanox™ coverslips placed in 24-well plates containing 500 µL of artificial saliva (0.25% w/v porcine stomach mucins, 0.35 w/v sodium chloride, 0.02 w/v potassium chloride, 0.02 w/v calcium chloride dihydrate, 0.2 w/v yeast extract, 0.1 w/v Lab Lemco Powder, 0.5 w/v proteose peptone. After sterilization, 40% urea was added). Overnight bacterial cultures, prepared on their designated day of use, were washed twice with PBS and standardized to an OD_{600}= 0.5 (*S. oralis*) and OD_{600}= 0.2 (*F. nucleatum* and *P.*

gingivalis), to achieve a 10^8 CFU/mL inoculum. Next, the inoculum was diluted in artificial saliva to obtain a working culture containing 10^7 CFU/mL of the microorganisms.

On the first day, *S. oralis* was added and incubated for 24 h in 5% CO_2. On the second day, the supernatant was removed and *F. nucleatum* was added and incubated anaerobically for a further 24 h. On the third day, the supernatant was again removed and *P. gingivalis* was added and incubated anaerobically for a further 24 h. During the next 4 days of biofilm growth, the artificial saliva was changed every day.

The treatments were added on the first day of biofilm growth and replaced with each change of artificial saliva. The concentration of morin used, both in its free form and encapsulated within the polymer formulation, was 1 mg/mL. The dose of morin chosen was based on the MIC (approximately 10 times the MIC value - Supplementary Material). To evaluate antibiofilm activity, biomass was assessed using a crystal violet assay, as described before (Muchova et al., 2022). Briefly, after the incubation period, the biofilm was washed with PBS, dried for 2 h at 37 °C and stained with crystal violet solution, diluted in water (0.05 % w/v), for 30 min. After this period, the biofilm was washed again with PBS, dried for a further 2 h and 100% ethanol was used to destain the biofilm. The absorbance was measured at 600 nm (Microplate reader Spark®, Tecan). The results were expressed in absorbance intensity (A_{590}). To determine bacterial viability after the treatment, colony forming units (CFU) were counted and live/dead staining (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, ThermoFisher Scientific, USA) was performed, followed by confocal laser scanning microscopy. For CFU counting, the biofilm was dispersed in PBS by pipetting with up and down movements. For *S. oralis* counting, biofilm dispersion was plated on BHI agar and incubated at 37 °C in 5% CO_2 for 24 h. For *F. nucleatum* and *P. gingivalis* counting, biofilm dispersion was plated on blood agar plates, incubated anaerobically (80% N_2, 10% CO_2, and 10% H_2; Don Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK) at 37 °C. Differential counting between *F. nucleatum* and *P. gingivalis* was carried out considering colony morphology (*F. nucletaum*: white colour and *P. gingivalis*: black colour colonies) and confirmed by Gram stain. The results were expressed in log (1+ CFU/mL). Live/dead images were obtained using a confocal laser scanning microscope (CLSM) (LSM 700, Zeiss, Germany) with a ×40 oil immersion objective (Zeiss Objective EC Plan-Neofluar 40×/1.30 Oil DIC M27, FWD = 0.21 mm). The excitation/emission was 488

nm<550 nm for SYTO9, 555 nm/> 550 nm, for propidium iodide and 490 nm/<525 nm for (WGA) Alexa Fluor$_{TM}$ 48. For 3D analysis Z-stacks were taken at 1.2 µm increments from the surface. To examine the biofilm architecture and the treatment effect, a scanning electron microscope (Zeiss EVO MA10) was used to obtain the images. The images were recorded at 1,000× and 5,000× magnification. The experiments were performed in triplicate.

2.6 Statistical analysis

The sample size was determined according to previous studies (Grant et al., 2023; Matthews et al., 2007; Milward et al., 2007). The data was analyzed using GraphPad Prism (version 10.0.2 for Windows, GraphPad Software, San Diego, California, USA). As data showed normal distribution (Shapiro-Wilk) and had equal variances (Levene's Test), One way ANOVA was performed, followed by Tukey's post-hoc test when $p<0.05$. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Evaluation of anti-inflammatory activity

The preliminary tests showed unexpected relatively high basal levels of IL-8, even in the groups without stimulation and in the groups grown with hydrocortisone-supplemented culture medium (Supplementary Material). Thus, for subsequent experiments, only GM-CSF expression was evaluated, as they presented more consistent values in the preliminary tests.

To analyse the anti-inflammatory activity of the system containing morin, the potential to reduce GM-CSF cytokine production (Figure 1) and gene expression levels of IL-8, GM-CSF, IL1B, NF-kB and NLRP3 (Figure 2 and 3) in response to *F. nucleatum* (Figure 2) and *P. gingivalis* (Figure 3) were examined.

