



Anti-inflammatory and macrophage polarization effects of Cranberry Proanthocyanidins (PACs) for periodontal and peri-implant disease therapy

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Abstract

Background and Objective: Macrophages' cytokine expression and polarization play a substantial role in the host's "destructive" inflammatory response to periodontal and peri-implant pathogens. This study aimed to evaluate cell viability, anti-inflammatory activity, and macrophage polarization properties of different cranberry concentrates.

Methods: THP-1 cells (monocytic line) were treated with phorbol myristic acid to induce macrophage differentiation. Human gingival fibroblasts (HFIB-G cell line), osteosarcoma-derived osteoblasts (SAOS-2 cell line), and induced macrophages were treated with cranberry concentrates at 25, 50, and 100 µg/mL for 120 seconds, 1 hour and 24 hours. Untreated cells at the same time points served as controls. For anti-inflammatory analysis, induced macrophages exposed to cranberry concentrates (A-type PACs) were stimulated with lipopolysaccharides (LPS) derived from *E coli* for 24 hours. Cell viability, interleukin (IL)-8, IL-1 β, IL-6, and IL-10 expression of LPS-stimulated macrophages, and macrophage polarization markers were evaluated through determination of live-cell protease activity, enzyme-linked immunosorbent assay, and immunofluorescence staining semi-quantification.

Results: Cranberry concentrates (A-type PACs) did not reduce HGF, SAOS-2, and macrophage viability after 24 hours of exposure. Pro-inflammatory cytokine expression (ie IL-8 and IL-6) was downregulated in LPS-stimulated macrophages by cranberry concentrates at 50 and 100 µg/mL. Anti-inflammatory IL-10 expression was significantly upregulated in LPS-stimulated macrophages by cranberry concentrates at 100 µg/mL after 24 hours of exposure. M1 polarization significantly decreased when LPS-stimulated macrophages were exposed to cranberry concentrates. High levels of positive M1 macrophages were present in all untreated control groups. M2 polarization significantly increased at all LPS-stimulated macrophages exposed to cranberry concentrates for 1 and 24 hours.

Shahram Ghanaati and Frank Schwarz contributed equally.

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Conclusion: Cranberry-derived proanthocyanidins may have the potential to act as an anti-inflammatory component in the therapy of periodontal and peri-implant diseases.

KEYWORDS

anti-inflammatory agents, cranberry, interleukins, macrophage polarization, peri-implantitis, periodontitis, proanthocyanidin

1 | INTRODUCTION

Periodontitis is an inflammatory disease caused by anaerobic gram-negative pathogens involving the active production of pro-inflammatory cytokines which modulate periodontal tissue destruction and trigger bone resorption.¹⁻³ Comparably, peri-implantitis is a pathologic condition associated with biofilm formation and is characterized by “inflammation in the peri-implant mucosa and a progressive loss of supportive bone.”⁴⁻⁸ Strong evidence suggests that periodontitis is a risk factor for peri-implant disease.^{5,9} Periodontitis and peri-implantitis have similar clinical and radiographic features but evident molecular and histopathological differences.¹⁰⁻¹³ The reported distribution of inflammatory cells in periodontitis and peri-implantitis lesions exhibited that macrophages represent 6% and 11% of the inflammatory cell population, respectively.¹²

Even though macrophages are present in moderate proportions at periodontitis and peri-implantitis sites, they play a substantial role in the host's inflammatory response and are considered as the “bridge between inflammation resolution and tissue repair.”¹⁴ The dual mechanisms of macrophages concerning turn over and tissue repair might govern homeostasis and disease progression at periodontal and peri-implant tissues¹⁴⁻¹⁶. In this setting, macrophages activated by bacteria sub-products like lipopolysaccharides (LPS) present a M1 phenotype and are associated with the secretion of pro-inflammatory cytokines (ie, IL-1 β , IL-6, IL-8) which trigger a “destructive osteolytic inflammation.” Conversely, macrophages activated by alternative ways present a M2 phenotype and are associated with “constructive inflammation” involving the secretion of anti-inflammatory cytokines and growth factors which enhance tissue repair.¹⁷⁻¹⁹ Macrophage phenotype and cytokine expression may imply the host's susceptibility to inflammation and response to periodontal and peri-implant therapy.^{15,20} Local delivery of host-modulating agents can be a favorable strategy to counteract the pathogenesis and destructive mechanisms of periodontal and peri-implant diseases^{2,20-24}. Particularly, cranberry (*Vaccinium macrocarpon*) polyphenolic compounds named proanthocyanidins (PACs) are reported to inhibit oral biofilm adherence, stimulate antibiotic effects, and be potential anti-inflammatory macrophage modulators.²⁴⁻³¹ Accordingly, it is hypothesized that cranberry concentrates may have potential anti-inflammatory effects able to counteract macrophage's “destructive” inflammatory response. The aim of this in vitro study was to evaluate cell viability, anti-inflammatory activity, and macrophage polarization effects of cranberry-derived concentrates.