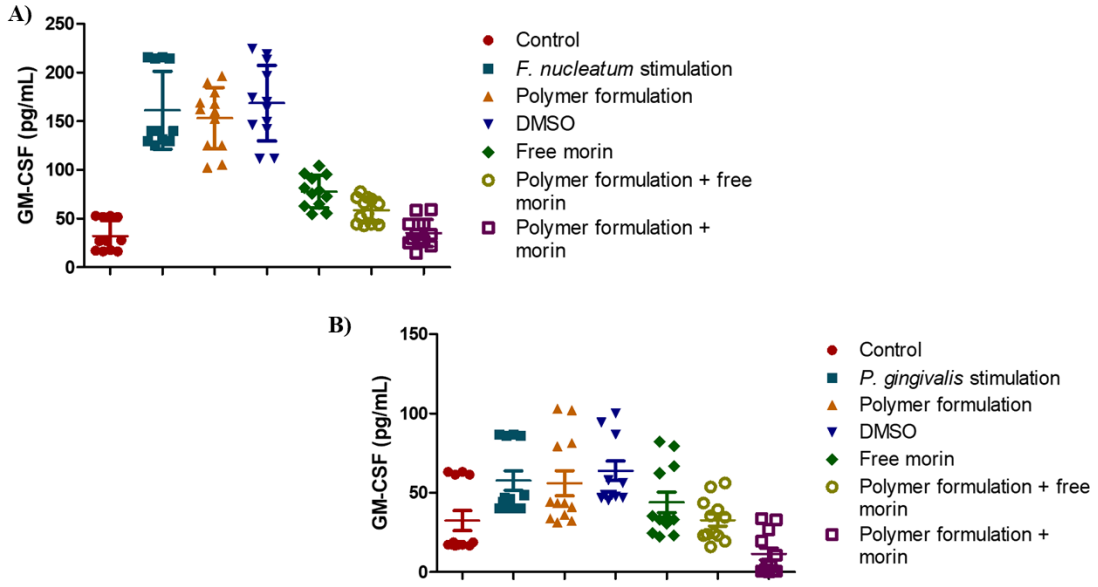

Figure 1. Anti-inflammatory effect of the polymer formulation without morin (0.82 mg/mL), DMSO (2 µl/mL), free morin (0.125 mg/mL), polymer formulation without morin (0.82 mg/mL) + free morin (0.125 mg/mL) and polymer formulation + morin (morin final dose: 0.125 mg/mL) by GM-CSF production by H400 for 8h and challenged with heat-killed *F. nucleatum* **(A)** and *P. gingivalis* **(B)** for 24h, MOI 100:1. Media were measured by ELISA. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 4 replicates). p-value<0.05.

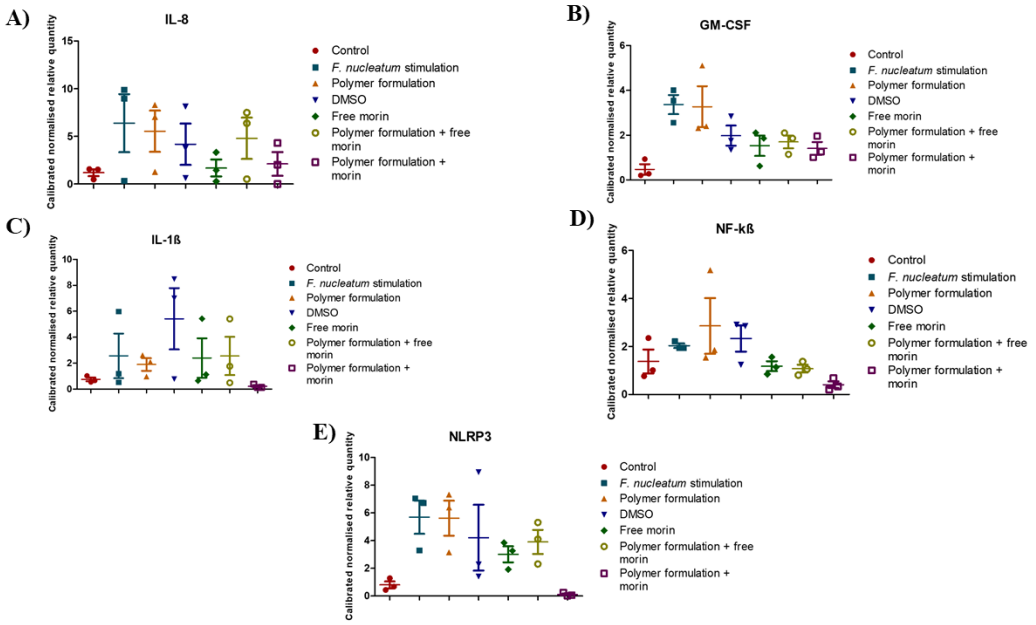

Figure 2. Anti-inflammatory effect of the polymer formulation without morin (0.82 mg/mL), DMSO (2 µl/mL), free morin (0.125 mg/mL), polymer formulation without morin (0.82 mg/mL) + free morin (0.125 mg/mL) and polymer formulation + morin (morin final dose: 0.125 mg/mL) by genes expression levels of IL-8 **(A)**, GM-CSF **(B)**, IL1B **(C)**, NF-kB **(D)** and NLRP3 **(E)** by H400 for 8h and challenged with heat-killed *F. nucleatum* for 24h, MOI 100:1. Genes expression levels were evaluated by RT – PCR. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 3 replicates).