2 | MATERIAL AND METHODS

2.1 | Cranberry concentrate preparation

Cranberry (*Vaccinium macrocarpon*) capsules (Uriach-Aquilea OTC) containing 130 mg (A-Type PACs) were previously evaluated and determined with the standard 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method using a commercially available standard (procyanidin A2), for the standard method and quantification of A-type PACs in cranberry capsules. The cranberry concentrates had a composition of 61% carbon and 5% hydrogen. The concentrates were dissolved in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) at concentrations of 25, 50, and 100 $\mu\text{g}/\text{mL}$ under constant stirring for 4 hours, at room temperature. Dilutions were sterilized through filtration (0.2 μm ; MILLEX-GS; Millipore Corp.), centrifuged for 5 minutes at 241.5 g (Labofuge 400, Thermo Scientific), and stored at 0°C. Cranberry dilutions were stirred over under constant agitation for a 30-minute period before application.

2.2 | Cell cultures

Human gingival fibroblasts (HGF) (HFIB-G cell line, fifth passage, Provitro) ($5 \times 10^3/\text{well}$) and human osteosarcoma-derived osteoblasts (SAOS-2) (Acc 243 cell line, fourth passage, German Collection of Microorganisms and Cell Cultures) ($1 \times 10^5/\text{well}$) were seeded in 96-well plates (Corning Inc) and cultured for 3 days in 2 mL of Dulbecco's modified Eagle's medium (DMEM, high glucose, GlutaMAX; Sigma-Aldrich) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Sigma Aldrich, Inc). Cell culture conditions were set at a temperature of 37°C and a humidified atmosphere of 5% CO_2 .

2.3 | Inducement of monocytes to macrophages

THP-1 cells (THP-1, DMSZ), a monocytic leukemia cell line, were cultivated at 37°C in a 5% CO_2 atmosphere in Roswell Park Institute medium (RPMI) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin for three days. Monocytes (5×10^3 cells/well) were seeded on 96-well plates and incubated in RPMI/10% FCS containing 10 ng/mL of phorbol myristic acid (PMA)^{32,33} (Sigma Aldrich, Inc). After 72 hours of PMA-induction, non-attached cells

were removed by aspiration and adherent cells were cultured in fresh RPMI medium, 10% FCS, for 24 hours at 37°C in 5% CO₂ atmosphere.

2.4 | Cell viability assay

Cultured SAOS-2 cells, HGF, and PMA-induced macrophages were exposed to cranberry concentrates at 25, 50, and 100 µg/mL for 2 minutes, 1 hour, and 24 hours and incubated after exposure for 24 hours at 37°C in a humidified 5% CO₂ atmosphere. Cranberry untreated cells at the same time points served as controls. Cell viability for SAOS-2 cells, HGF, and macrophages was measured at days 0 (immediately after treatment), 3, and 7 after the corresponding exposure periods by the use of a luminescence assay according to the manufacturer's instructions (CellTiter-Glo, Promega) evaluating the live-cell protease activity in an illuminometer (Victor X3; Perkin-Elmer) at a wavelength of 490 nm.

2.5 | Treatment of macrophages and LPS stimulation

Previously seeded PMA-induced macrophages were exposed to cranberry concentrations at 25, 50, and 100 µg/mL for 2 minutes, 1 hour, and 24 hours. Cells incubated without cranberry concentrates were used as controls. After exposure to cranberry concentrates, the culture supernatants were replaced with fresh serum-free RPMI medium and macrophages were stimulated with 1 µg/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, Inc) derived from *E coli* for 24 hours. Macrophages with no LPS stimulation served as an additional control.

2.6 | Macrophage cytokine expression

Following 24 hours of LPS of stimulation, the culture supernatants of control and test groups were collected and stored at -20°C. The supernatants were assayed for pro- and anti-inflammatory cytokine expression. Enzyme-linked immunosorbent assay (DuoSet® ELISA Development Systems) was used to determine IL-8, IL-1 β, IL-6, and IL-10 concentrations according to the manufacturer's protocol (R&D Systems). Optical densities of each well were measured using a microplate reader (Infinite200, TECAN) at a wavelength of 450 nm.