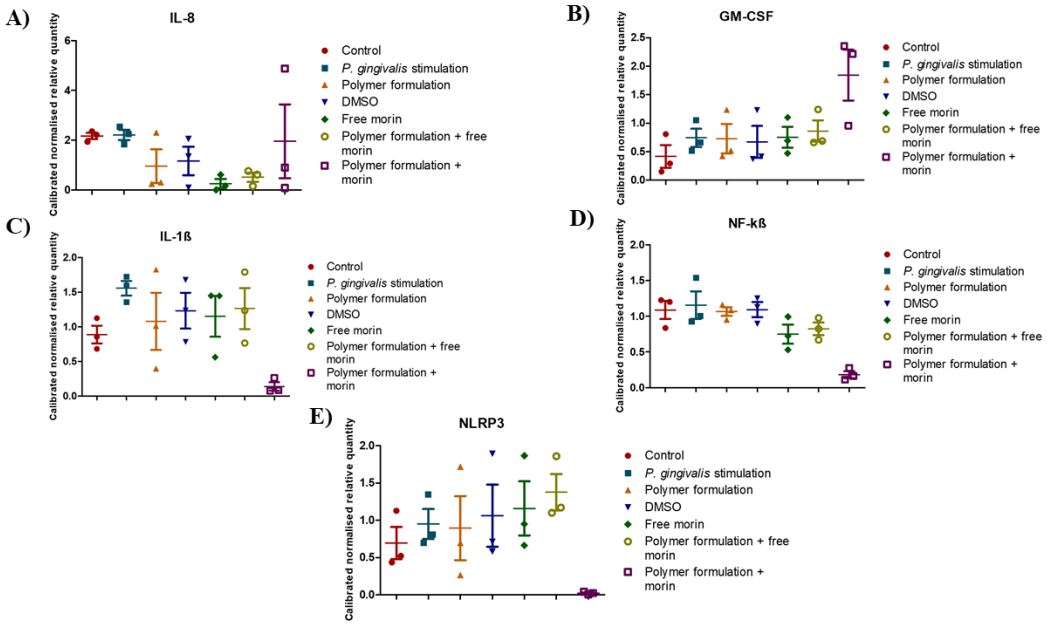

Figure 3. Anti-inflammatory effect of the polymer formulation without morin (0.82 mg/mL), DMSO (2 µl/mL), free morin (0.125 mg/mL), polymer formulation without morin (0.82 mg/mL) + free morin (0.125 mg/mL) and polymer formulation + morin (morin final dose: 0.125 mg/mL) by genes expression levels of IL-8 **(A)**, GM-CSF **(B)**, IL1B **(C)**, NF-kB **(D)** and NLRP3 **(E)** by H400 for 8h and challenged with heat-killed *P. gingivalis* for 24h, MOI 100:1. Genes expression levels were evaluated by RT – PCR. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 3 replicates).

Free morin and polymer formulation + morin showed a significant reduction in GM-CSF production in H400 cells stimulated with either *F. nucleatum* or *P. gingivalis* when compared to the control, to the polymer formulation (without morin) and to DMSO. The polymer formulation containing morin proved to be more effective in reducing GM-CSF production when compared with free morin and polymer formulation+free morin (Figure 1).

The gene expression experiments revealed no significant differences between the tested groups in the expression of all genes (IL-8, GM-CSF, IL1B, NF-kB and NLRP3) when cells were stimulated with *P. gingivalis* (Figure 3). In general, *F. nucleatum* caused a greater stimulus in H400 cells when compared to *P. gingivalis*, which correlated to ELISA results - Figure 1.

F. nucleatum stimulation (Figure 2A) showed no significant difference in IL-8 expression among the tested groups, corroborating with the results from ELISA test

(Figure S3A, C). However, when cells were stimulated with *F. nucleatum*, the polymer formulation + morin group significantly decreased GM-CSF gene expression levels compared to the stimulation and polymer formulation groups (Figure 2B). There was no statistically significant difference between the control group (without stimulation), free morin, formulation + free morin, or formulation + morin. In contrast, free morin and polymer formulation + free morin groups showed a significant difference when compared to the control group. These findings for GM-CSF gene expression levels are also in line with those found by ELISA (Figure 1).

For the IL1β gene (Figure 2C), stimulated by *F. nucleatum*, the polymer formulation containing morin significantly decreased gene expression levels compared to the DMSO group. However, no statistically significant differences were observed between formulation+morin and control groups. Free morin and polymer formulation + free morin groups did not reduce gene expression levels when compared to the other groups. For the NF-kB gene (Figure 2D), stimulated by *F. nucleatum*, the polymer formulation containing morin proved to be significantly efficient in reducing gene expression levels when compared to the other groups. Free morin and polymer formulation + free morin groups did not significantly reduce gene expression levels when compared to the stimulation and DMSO group. For the NLRP3 gene, (Figure 2E), stimulated by *F. nucleatum*, the polymer formulation containing morin also showed a significant reduction in gene expression levels when compared to the stimulation-only, polymer formulation (without morin) and DMSO groups. However, there was no statistically significant difference when compared to the free morin group and the polymer formulation + free morin group. On the other hand, free morin and polymer formulation + morin did not decrease gene expression when compared to the stimulation, polymer formulation and DMSO group.

3.2 Evaluation of antioxidant activity

The chosen concentration (MOI 100:1) of heat-killed periodontal pathogens, *F. nucleatum* and *P. gingivalis,* were non cytotoxic to neutrophils (Figure 4A). Free morin, polymer formulation + free morin and polymer formulation + morin reduced ROS generation, with no significant difference between these three groups when stimulated with *F. nucleatum* (Figure 4B). For *P. gingivalis*-infected groups, there was no significant reduction in ROS generation between the treatment groups (Figure 4C).