2.7 | Macrophage immunofluorescence stain

After supernatant removal, wells with adhered LPS-stimulated macrophages were three times (3x) rinsed with PBS and fixed with 4% buffered formalin (Roti-Histofix 4% acid-free pH7, Carl-Roth) for 10 minutes at room temperature. After fixation, wells

were rinsed with PBS (3x) and permeabilized with 0.1% Triton X/PBS. The mentioned rinsing step (3x) was repeated and double immunofluorescence staining was performed per group. Cells were incubated with the following primary antibodies: mouse anti-human CD68 (1:200, Dako M0814) and rabbit anti-human CCR7 (M1 marker) (1:200, R&D system) or mouse anti-human CD68 and rabbit anti-human CD206 (M2 marker) (1:200, R&D system) in 1% bovine serum albumin (BSA) in PBS for 60 minutes at room temperature. Cells were then washed with PBS (3x) and incubated with the secondary antibodies: Alexa Fluor® 488 goat anti-mouse (Molecular Probes, MoBitec) (dilution 1:1000) and Alexa Fluor® 546 goat anti-rabbit (Molecular Probes, MoBitec) (dilution 1:1000) in 1%BSA/PBS for 60 minutes at room temperature avoiding light exposure. Cranberry untreated LPS-stimulated macrophages serve as control group.

2.8 | Semi-quantification of M1 and M2 macrophage polarization

Microscopic images were acquired using an inverted microscope (Nikon Eclipse TS100). Images were extracted using fluorophore channels matching to CD68 (total macrophages), CCR7 (M1 polarization), and CD206 (M2 polarization). Representative areas of each group were photographed (10 independent fields per group) (10x objective). The extracted images were analyzed using a software program (ImageJ software, 1.52a). Macrophage polarization semi-quantification was analyzed as percentages of positive double-stained cells for CD68 and CCR7 (M1) or CD68 and CD206 (M2) over the total number of CD68 positive cells.

2.9 | Statistical analysis

Statistical analysis was performed using a commercially available software program (SPSS 26, 19.0). All experiments were performed in triplicate samples. Data were analyzed in terms of means and standard deviations (SD). One-way analysis of variance (ANOVA) was used for comparisons between groups. Post hoc test was performed, and differences were considered as statistically significant at $P < .05$ and $P < .01$.

3 | RESULTS

3.1 | Cell viability

Cell viability of HGF, SAOS-2 cells, and PMA-induced macrophages exposed to cranberry concentrates for 24 hours is presented in Figure 1. No significant effects on HGF viability were detected in response to any of the cranberry tested concentrations. SAOS-2 cell viability was not affected after 24 hours of exposure at day 0 and day 3 of culture; however, it increased at all cranberry exposed groups at

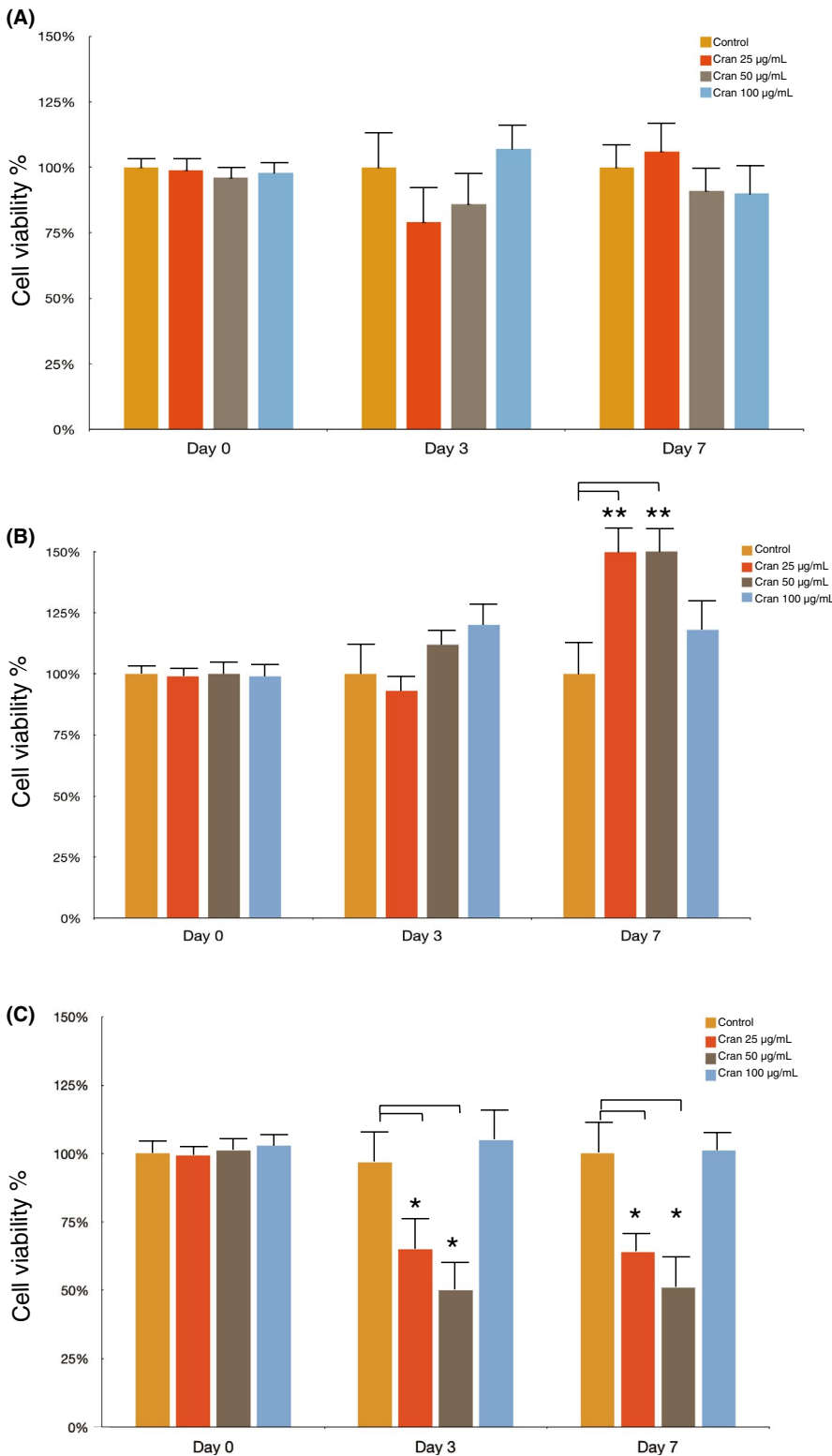


FIGURE 1 Effect of cranberry PACs concentrates on cell viability of (A) HGF, (B) SAOS-2, and (C) macrophages after 24 h of exposure, measured at day 0 (immediately after treatment), day 3, and day 7 of culture. The bars represent the percentage of cell viability mean values in comparison with the cranberry untreated control group, representing 100% cell viability. $P < .05$ * and $P < .01$ **, $n = 3$

day 7, presenting statistical differences ($P < .01$) for 25 and 50 µg/mL when compared to the control group. Induced macrophages' viability was not affected by cranberry concentrates at day 0; however, it was significantly reduced at days 3 and 7 after exposure to cranberry concentrates at 25 and 50 µg/mL for 24 hours.

3.2 | Cranberry PACs' effects on cytokine production

Cranberry A-type PACs' effects on cytokine production of LPS-stimulated macrophages are depicted in Figure 2. IL-8 and IL-6

pro-inflammatory expression was significantly downregulated ($P < .01$) when exposed to cranberry concentrates at 50 and 100 $\mu\text{g}/\text{mL}$ for 1 and 24 hours ($P < .01$). Only the cranberry concentrate at 100 $\mu\text{g}/\text{mL}$ revealed an antagonist effect, increasing IL-6 expression after 120 seconds of exposure ($P < .05$). No significant differences were detected for IL-1 β expression among the control and test groups. Anti-inflammatory IL-10 expression was significantly increased at cranberry 100 $\mu\text{g}/\text{mL}$ concentrate when compared to the cranberry untreated control group after 24 hours of exposure ($P < .01$).

3.3 | Effect of cranberry PACs on macrophage polarization

Macrophage M1 and macrophage M2 semi-quantification is presented in Figures 3 and 4. LPS-stimulated macrophages revealing a positive M1 phenotype (assessed via immunofluorescence staining for CCR7 marker) were highly expressed at the cranberry untreated control groups and significantly reduced when exposed to all tested cranberry concentrates for 1 and 24 hours ($P < .01$). Parallel to the decrease in M1 expression, LPS-stimulated macrophages expressing a positive M2 phenotype (evaluated via immunofluorescence

staining for CD206 marker) significantly increased in all cranberry groups after 2 minutes, 1 hour, and 24 hours of exposure to concentrations of 50 and 100 $\mu\text{g}/\text{mL}$ ($P < .01$). Unlikely, only 25 $\mu\text{g}/\text{mL}$ concentrate decreased M2 expression after 120 seconds of exposure. Representative immunofluorescence images of macrophage polarization at control and test groups are presented in Figures 3 and 4.