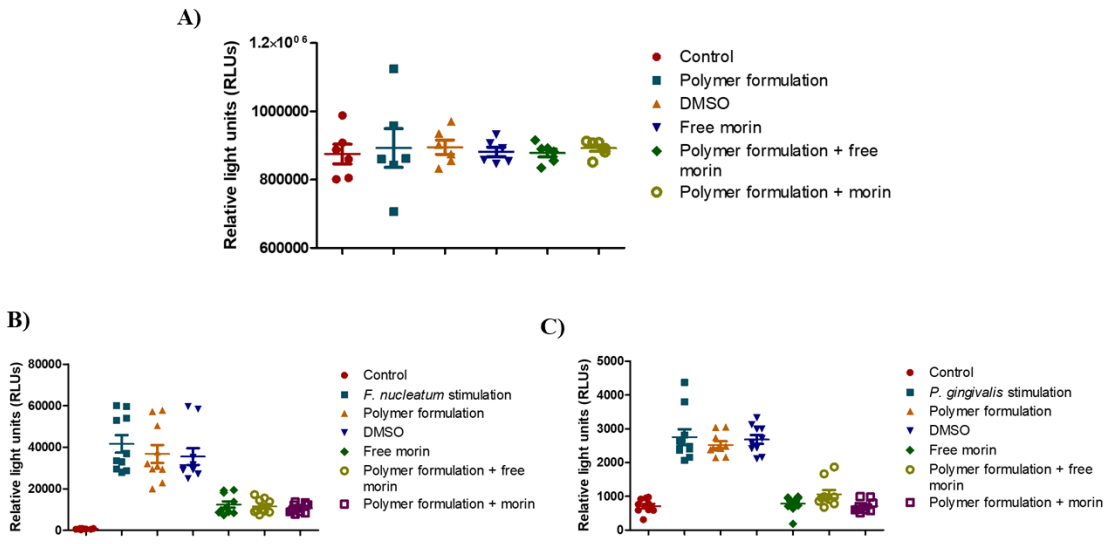

Figure 4. (A) Cytotoxicity of neutrophils (luminescence viability) after 2.5 hours of incubation with polymer formulation without morin (0.07 mg/mL), DMSO (0.165 µl/mL), free morin (0.01 mg/mL), polymer formulation (0.07 mg/mL) + free morin (0.01 mg/mL)] and polymer formulation + morin (morin final dose: 0.01 mg/mL) measured using Cell Titre Glo. One way ANOVA (mean ± SD; results represent two independent experiments with n = 3 donors in each experiment). Antioxidant effect, through chemiluminescent ROS generation (RLUs), of the polymer formulation without morin (0.82 mg/mL), DMSO (2 µl/mL), free morin (0.125 mg/mL), polymer formulation without morin (0.82 mg/mL) + free morin (0.125 mg/mL) and polymer formulation + morin (morin final dose: 0.125 mg/mL) by neutrophils incubated for 2.5 hours without bacteria challenged, immediately challenged with heat-killed *F. nucleatum* **(B)** or *P. gingivalis* **(C)**, MOI 100:1. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent two independent experiments with n = 5 donors in each experiment).

3.3 Evaluation of antimicrobial activity

3.3.1 Planktonic culture assay

The initial assessment of the antimicrobial effect of morin was conducted on planktonic cultures of periodontopathogens. According to Figure 5, free morin and the polymer formulation containing morin (1 mg/mL) significantly inhibited the growth of *F. nucleatum* and *P. gingivalis* ($p<0,001$).

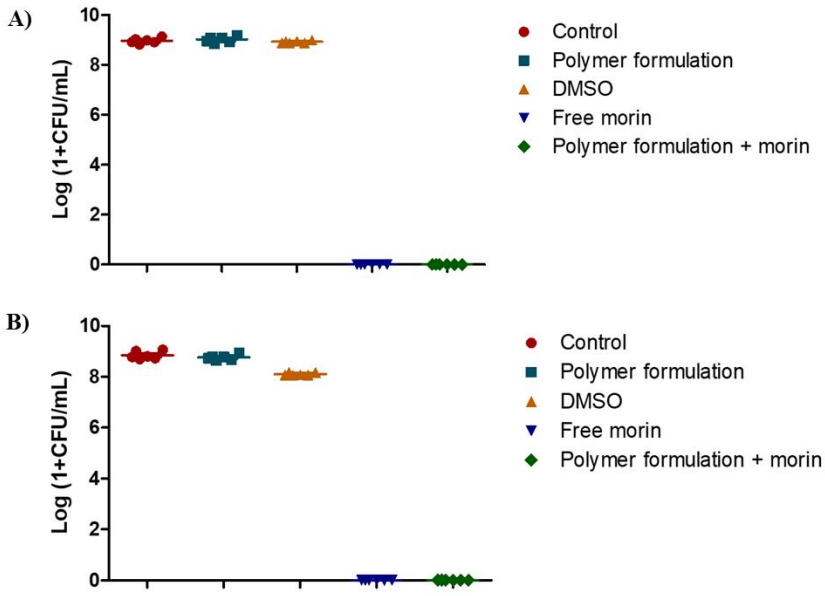

Figure 5. Antimicrobial effect of the polymer formulation without morin (6.58 mg/mL), DMSO (16 µl/mL), free morin (1 mg/mL) and polymer formulation (6.58 mg/mL) + morin (1 mg/mL), on planktonic culture of *F. nucleatum* **(A)** and *P. gingivalis* **(B)**, after 24 h of exposure. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 2 replicates).