4 | DISCUSSION

This study aimed at investigating cell viability, anti-inflammatory activity, and macrophage polarization properties of different cranberry-derived PACs' concentrations in vitro. Within its limitations, the presented data suggest that cranberry concentrates may not interfere negatively with cell viability (ie, fibroblasts, SAOS-2, and macrophages) and can have a potential anti-inflammatory activity and polarization effect on macrophages. This was evidenced by no-cytotoxic effects after 24 hours of exposure on cells that are involved in periodontal and peri-implant tissues. Also, by a substantial increase of SAOS-2 viability after seven days of culture that could be possibly induced by the reported cranberry antioxidant and bioactive features.³⁴ Furthermore, the reported outcomes demonstrated a significant downregulation of pro-inflammatory cytokines, an

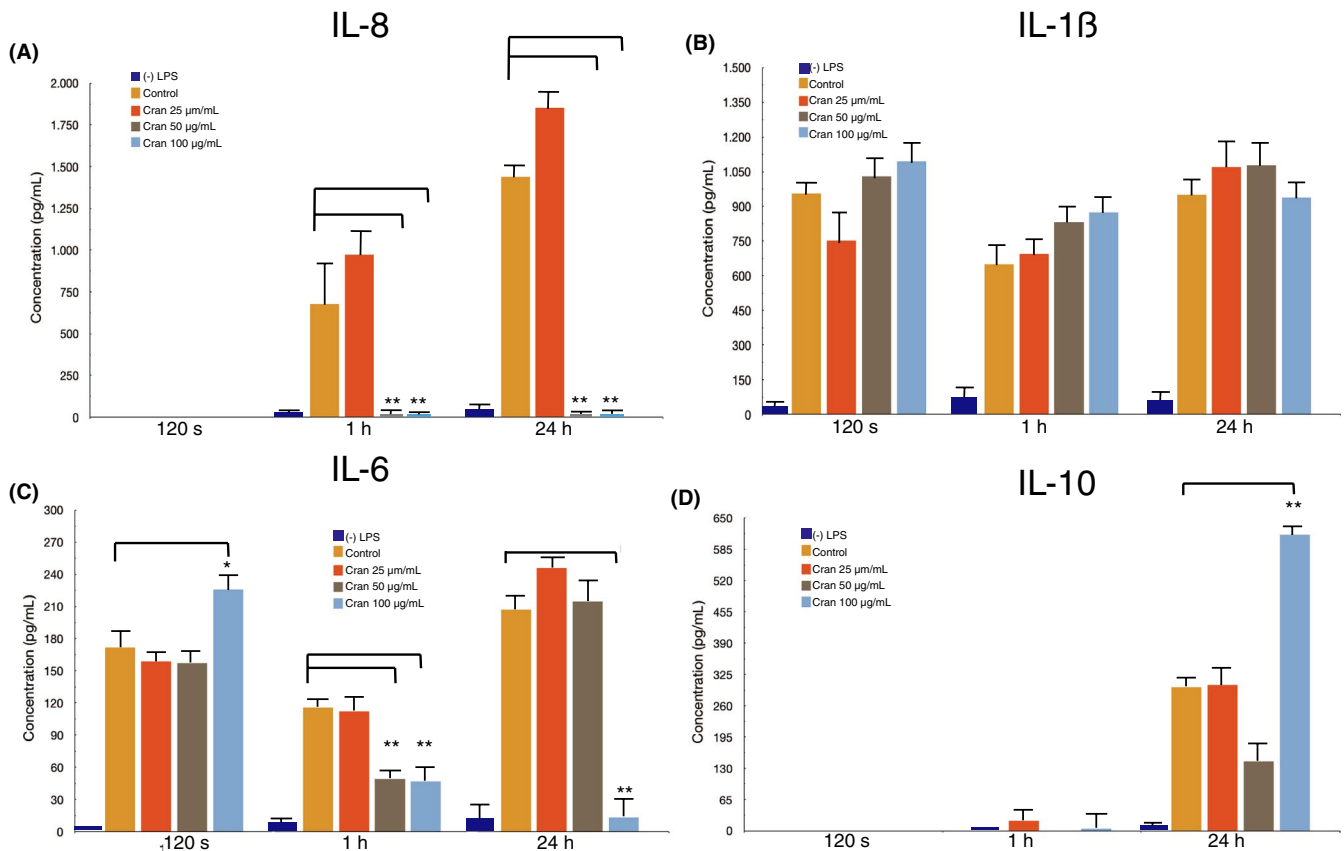


FIGURE 2 Effect of cranberry PACs concentrates on LPS-stimulated macrophage secretion of pro- and anti-inflammatory cytokines (pg/mL): (A) IL-8, (B) IL-1 β , (C) IL-6, and (D) IL-10 after 120 s, 1 and 24 h of exposure. Control group represents cranberry untreated LPS-stimulated macrophages. (-) LPS group represents macrophages that were not exposed to cranberry concentrates and LPS $P < .05$ * and $P < .01$ **, $n = 3$