3.3.2 Multi-species biofilm assay

To confirm the findings of previous tests, the effect of morin was tested on mature multi-species periodontal biofilms. Free morin, polymer formulation+free morin and the polymer formulation containing morin significantly reduced the viability of *S. oralis* and *F. nucleatum* in multispecies biofilms when compared to the control, to the polymer formulation (without morin) and to DMSO (Figure 6AB). *P. gingivalis* levels were below the detection limit but its presence was confirmed in SEM and confocal images (Figures 7 and 8). Free morin, polymer formulation+free morin and the polymer formulation containing morin also significantly reduced the biomass of the multispecies biofilm (Figure 6C).

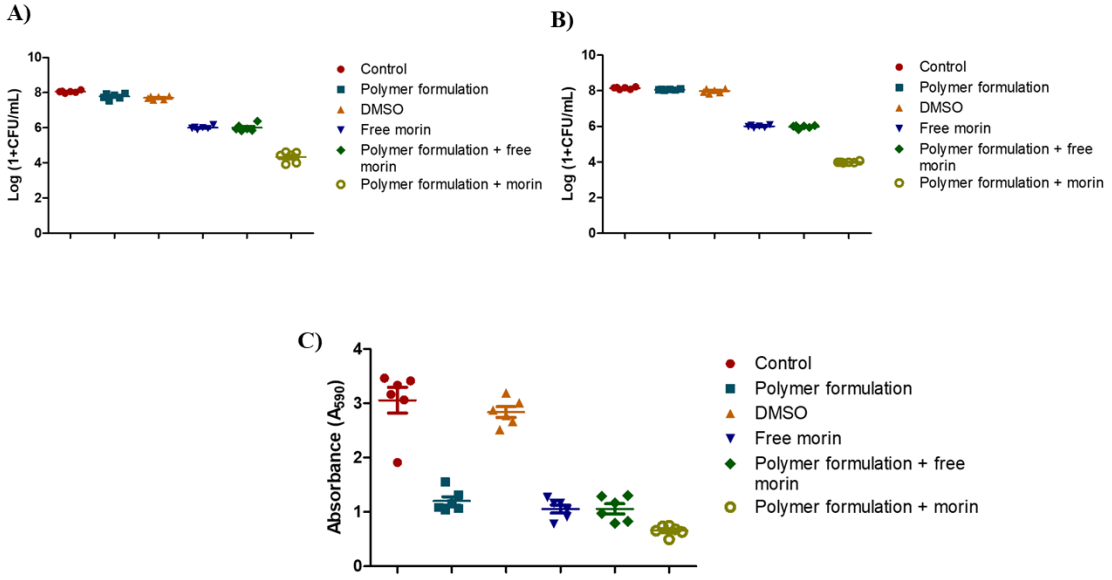

Figure 6. Antimicrobial effect, after 7 days, of the polymer formulation, without morin (6.58 mg/mL), DMSO (16 µl/mL), free morin (1 mg/mL), polymer formulation (6.58 mg/mL) + free morin (1 mg/mL) and polymer formulation + morin (1 mg/mL) on *S. oralis* **(A)** and *F. nucleatum* **(B)** . One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 2 replicates). **C)** Biomass after 7 days of exposure to the groups shown. One way ANOVA with Tukey's post-hoc test (mean ± SD; results represent three independent experiments with n = 2 replicates).

The polymer formulation containing morin proved to be more effective in terms of antimicrobial and antibiofilm activity when compared to morin in its free form. The polymer formulation without morin also significantly reduced the biomass of the multispecies biofilm when compared to the control (Figure 6C). These findings were also confirmed by the SEM and confocal images (Figure 7 and 8).

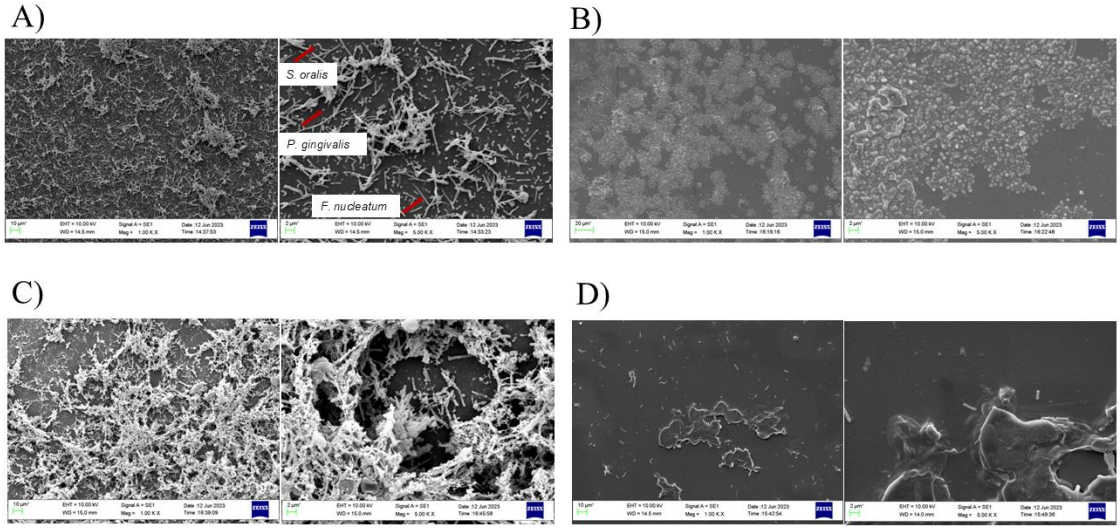

Figure 7. SEM photomicrographs of the groups **(A)** control - multispecies biofilm **(B)** control – *P. gingivalis* biofilm **(C)** free morin - multispecies biofilm **(D)** polymer formulation + morin - multispecies biofilm. X 1,000 and X 5,000 magnification.

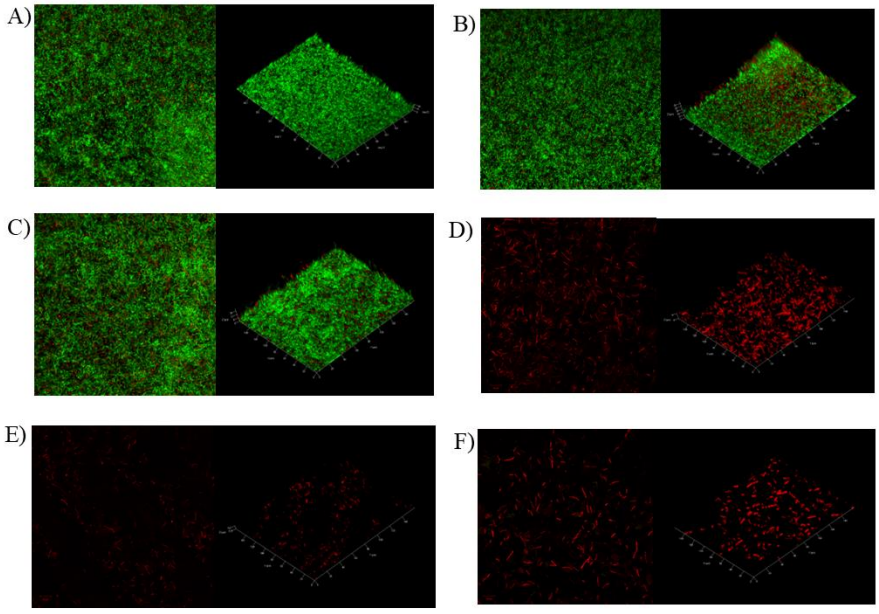

Figure 8. CLSM images of live/dead cells for each group obtained from multispecies biofilm exposed to different experimental treatments. The viable cells emit green fluorescence while dead cells emit red fluorescence. **(A)** Control **(B)** polymer formulation **(C)** DMSO **(D)** free morin **(E)** polymer formulation + free morin **(F)** polymer formulation + morin.

SEM images (Figure 7) indicated the presence of all three bacterial species in the control group biofilm, with *F. nucleatum* being the most dominant. In the polymer

formulation containing morin, the three species were observed, but more isolated from each other, with no biofilm formation. It was also possible to observe an amorphous mass compatible with the polymeric formulation. Similar findings were observed in the confocal images. In the control, in the polymer formulation (without morin) and in DMSO groups, a thick biofilm with live bacteria was evident. In the treatment groups - free morin, polymer formulation + free morin and polymer formulation containing morin- no biofilm formation was observed, and the bacteria were dead.

4. Discussion

Periodontitis is the result of dysregulated and exaggerated inflammation, which is associated with neutrophil infiltration and excess ROS release in periodontal tissues in response to periodontal bacteria, causing collateral tissue damage. The oral epithelium is responsible for initiating and propagating the cytokine cascade and neutrophil recruitment when exposed to periodontal bacteria (Matthews et al., 2007; Milward et al., 2013). Hence, an essential therapeutic approach involves regulating the epithelial responses to inflammatory stimuli. Therefore, we explored the role of morin, with or without a polymer-based formulation for controlled release, as a potential modulator of inflammatory cytokines and of ROS production, as well as their antimicrobial effect against microorganisms associated with periodontitis.

This study showed that there was a difference in secretion of inflammatory cytokines and gene expression levels when the cells were stimulated with either *F. nucleatum* or *P. gingivalis*, which is in agreement with the literature (Abdulkareem et al., 2020; Milward et al., 2007; Milward et al., 2013). *F. nucleatum* is an important bridging bacteria between health and pathogenic biofilm and this difference between species may be attributed to more potent virulence factors expressed by *F. nucleatum*, such as higher adherence to epithelial cells in comparison to *P. gingivalis* (Huang et al., 2001; Li et al., 2015; Zhang et al., 2022). Although *P. gingivalis* is strongly associated with periodontal disease, the efficient adhesion of *F. nucleatum* facilitates colonization and biofilm formation in the oral cavity. Consistent with our results, Martinho et al. (2016), showed that various bacteria have distinct lipopolysaccharide (LPS) structures capable of inducing different levels of inflammation. Their work also confirmed that *F. nucleatum* LPS secretion leads to greater inflammatory cytokine release compared to that of *P. gingivalis*. Thus, the results of this study suggest that differences in the inflammatory responses induced by various periodontal pathogens

contribute to a better understanding of periodontal disease. Furthermore, this study validates the use of a robust cell stimulation model using heat-killed, periodontally relevant bacteria, specifically *F. nucleatum* and *P. gingivalis*. The use of heat-killed bacteria is a safe approach, eliminating the need to expose cells to live bacteria and reducing the risk of contamination. Moreover, it enhances experimental standardization, offering greater control over experimental conditions. This model can be used in future studies to evaluate the anti-inflammatory effect of other substances. However, a limitation of the present study was the use of a traditional monolayer cell culture model. Although widely employed, this model does not accurately replicate the complex and dynamic *in vivo* tissue environment. Given the promising results obtained, and to further advance this line of research, we recommend that future studies adopt a three-dimensional coculture model to better reproduce a more physiologically representative experimental environment.