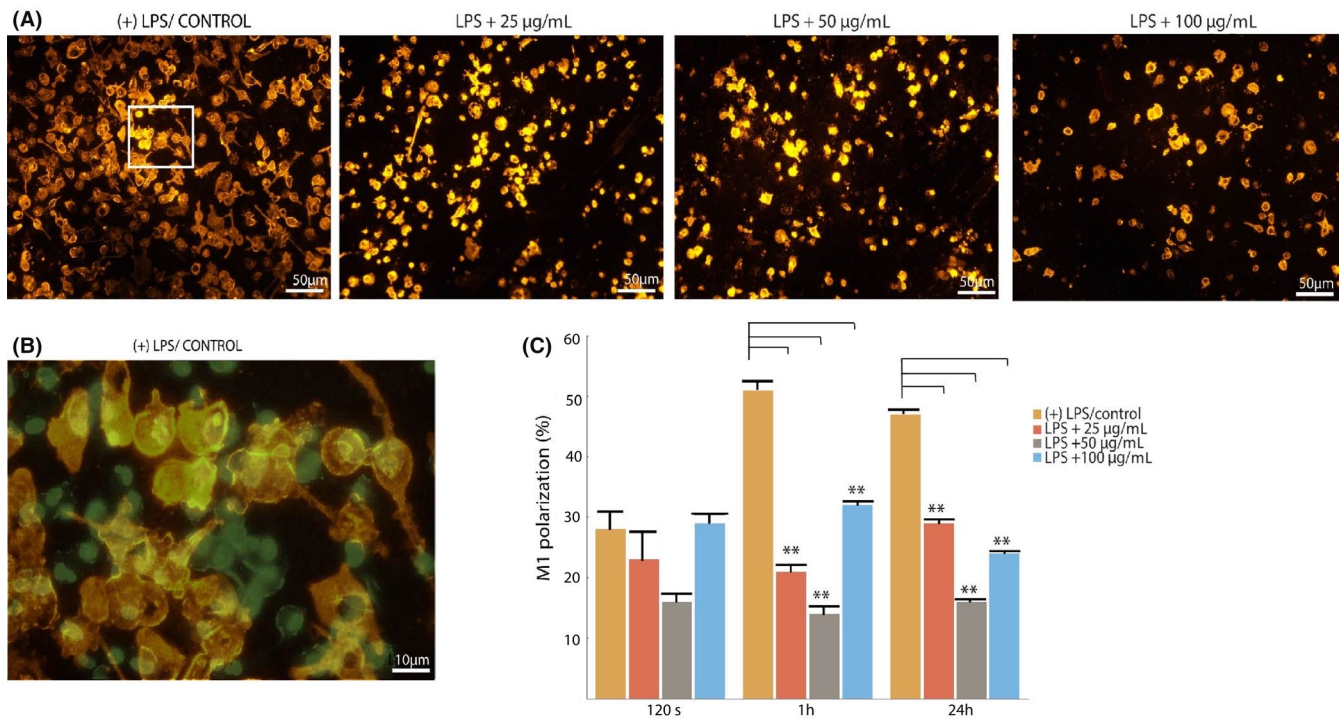


FIGURE 3 A, Representative immunofluorescence images (10x) of macrophage M1 polarization at control and test groups after 24 h of exposure. Control group represents LPS-stimulated macrophages without cranberry PACs exposure. B, Magnification (40x) of the white frame at control group depicts merged staining of positive CD68 (green) and CCR7 (yellow) macrophages. C, Macrophage M1 polarization quantification expressed as percentages of positive double-stained cells for CD68 and CCR7 (M1 marker) among the total number of CD68 positive cells. $P < .05$ * and $P < .01$ **, $n = 3$

upregulation of anti-inflammatory cytokines, and high M2 polarization levels triggered by cranberry concentrates composed of A-type PACs.

On the basis of cytokine expression analysis, previous studies have evaluated the effect of cranberry concentrates on the production of inflammatory interleukins.^{25,35-37} Bodet et al reported that cranberry non-dialyzable material (NDM) at 25 and 50 µg/mL decreased IL-8 macrophage expression.²⁵ Comparably, Tipton et al showed that cranberry NDM at concentrations of 10, 25, and 50 µg/mL significantly decreased IL-8 expression of gingival fibroblasts previously stimulated with IL-17.³⁵ Additionally, a similar study exposed a significant downregulation of IL-8 expression in three synovial fibroblasts lines exposed to cranberry NDM at concentrations of 25, 50, 100 and 250 µg/mL.³⁸ The aforementioned studies correlate with the present results, also pointing to a significant downregulation of IL-8 when macrophages were exposed to cranberry concentrates at 50 and 100 µg/mL for 1 and 24 hours. There is limited evidence evaluating pro-inflammatory IL-1 β expression after cranberry stimulation. Bodet et al exhibited an antagonist effect of cranberry NDM when exposed to LPS-stimulated macrophages. The cytokine expression increased when macrophages were treated with cranberry NDM at 10 and 25 µg/mL; however, at a concentration of 50 µg/mL, there was a significant reduction of IL-β secretion.²⁵ In contrast, the present analysis revealed that cranberry concentrates did not induce any significant effect on IL-1 β production. IL-6 expression was also evaluated in previous studies.^{33,35} A prior study