It was initially demonstrated that morin has an anti-inflammatory effect by significantly reducing the expression levels of pro-inflammatory cytokines. The findings of this study agree with existing literature, which has demonstrated anti-inflammatory effects of morin across multiple cell lines using different concentrations and treatment duration. It has been postulated that morin may inhibit pro-inflammatory mediator production, modulate cell signaling pathways and inhibit immune cell activation (Rajput et al., 2021; Singh et al., 2018; Wang et al., 2016). Although its precise mechanism of action remains to be fully elucidated, the present study brings valuable insights into its mechanisms of action and supports morin as an emergent natural alternative for managing exaggerated inflammation characteristic of periodontitis.

The results of this study showed unexpected relatively high basal levels of IL-8, even in the groups without stimulation and in the groups grown with hydrocortisone-supplemented culture medium (Supplementary Material). The hypotheses for these findings are that H400 cells are more sensitive and respond more intensely and more rapidly with IL-8 cytokine production (Grant et al., 2010, 2023). It is known that H400 cells rapidly secrete the pro-inflammatory cytokines IL-8 and GM-CSF after stimulation with heat-killed *F. nucleatum* and *P. gingivalis*. However, IL-8 production occurs more quickly, immediately after stimulation, compared to GM-CSF production, which takes a little longer to be secreted (Milward et al., 2007). For this reason, subsequent experiments were carried out without hydrocortisone and only

GM-CSF secretion was used as the cytokine marker. GM-CSF is an important pro-inflammatory cytokine because it is the initial part of the first line epithelial response to pathogen stimulation and causes a number of downstream sequelae important in the innate inflammatory response. After stimulation, H400 cells respond rapidly and generate a pro-inflammatory response characterized by the activation of NF- κB. NF-κB translocates to the nucleus, where it binds to κB binding sites in the promoter region of target genes, which encode pro-inflammatory cytokines such as GM-CSF (Grant et al., 2010, 2023). This study showed that morin significantly reduces GM-CSF levels, with the effect being more pronounced when morin was incorporated into the polymer-based formulation. One of the hypotheses for these results is the limited stability of morin (Jangid et al., 2018; Yazdanshenas & Gharib, 2017). To overcome this challenge and to increase its anti-inflammatory effect, we used a polymeric controlled release delivery formulation, recently developed by Sales et al. (2023). This formulation is water-soluble, stable, delivers controlled release of morin and shows antimicrobial properties against *S. mutans*. Therefore, the formulation proved to be capable of enhancing the anti-inflammatory effect of morin, as observed by decreased GM-CSF levels in the group polymer-based formulation containing morin. This is the first study to investigate the anti-inflammatory effects of morin encapsulated in a polymer-based formulation as a potential novel therapy in Periodontitis.

It is worth noting that the optimal treatment time (8 h) and the stimulation time of 24 h were chosen based on pro-inflammatory cytokines production measured by ELISA. These findings corroborate with previous studies indicating that morin treatment beyond 24 h does not provide anti-inflammatory benefits (Rajput et al., 2021; Singh et al., 2018; Wang et al., 2016). However, it is very likely that gene expression occurs earlier than cytokine production. Therefore, the optimal time point for detecting gene expression changes may have been missed, with gene expression returning to baseline levels. Despite this, the PCR-based gene expression provided valuable insights into the behavior of H400 cells when treated with morin or the polymer-based formulation containing morin, followed by immediate stimulation with heat-killed periodontal bacteria. According to the results obtained, we emphasise the importance of precise timing in experimental design. Additional experiments should be carried out with different treatment times and stimuli in order to better understand the time point of gene expression by H400 cells.

One of the parameters for evaluating the antioxidant effect of a substance is the reduction in the generation of ROS by neutrophils, as this enables the death and removal of pathogens, which is essential in the innate immune response (Roberts et al., 2015). Studies show that ROS contribute to periodontitis progression by causing tissue damage, lipid peroxidation, activation of chronic ROS production and increased osteoclast differentiation (Dahiya et al., 2013; Waddington et al., 2000). The results of this study show that the periodontal relevant bacteria, *F. nucleatum* and *P. gingivalis* stimulate neutrophils to release ROS. However, as with H400 cells, *F. nucleatum* causes higher levels of ROS production by neutrophils. These findings are consistent with the literature, which shows that the release of ROS by neutrophils varies for each species of periodontal bacteria, with *F. nucleatum* as the species that most stimulates neutrophils to produce ROS (Hirschfeld et al., 2017). Although no difference was found between the treatment groups, morin was shown to have an antioxidant effect by reducing ROS generation, corroborating the literature (Mo et al., 2019; Rajput et al., 2021; Wang et al., 2006). In addition, the lack of differences observed between morin and the polymer-based formulation containing morin is potentially due to the fact that the polymer-based formulation was vortexed before being added to the 96-well plates containing the neutrophils, which led to immediate release of morin. Future studies with different methodologies need to be carried out to better elucidate the antioxidant effect of the systems containing morin.

In addition to its anti-inflammatory and antioxidant effect, morin also showed an antimicrobial effect against periodontopathogens. Morin has been demonstrated to be an effective inhibitor of various microorganisms, such as *Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans* (Abirami et al., 2020; Huang et al., 2014; Rajput et al., 2021). Although its mechanism of action has not been fully elucidated, it has been proposed that it may involve facilitating bacterial aggregation, induction of oxidative stress in bacteria and interaction with the lipids of the phospholipid bilayer of the bacterial membrane. This interaction increases membrane permeability and alters membrane structures such as thickness and indirectly modulates the distribution and function of membrane proteins. This facilitates the entrance of toxic compounds and the exit of essential substances, resulting in loss of cell integrity and bacterial death. Morin is also known to inhibit bacterial toxins and nucleic acid synthesis by hydrogen bonding with the nitrogenous bases of DNA, interfering with gene replication and transcription (Górniak et al., 2019; Rajput et al.,

2021; Yang et al., 2012). These inhibitory characteristics of morin are attributed to its chemical structure. It is known that the antimicrobial activity of flavonoids increases with the number of hydroxyl groups present in the chemical structure (Górniak et al., 2019). This characteristic is a key differentiator of morin compared to other natural substances and enhances its effect. Morin has a structure rich in aromatic rings and hydroxyl groups, which allows it to strong interactions with bacterial cell membrane, leading to cell death (Kováč et al., 2022). Moreover, this structure enhances morin's ability to penetrate dense and resistant bacterial biofilm, unlike other substances that exhibit only superficial effects (Bouchelaghem et al., 2022). In addition to morin's multiple mechanisms of action, downregulation of the PBP2a-mediated resistance pathway makes the development of antimicrobial resistance less likely. This provides a significant advantage over local antibiotics commonly used as adjunctive therapy in periodontal instrumentation (Rajput et al., 2021; Yang et al., 2012). Furthermore, unlike the commercially available non-antibiotic local device "Periochip", which offers solely antimicrobial activity, morin combines antimicrobial, anti-inflammatory, and antioxidant properties, offering a broader therapeutic benefit for periodontal treatment. Nevertheless, while the antimicrobial properties of morin are well-documented, there is a lack of literature examining the impact of morin on microorganisms associated with periodontitis.

Morin was evaluated on planktonic cultures of *F. nucleatum* and *P. gingivalis* and on multispecies biofilms at a concentration approximately 10 times the MIC value. The treatments proved to be effective in eradicating both *F. nucleatum* and *P. gingivalis* species and in reducing biofilm and microbial viability (Figure 7). The observed anti-biofilm effect is attributed to morin's mechanism of action, which inhibits the initial adhesion of bacterial cells by modulating the expression of adhesion proteins, thereby blocking one of the earliest steps in biofilm formation (Huang et al., 2014), and to the bioadhesive characteristics of the polymeric formulations, precisely due to the presence of sodium alginate and gellan gum. Both polymers have been shown to have bioadhesive properties (Kesavan et al., 2010; Lee & Mooney, 2012; Meneguin et al., 2018; Osmałek et al., 2014). This is the potential reason why the polymer-based formulation (without morin) also had an anti-biofilm effect, as they adhered to the glass coverslips, preventing biofilm formation. These findings are further supported by SEM images (Figure 8), wherein residual biopolymers are evident, aligning with findings in the literature (de Farias et al., 2020). A limitation of

the study was that the antimicrobial effect was evaluated in a preventive rather than therapeutic manner. The treatments were added at the beginning of biofilm formation and replaced with each change of culture medium. However, the study is in line with previous studies that showed that one of the most important antimicrobial effects of morin is its anti-adhesive activity, interfering with biofilm formation and growth (Rajput et al., 2021; Yang et al., 2012). As this is the first study to evaluate the effect of morin on periodontal pathogens, the effect found shows that morin and the polymer-based formulation containing morin have potential for controlling periodontal pathogens. Future studies to evaluate morin's therapeutic effect on established biofilms are required.

One limitation of using morin in dentistry is its yellow colour, which can potentially cause tooth and restoration discoloration. In this context, the polymer-based formulation containing morin, besides showing a greater antimicrobial and antibiofilm effect when compared to free morin, demonstrated no observable colour change in biofilms (data not shown). Clinically, this is another advantage of the polymer-based formulation containing morin compared to free morin. Future clinical studies are essential to assess the influence of morin and polymer-based formulation containing morin on potential tooth and dental restoration colour changes.

This study effectively explored a new application of morin and the polymer-based formulations containing morin developed by Sales et al. (2023). The findings serve as an incentive for further research to be carried out exploring natural compounds and microencapsulation of drugs for oral use and also morin effect on microorganisms that cause periodontal disease. Further studies to evaluate the therapeutic effect of morin-containing systems on mature biofilms, and *in situ* and clinical studies should be carried out to further explore its therapeutic potential in periodontitis.

5. Conclusion

In conclusion, morin showed an anti-inflammatory and antioxidant effect by modulating pro-inflammatory cytokine production and their gene expression levels and by reducing extracellular and total ROS, respectively, produced by H400 cells in response to *F. nucleatum* and *P. gingivalis* stimulation. Furthermore, morin showed an antimicrobial effect, as it was effective in combating periodontal pathogens in planktonic culture and in biofilm formation. In addition, the polymeric formulations

with controlled release of morin, enhanced these effects. These findings could offer a promising strategy to combat the deregulated and exaggerated inflammation caused by periodontal disease.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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