revealed a significant reduction of IL-6 expression when LPS induced macrophages were exposed to cranberry NDM concentrations at 25 and 50 µg/mL for 24 hours.²⁵ Similarly, Tipton et al revealed a reduction of IL-6 expression by synovial fibroblasts exposed to cranberry NDM at concentrations of 25, 50, 100, and 250 µg/mL.³⁸ Moreover, Denis et al reported that LPS-stimulated human epithelial colorectal adenocarcinoma cells decreased IL-6 expression when exposed to low, medium, and high molecular mass (250 µg/mL) cranberry compounds.³⁶ The latter results correlate with the present findings, pointing to a significant downregulation of IL-6 secretion when exposed to cranberry concentrates (A-type PACs) at 50 and 100 µg/mL for 1 and 24 hours. Even though cranberry-derived concentrates induced a significant downregulation of pro-inflammatory cytokines in the present in vitro study, it is imperative to consider that inflammation on macrophages was induced by a commercially available LPS (*E coli*) agent which does not resemble the real conditions that exacerbate inflammation at periodontal and peri-implant tissues. Bearing in mind this limitation, future studies should consider to use LPS derived from biofilm involving periodontitis and peri-implantitis pathogens to mimic a more realistic condition.

Considering the anti-inflammatory cytokine expression, IL-10 was reported to have potential anti-inflammatory properties associated with M2 macrophage activation.^{14,15} The present study revealed a significant upregulation of IL-10 expression when macrophages were exposed to the highest cranberry concentrate for 24 hours. These effects need to be further investigated in future studies.

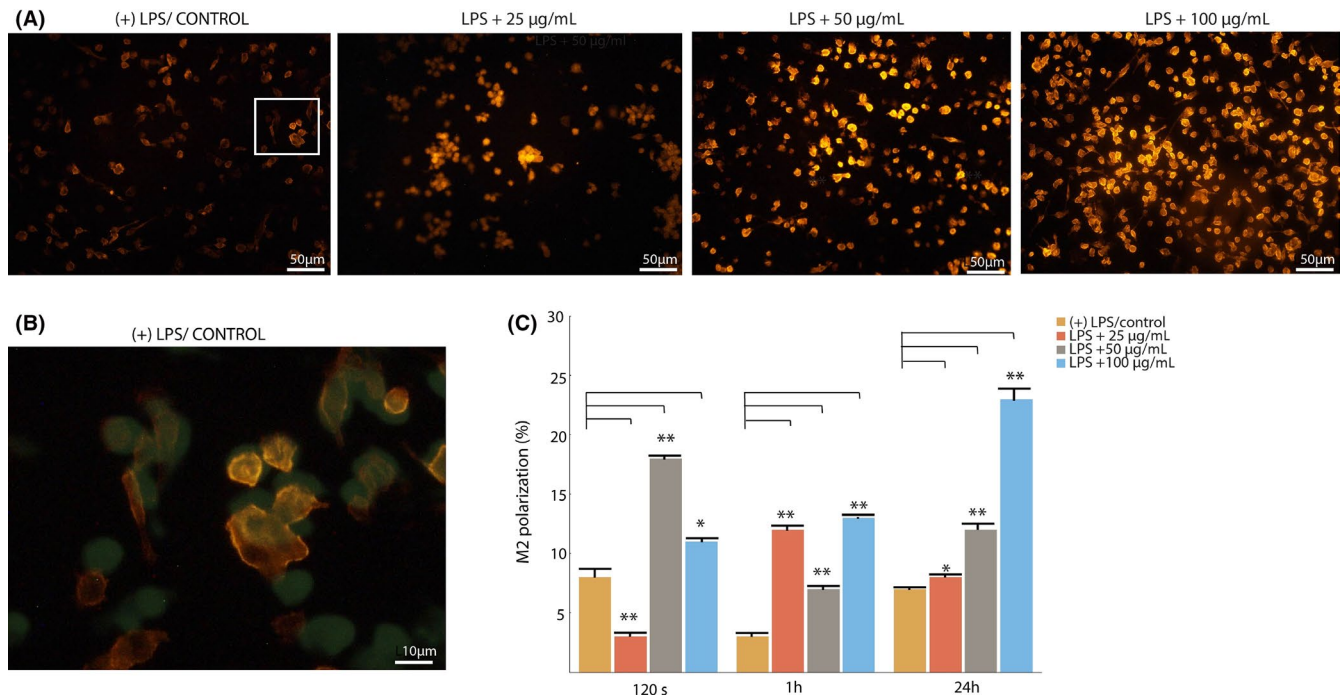


FIGURE 4 A, Representative immunofluorescence images (10x) of macrophage M2 polarization at control and test groups after 24 h of exposure. Control group represents LPS-stimulated macrophages without cranberry PACs exposure. B, Magnification (40x) of the white frame at control group depicts merged staining of positive CD68 (green) and CD206 (yellow) macrophages. C, Macrophage M2 polarization quantification expressed as percentages of positive double-stained cells for CD68 and CD206 (M2 marker) among the total number of CD68-positive cells. $P < .05$ * and $P < .01$ **, $n = 3$

To the authors' best knowledge, this is the first in vitro study that provides evidence on the effect of cranberry concentrates on macrophage M1 and macrophage M2 polarization. In chronic inflammatory conditions, CD4-T-cell-centered processes can induce T-helper cells to stimulate or counteract inflammation and osteolysis, playing a substantial role on macrophage polarization effects.¹⁴ In this hallmark, Th1 and Th17 cells have been related to disease progression, upregulating inflammation and osteolytic effects. On the other hand, Th2 cells and Tregs have been described to counteract disease progression and to downregulate inflammation. In this context, the Th transition from Th1 to Th2/Tregs has been associated to M2 polarization induction and the arrest of disease progression.^{14,39} Moreover, the host's inflammatory response to bacteria endotoxins as LPS is a critical factor activating "destructive" inflammation at periodontal and peri-implant tissues. Several studies reported that LPS is a potential M1 inducer.⁴⁰⁻⁴² Accordingly, the present study showed high levels of positive M1 macrophages at all untreated control groups after LPS stimulation. Furthermore, a previous study evaluating the effect of cranberry PACs on LPS-stimulated macrophages reported that cranberry PACs significantly attenuated the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).²⁸ In particular, the expression of iNOS is considered as a M1 marker,¹⁵ which also correlates with the results of the present study suggesting that M1 polarization was significantly reduced when exposed to cranberry concentrates. Prominently, M2 phenotype levels increased at all cranberry concentrates, conveying an important immune modulation action. Considering that M2 activation has been

associated with IL-10 expression,^{14,40} the present study supported this association showing that the highest percentage of positive M2 macrophages was related to a significant expression of IL-10 after exposure to cranberry A-type PACs maximum concentration for 24 hours. Nevertheless, future studies should further evaluate the effect of cranberry concentrates on the T cell-centric framework and immune-regulatory properties of Th cells, which are essential to comprehend M1 and M2 polarization mechanisms.

Considering the limitations of an in vitro model, the present study suggests that cranberry concentrates of 50 and 100 µg/mL at exposure periods of 1-24 hours could be beneficial to counteract inflammation and destructive processes at periodontal and peri-implant diseased sites. From a clinical perspective, cranberry concentrates could be applied locally at the affected sites as a subgingival gel acting as a controlled drug delivery system as exemplified by Hirasawa et al,⁴³ as a mesoporous biomaterial for regenerative purposes embedding cranberry biomolecules as reported by Galarraga et al,²⁶ or as cranberry nano-particles having a high bioavailability as described by Zou et al⁴⁴ The mentioned strategies could possibly allow an effective localized action of cranberry PACs on the treated area. Still, these clinical indications sustained on in vitro observations need to be proven in future long-term clinical studies evaluating efficacy of cranberry drug delivery systems and concomitant anti-inflammatory effects.

Bearing in mind that cranberry-derived PACs are reported to have multifunctional anti-biofilm, analgesic and macrophage modulation effects, the present study suggests that cranberry-derived

PACs may serve as a potential anti-inflammatory component that can target macrophages enhancing “constructive inflammation” at diseased tooth and implant sites. Nevertheless, future clinical research is indispensable to evaluate the “in vivo” anti-inflammatory effects of cranberry agents.

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CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

AUTHORS CONTRIBUTIONS

ME Galarraga-Vinueza contributed to conception, design, data acquisition, analysis, interpretation, and drafted and critically revised the manuscript. A. Ramanauskaite, K. Obreja, S. Al-Maawi, R. Magini, and R. Sader contributed to data acquisition and analysis, and critically revised the manuscript. E. Dohle, S. Ghanaati, and F. Schwarz contributed to data analysis and interpretation, and drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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